بسم الله الرحمن الرحيم

Sudan University of Science and Technology

College of Graduate Studies

Molecular Detection of Carbapenem Resistance Genes of *Klebsiella pneumoniae* Isolated from Clinical Specimens in Khartoum State

الكشف الجزيئي لجينات مقاومة الكاربابينيم للكليبسيلا الرئوية المعزولة من عينات سريرية في ولاية الخرطوم

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الآيـــــة

بِسَمِ ٱللَّهِ الرَحمنِ الرَحِيم

قال تعالى:

﴿ اللَّهُ لا إِلَهَ إِلَّا هُوَ الْحَيُّ الْقَيُّومُ لا تَأْخُذُهُ سِنَةٌ وَلا نَوْمٌ لَهُ مَا فِي السَّمَوَاتِ وَمَا فِي الأَرْضِ مَنْ ذَا الَّذِي يَشْفَعُ عِنْدَهُ إِلَّا بِإِذْنِهِ يَعْلَمُ مَا بَيْنَ أَيْدِيهِمْ وَمَا خَلْفَهُمْ وَلا يُحِيطُونَ بِشَيْءٍ مِنْ عِلْمِهِ إِلَّا بِمَا شَاءَ وَسِعَ كُرْسِيُّهُ السَّمَوَاتِ وَالأَرْضَ وَلا يَئُودُهُ حِفْظُهُمَا وَهُوَ الْعَلِيُّ الْعَظِيمُ ﴾

سورة البقرة (255)

DEDICATION

To my beloved family

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ABSTRACT

Carbapenems are the most powerful β-lactam antibiotics against bacteria especially multidrug resistant isolates like *Klebsiella pneumoniae* (*K. pneumoniae*). Since there were no comprehensive studies about prevalence of carbapenem resistance genes in Sudan, so this study was conducted to detect carbapenem resistance genes of *K. pneumoniae* from clinical specimens in Khartoum State during the period from March to November 2018.

A total of sixty (n=60) *K. pneumoniae* were included in this descriptive-cross sectional laboratory based study. *K. pneumoniae* were isolated from urine, blood, wound swab and sputum samples, which were obtained from different hospitals in Khartoum State. The samples were collected from both males and females with different age using non-self-constructing information list. The isolated organism was stored in 20% Glycerol peptone media and was inoculated in MacConkey's agar. Antibiotic susceptibility tests were carried out using Kirby Bauer technique. DNA was extracted using guanidine chloride method. The isolates were detected using PCR based method targeting 16S rRNA gene then were tested for the presence of carbapenem resistance genes (*NDM*, *IMP*, *SPM*, *OXA-48* and *KPC*) using Multiplex PCR.

Out of 60 *K. pneumoniae* isolates, 48 (80%) were positive for one or more carbapenem resistance genes; 41 (68.3%) were positive for *OXA-48* gene, 6 (10%) were positive for *NDM* gene, 5 (8.4%) were positive for *SPM* gene, 5 (8.4%) were positive for *KPC* gene and 2 (3.3%) were positive for *IMP* gene. Ten isolates possess more than one gene. Thirty-eight isolates possess one gene and the remaining twelve isolates were free from these genes.

It is concluded that, there is a high percentage and multiplicity of carbapenem resistance genes among *K. pneumoniae* isolates in Khartoum State. In addition, this study shown for the first time, *K. pneumoniae* carried *SPM* gene.

ملخص الأطروحة

الكاربابينيمات (بيتا لاكتام) من أقوى المضادات الحيوية ضد البكتيريا وخاصة المقاومة للأدوية مثل الكليبسيلا الرئوية. ونظراً لعدم وجود دراسات شاملة حول انتشار الجينات المقاومة للكاربابينيم في السودان لذا أجريت هذه الدراسة للكشف عن جينات مقاومة الكاربابينيم للكليبسيلا الرئوية المعزولة من عينات سريرية في ولاية الخرطوم في الفترة من مارس الى نوفمبر 2018.

شملت هذه الدراسة الوصفية ستين (ن = 60) من الكليبسيلا الرئوية المعزولة من عينات البول، الدم، الجروح والبلغم التي جمعت من مختلف المستشفيات في ولاية الخرطوم. تم عزل الكليبسيلا الرئوية من الذكور والإناث من مختلف الأعمار. ومن ثم تم حفظها في وسط الجليسرول بيبتون بتركيز 20% لحين زرعها في الاجار المناسب لها. أجريت اختبارات الحساسية للمضادات الحيوية باستخدام تقنية كيربي باور. استخلص الحمض الوراثي النووي باستخدام طريقة الجوانودين كلورايد. حيث تم التعرف عن جميع السلالات المعزولة باستخدام تفاعل البلمرة التسلسلي ثم تم اختبارها لوجود جينات مقاومة الكاربابينيم (*NDM*، *IMP*، *SPM*، *OXA-48* و*KPC*) باستخدام تفاعل البلمرة التسلسلي المتعدد.

من بين ستين كليبسيلا رئوية معزولة، 48 (80%) أعطت نتيجة إيجابية لجين واحد أو أكثر من جينات مقاومة الكاربابينيم: 41 (68.3%) كانت إيجابية لجين *OXA-48*، 6 (10%) كانت إيجابية لجين *NDM*، 5 (8.3%) كانت إيجابية لجين *SPM*، 5 (8.3%) كانت إيجابية لجين *KPC* و2 (3.3%) كانت إيجابية لجين *IMP*. وقد تبين ان هناك عشره عزلات تمتلك أكثر من جين واحد. ثمانية وثلاثون عزلة تمتلك جين واحد فقط. اثنتا عشر عزلة خالية من جميع الجينات.

من النتائج أعلاه خلصت الدراسة إلى أن هناك نسبة عالية وتعددية في جينات مقاومة الكاربابينيم في الكليبسيلا الرئوية المعزولة في ولاية الخرطوم. بالإضافة إلى ذلك، أظهرت هذه الدراسة لأول مرة كليبسيلا رئوية حاملة لجين *SPM*.

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ABBREVIATIONS

API	Analytical profile index
BTB	Bromothymol blue
CAZ-AVI	Ceftazidime-avibactam
CF	Complement fixation
CLSI	Clinical and Laboratory Standards Institute
CP	Carbapenemase-producing
CRE	Carbapenem Resistant *Enterobacteriaceae*
CRKP	Carbapenem Resistant *K. pneumoniae*
EDTA	Ethylenediamainetetraacetic acid
EMB	Eosin-methylene blue
ESBLs	Extended spectrum beta-lactamases
GES	Guiana extended spectrum
GIM	German imipenemase
HV	Hypervirulent
ICU	Intensive Care Units
ID	Immunodiffusion
IMI	Imipenem-hydrolyzing β-lactamase
IMP	Imipenemase
KPC	*Klebsiella pneumoniae* Carbapenemase
LPS	Lipopolysaccharide
MBLs	Metallo-beta-lactamases
MDR	Multidrug resistant
MHT	Modified Hodge test
MIC	Minimum Inhibitory Concentration
MRSA	Methicillin-resistant *S. aureus*
NDM	New Delhi Metallo-betalactamase
NMC-A	Not metalloenzyme carbapenemase

OMPs	Outer membrane proteins
OXA-48	Oxacillinase-48
PBPs	Penicillin binding proteins
PCR	Polymerase Chain Reaction
SIM	Seoul imipenemase
SME	*Serratia marcescens* enzyme
TBE	Tris Base EDTA
UTI	Urinary tract infections
VFs	Virulence factors
VIM	Verona Integron Metallo-betalactamases

CHAPTER ONE

INTRODUCTION AND OBJECTIVES

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1.1. Introduction

The genus Klebsiella belongs to the tribe Klebsiellae, a member of the family *Enterobacteriaceae* (Qureshi, 2017). *K. pneumoniae* is a Gram-negative, nonmotile, encapsulated, lactose-fermenting, facultative anaerobic, rod-shaped bacterium (Paczosa and Mecsas, 2016). It possess many virulence factors that play a major role in the severity level of infection (Gharrah *et al.,* 2017). It is both a common hospital-acquired and potential community-acquired pathogen, causing urinary tract infections (UTI), nosocomial pneumonia, and intraabdominal infections (Ko *et al.,* 2002).

Beta-lactam (β-lactam) antibiotics are commonly used to treat infectious diseases and include the penicillins, cephalosporins, monobactams and carbapenems (Meletis, 2016). They all share a common beta-lactam ring and act similarly by binding to and inactivating the penicillin-binding proteins (PBPs), which are responsible for the formation of the bacterial cell wall (Meletis, 2016). Carbapenems (imipenem, meropenem, biapenem, ertapenem, and doripenem) were used for the treatment of severe infections caused by multi-resistant *Enterobacteriaceae*, such as *K. pneumoniae* (Lee *et al.*, 2016).

Klebsiella pneumoniae have acquired carbapenemases, which are enzymes capable of breaking down most β-lactams including carbapenems, and thus conferring resistance to these drugs (Lee *et al.*, 2016). Ambler molecular class A *K. pneumoniae* carbapenemase (KPC), class B; Verona integron metallo-betalactamases types (VIM), Imipenemase (IMP) and New Delhi metallo-betalactamase (NDM) and class D oxacillinase-48 (OXA-48) are the most often found among *K. pneumoniae* isolated during serious nosocomial infections (Pollett *et al.*, 2014). While other carbapenemases,

such as SPM have been not found in *K. pneumoniae* (Lee *et al.*, 2016). Genes coded for these carbapenemases were located on transferable plasmids and can be freely transferred between bacteria, from one region to another and from one country to the other and then spread all over the world (Satir *et al.*, 2016).

Recently, increasing resistance to carbapenems in health care associated infections has been reported worldwide (Mushi *et al.*, 2014). KPC-producing *K. pneumoniae* was first noticed in the northeastern parts of the United States during the first decade of the 21st century (Tzouvelekis *et al.*, 2012). The NDM-1 enzyme is a more recently reported class B carbapenemase arose from India in 2008 and spread rapidly worldwide (Girmenia *et al.*, 2016). OXA-48-producing *K. pneumoniae* was first detected sporadically in Turkey, in 2001. About the same time, isolates were also identified in other Middle Eastern and North African countries (Mushi *et al.*, 2014). IMP type beta-lactamases were dominant in the Asian continent and Australia and it has been only sporadically detected in Europe and the Mediterranean area (Girmenia *et al.*, 2016).

In Sudan, a study by Ali and Omer, (2017) revealed that seventy-two percent of *K. pneumoniae* isolates were positive for carbapenemase genes and the most common type is *KPC* gene. Also they detected co-production of *KPC, VIM, NDM* and *OXA-48* genes in *K. pneumoniae*. Another study also from Sudan by Satir et al., (2016) which indicated widespread prevalence and multiplicity of these genes in carbapenem resistance Gram-negative isolates. In addition, Dahab *et al.*, (2017) revealed that the percentage of resistance to carbapenems due to production of carbapenemase enzymes is very high in Sudan and *OXA-48* gene is more predominant one.

1.2. Rationale

The emergence and spread of carbapenem resistance *Enterobacteriaceae* (CRE) have become an increasing concern for healthcare services worldwide, especially when mediated by transferable carbapenemase-encoding genes (Baran and Aksu, 2016; Meletis, 2016). Infections caused by these bacteria have been associated with significant morbidity and mortality and treatment options have been limited (Baran and Aksu, 2016). *K. pneumoniae* is now recognized as an urgent threat to human health because of the emergence of multidrug-resistant strains associated with hospital outbreaks and hypervirulent strains associated with severe community-acquired infections (Holt *et al.*, 2015). Resistance to antimicrobial agents is often associated with the spread of transmissible plasmids, which may also carry virulence determinants (Derakhshan *et al.*, 2016). In Africa, data on the prevalence and distribution of carbapenem resistance among the multidrug resistant Gram-negative bacteria is still limited (Mushi *et al.*, 2014). In addition, detection of carbapenem resistance genes producing bacteria may be difficult based on routine antibiotic susceptibility testing (Satir *et al.*, 2016).

Considering the fact that there were no comprehensive studies about prevalence of carbapenem resistance genes in Sudan so detection of those genes in *Klebsiella pneumoniae* from clinical specimens will be of a great value. Such data serve an important role in understanding the spread of this pathogen.

1.3. Objectives

1.3.1. General objective

To detect carbapenem resistance genes of *Klebsiella pneumoniae* from clinical specimens in Khartoum State.

1.3.2. Specific objectives

1- To identify the organism under study using PCR based method targeting 16S rRNA gene.

2- To assess the susceptibility of *K. pneumoniae* to antimicrobial agents using disc diffusion method.

3- To detect the carbapenem resistance genes of *K. pneumoniae* (*KPC*, *OXA48, NDM, IMP*, and *SPM*) using multiplex PCR.

4- To associate between resistance to antimicrobial agents and the presence of carbapenem resistance genes of *K. pneumoniae.*

5- To correlate between the presences of carbapenem resistance genes with different clinical specimens and age groups.

CHAPTER TWO

LITERATURE REVIEW

CHAPTER TWO

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2.1. *Klebsiella pneumoniae (K. pneumoniae)*

2.1.1. General characteristics

In 1882, Carl Friedlander first described *K. pneumoniae* as an encapsulated bacillus after isolating the bacterium from the lungs of those who had died from pneumonia (Ashurst and Dawson, 2018). Originally named Friedlander's bacillus, it was not until 1886 when the bacterium garnered the name *Klebsiella* (Ashurst and Dawson, 2018).

Klebsiella pneumoniae is a Gram-negative pathogenic bacterium. On agar media, it has a mucoid phenotype that is conferred by the polysaccharide capsule attached to the bacterial outer membrane, and ferments lactose (Martin and Bachman, 2018). The organism is part of the *Enterobacteriaceae* family, which is comprised of other familiar pathogens such as *Escherichia coli*, *Yersinia* species, *Salmonella* species, and *Shigella* species (Martin and Bachman, 2018).

Humans serve as the primary reservoir for *K. pneumoniae* (Ashurst and Dawson, 2018). In the general community, 5% to 38% of individuals carry the organism in their stool and 1% to 6% in the nasopharynx (Ashurst and Dawson, 2018). However, higher rates of colonization have been reported in those who experience chronic alcoholism (Ashurst and Dawson, 2018). In hospitalized patients, the carrier rate for *K. pneumoniae* is much higher than that found in the community (Ashurst and Dawson, 2018).

2.1.2. Culture and metabolic characteristics

Klebsiella species are easily cultured on media suitable for *Enterobacteriaceae* bacteria, including: nutrient agar, tryptic casein soy agar, bromocresol purple lactose agar, Drigalski agar, MacConkey agar, eosin-methylene blue (EMB) agar and bromothymol blue (BTB) agar (De Jesus *et al.*, 2015). No additional growth factors are required by *K.*

pneumoniae, which is capable of both fermentative and respiratory metabolism (De Jesus *et al.*, 2015). The facultative anaerobe can have a variable mucoid appearance, which may vary between different strains and be influenced by the composition of the medium used (De Jesus *et al.*, 2015). All *Klebsiella* strains are capable of utilising L-arabinose, D-arabitol, D-cellobiose, citrate D-fructose, D-galactose, D-glucose, 2-ketogluconate, maltose, D-mannitol, D-melibiose, Draffinose, D-trehalose and D-xylose, whilst lactose and D-sorbitol can be used as a carbon source by all strains, except *K. pneumoniae* subsp. *ozaenae* and *K. pneumoniae* subsp. *Ornithinolytica* (De Jesus *et al*., 2015).

Klebsiella species are oxidase negative, catalase positive and often Voges-Proskauer test positive, with the exception of *K. pneumoniae* subsp. *ozaenae* and *K. pneumoniae* subsp. *Rhinoscleromatis*. Most strains can hydrolyze urea, reduce nitrates without the production of H_2S gas, as well as utilize glucose and citrate as carbon sources. In the case of fermentation of glucose, a gas and an acid are produced (De Jesus *et al*., 2015).

2.1.3. Clinical significance

Klebsiella pneumoniae colonizes the mucosal surfaces in humans, including the nasopharynx and the gastrointestinal tract (Martin and Bachman, 2018). Gastrointestinal carriage of the organism was significantly associated with subsequent infections in hospitalized patients, indicated the link between infecting and colonizing isolates of *K. pneumoniae* within infected patients (Martin and Bachman, 2018).

Klebsiella pneumoniae has recently gained notoriety as an infectious agent due to a rise in the number of severe infections (Paczosa and Mecsas, 2016). These concerning circumstances have arisen due to the emergence of *K. pneumoniae* strains that have acquired additional genetic traits and become hypervirulent (HV) (Paczosa and Mecsas, 2016). Infections including, but not limited to, pneumonia, sepsis, UTI, bacteremia,

meningitis, and pyogenic liver abscesses (Paczosa and Mecsas, 2016). When considering these infections, it is important to recall that HV strains are far more likely to cause community-acquired and systemic infections in otherwise healthy individuals (Paczosa and Mecsas, 2016). Primary infections caused by classical *K. pneumoniae* strains are usually pneumonias or UTIs (Paczosa and Mecsas, 2016). Classical *K. pneumoniae* strains also cause very serious infections such as bacteremia, and can be either primary bacteremias or secondary bacteremias that arise from secondary spread from a primary infection in the lungs or bladder (Paczosa and Mecsas, 2016).

2.1.4. Virulence factors

Virulence factors (VFs) comprise mechanisms allowing pathogenic bacteria to cause infections (Gharrah *et al.*, 2017). Virulence factors in *K. pneumoniae* include capsule, lipopolysaccharide, siderophores, and pili, which are encoded by genes in both the core and accessory genomes (Martin and Bachman, 2018). Allantoin utilization, other iron uptake systems, efflux pumps, and a type VI secretion system have been identified as virulence factors more recently (Martin and Bachman, 2018). Genomics becomes a good tool for recognizing genes harboring specific virulence factors (Gharrah *et al.*, 2017). These genes may determine the severity of an infection, and therefore the virulence of the infecting strain and determine the ability of a colonizing strain to progress to infection, defining the pathogenic potential of a given strain (Martin and Bachman, 2018). Capsule, a polysaccharide matrix that coats the cell, is necessary for *K. pneumoniae* virulence (Paczosa and Mecsas, 2016). Hypervirulent *K. pneumoniae* strains produce a hypercapsule, also known as being hypermucoviscous, which consists of a mucoviscous exopolysaccharide bacterial coating that is more robust than that of the typical capsule (Paczosa and Mecsas, 2016). Both classical capsule and HV hypercapsule

are made up of strain-specific capsular polysaccharides termed K antigens (Paczosa and Mecsas, 2016).

Lipopolysaccharide (LPS), also termed endotoxin, is a major component decorating the outer membrane of Gram-negative bacteria (Martin and Bachman, 2018). It is widely recognized as the most powerful mediator of septic shock caused by bacteria (Martin and Bachman, 2018).

Siderophores are molecules that possess a higher affinity for iron than host transport proteins do and have ability to steal iron from host iron-chelating proteins or scavenge it from the environment (Paczosa and Mecsas, 2016). *K. pneumoniae* secrete multiple types of siderophores to evade the host immune response (Martin and Bachman, 2018).

Fimbriae represent another class of *K. pneumoniae* virulence factors and are important mediators of *K. pneumoniae* adhesion (Paczosa and Mecsas, 2016). They are filamentous structures extending from the surface of bacteria (Martin and Bachman, 2018). In *K. pneumoniae*, type 1 and 3 fimbriae are the major adhesive structures that have been characterized as pathogenicity factors (Paczosa and Mecsas, 2016).

Efflux pumps have frequently been associated with antibiotic resistance in *K. pneumoniae* due to their ability to export antibiotics from bacterial cells (Martin and Bachman, 2018). Interestingly, the efflux pump AcrAB contributes to virulence in murine respiratory infections caused by *K. pneumoniae* (Martin and Bachman, 2018). Overall, these AcrAB functions are likely mediated by the export of detrimental host molecules or antibiotics out of the bacterial cell (Paczosa and Mecsas, 2016).

The type VI secretion system (T6SS), first identified in *V. cholera*, is a syringe-like apparatus anchored within the bacterial cell membrane that serves to inject various effector molecules and toxins into other cells (Martin and Bachman, 2018).

Metabolism of allantoin is a method by which bacteria can obtain carbon and nitrogen from their environment (Paczosa and Mecsas, 2016). An operon containing genes involved in allantoin metabolism was identified in a search for *K. pneumoniae* genes whose transcription was upregulated in HV *K. pneumoniae* strains compared to classical strains (Paczosa and Mecsas, 2016).

2.1.5. Microbiological assays

2.1.5.1. Identification of *K. pneumoniae* using conventional method

Conventional methods that allow the detection of *K. pneumoniae* include microscopic observation, gram staining, traditional culture based techniques, biochemical tests, immunological assays etc. Microscopic examination remains the most commonly requested microbiological investigation but it could not distinguish between infection, colonization, and contamination when the specimen is collected through the oropharynx (Aurna, 2017). The Gram-stained smear is an essential and necessary part of evaluation of sputum and tracheal aspirates for determining the quality and acceptability of specimens for bacterial culture but may be difficult to interpret as *Klebsiella spp.* due to the presence of oropharyngeal commensals in lower respiratory tract (Aurna, 2017).

Microbiologic culture of specimens allows definitive identification of the suspected pathogens and permits determination of bacterial susceptibility to antimicrobial agents. Culture results must be correlated with the Gram stain findings (Aurna, 2017).

The serologic methods commonly used in diagnostic laboratories include enzyme immunoassay, immunoprecipitation, immunodiffusion (ID), complement fixation (CF), immunoblotting (including Western blot), agglutination, hemagglutination inhibition, and indirect immunofluorescence assay. The problems often associated with

serological detection assays are reduced reproducibility, sensitivity and specificity with high cost (Aurna, 2017).

K. pneumoniae can be identified by Analytical profile index system (API) or by traditional biochemical tests, and the whole process requires at least 24 to 48 hours (Aurna, 2017).

2.1.5.2. Identification of *K. pneumoniae* using PCR based method targeting 16S rRNA gene

Over the past several decades, a number of molecular markers that permit identification of specific microbial taxa and their phylogenetic classification have been identified (Srinivasan *et al.*, 2015). Among these molecular markers, 16S rRNA, about 1500 base pair gene coding for a catalytic RNA that is part of the 30S ribosomal subunit, has desirable properties that allowed it to become the most commonly used such marker (Srinivasan *et al.*, 2015).

Different studies had been carried out to successfully and rapidly detect *K. pneumoniae* based on PCR-mediated amplification of the 16S rRNA gene because the 16S rRNA gene is present in all bacteria (Aurna, 2017). The 16S rRNA gene consists of highly conserved nucleotide sequences, interspersed with variable regions that are genus- or species-specific (Aurna, 2017). PCR primers targeting the conserved regions of rRNA amplify variable sequences of the rRNA gene (Aurna, 2017).

2.1.6. Emergence of antibiotic resistance in *K. pneumoniae*

The explosion of antibiotic usage worldwide was always followed by the appearance of clinical infection with organisms resistant to commonly used antibiotics (Elhag, 2014). The global antibiotic resistance is rising, while development of new antimicrobials is lagging behind (Elhag, 2014). In Sudan, the situation is even more complicated by general inappropriate use of antibiotics that may have led to the development of resistance; imprudent use of antibiotics, availability of too many antibiotic brands for

the same generic with no clear quality control measures, routine use of antibiotics in livestock for treatment and use of sub therapeutic doses for animals' growth promotion (Ahmed, 2014). *K. pneumoniae* are one of several bacteria that have experienced a dramatic increase in antibiotic resistance in the past decades (Martin and Bachman, 2018). It possess three modes of antibiotic resistance, include drug modification or enzymatic inactivation, antibiotic target modification or decreased concentrations of antimicrobial drugs within cells (possible by reduced permeability) and increased efflux activity (De Jesus *et al.*, 2015). These modes of action are encoded either intrinsically or acquired through mutation and resistance gene acquisition (De Jesus *et al.*, 2015).

Resistance of bacteria to β-lactam antibiotics emerged before penicillin was widely used to treat infections, as in *K. pneumoniae*, resistance to some β-lactams is intrinsic since the enzyme is encoded in the core genome of the species like SHV which is consistently found in the chromosome (Martin and Bachman, 2018). This enzyme capable of hydrolysing third-generation cephalosporins and monobactams (De Jesus *et al.*, 2015). Other β-lactamases are part of the accessory genome like TEM-1 and AmpC enzymes that are plasmid-mediated β-lactamase (Martin and Bachman, 2018). The worrisome β-lactamases form are found among the extended-spectrum β-lactamases that are initially identified as variants of the common SHV-1 or TEM-1 β-lactamases (Bush, 2010). The genes that coded for these enzymes are generally found on plasmids that conferred resistance to multiple antibiotic classes and that are readily transferable among species (Bush, 2010).

Perhaps due to the selective pressure of treating ESBLs infections with carbapenems, carbapenem resistance has emerged and *K. pneumoniae* is the most common carbapenem-resistant *Enterobacteriaceae* (CRE) (Martin and Bachman, 2018). Carbapenem resistance in *K. pneumoniae*

can be mediated in part through up-regulation of efflux pumps and alteration of outer membrane porins in the core genome, and hyperproduction of ESBL enzymes or AmpC β-lactamases in the accessory genome (Martin and Bachman, 2018). Colistin has long been are serve drug used for the treatment of carbapenem resistant *Klebsiella pneumoniae* (Pragasam *et al*., 2017). Colistin resistance is mainly mediated by the alteration in the LPS of bacterial outer membrane (Pragasam *et al*., 2017). These alterations are mediated by mutations in several genes involved in lipid A modifications and most commonly mutations in *mgrB* gene has been reported (Pragasam *et al*., 2017). Recently there is emergence of plasmid mediated resistance due to *mcr-1* and *mcr-2* genes which poses a threat for the rapid global spread (Pragasam *et al*., 2017).

2.2. Carbapenems

In the late 1960s, as bacterial β-lactamases emerged and threatened the use of penicillin, the search for β-lactamase inhibitors began in earnest (Papp-Wallace *et al*., 2011). After a series of experiments, thienamycin was firstly serve as the parent or model compound for all carbapenems which is a natural product from *Streptomyces cattleya* bacterium (Rao, 2012). To date, more than 80 compounds with mostly improved antimicrobial properties, compared to those of thienamycin (Papp-Wallace *et al.*, 2011). The term "carbapenem" is defined as the 4:5 fused ring lactam of penicillins with a double bond between C-2 and C-3 but with the substitution of carbon for sulfur at C-1(Rao, 2012).

Since thienamycin displayed inhibitory microbiological activity against Gram-negative bacteria, unfortunately, it was found to be unstable in aqueous solution, sensitive to mild base hydrolysis, and highly reactive to nucleophiles, such as hydroxylamine, cysteine. The chemical instability of thienamycin stimulated the search for analogous derivatives with increased stability (Papp-Wallace *et al.*, 2011).

The first developed was the N-formimidoyl derivative, imipenem. Imipenem and a closely related carbapenem, panipenem, identified later, were more-stable derivatives of thienamycin and less sensitive to base hydrolysis in solution (Papp-Wallace *et al.*, 2011). In 1985, imipenem (originally called MK0787) became the first carbapenem available for the treatment of complex microbial infections (Rao, 2012). Like its parent, thienamycin, demonstrated high affinity for PBPs and stability against β-lactamases (Papp-Wallace *et al.*, 2011).

Along the journey to the discovery of more-stable carbapenems with a broader spectrum, the other currently available compounds, meropenem, biapenem, ertapenem, and doripenem, were developed, and several novel carbapenems were identified. These novel carbapenems included antipseudomonal carbapenems, anti-methicillin-resistant *S. aureus* (MRSA) carbapenems (i.e., cationic and dithiocarbamate carbapenems), orally available carbapenems, trinem carbapenems, a dual quinolonyl-carbapenem, and others (Papp-Wallace *et al.*, 2011).

Several chemical approaches were developed for the synthesis of carbapenems since fermentation was not an efficient method for production. Natural products were often used as starting material for production of carbapenems, and the synthetic approach was largely influenced by the desired stereochemistry of the final compound (Papp-Wallace *et al.*, 2011).

As a class of β-lactams, carbapenems are not easily diffusible through the bacterial cell wall. They enter Gram-negative bacteria through outer membrane proteins (OMPs), also known as porins (Rao, 2012). After transversing the periplasmic space, carbapenems “permanently” acylate penicillin binding proteins (PBPs) which are enzymes (i.e., transglycolases, transpeptidases, and carboxypeptidases) that catalyze the formation of peptidoglycan in the cell wall of bacteria. Carbapenems act as

mechanism-based inhibitors of the peptidase domain of PBPs and can inhibit peptide crosslinking as well as other peptidase reactions. A key factor of the efficacy of carbapenems is their ability to bind to multiple different PBPs (Papp-Wallace *et al.*, 2011).

In general, imipenem, panipenem, and doripenem are potent antibiotics against Gram-positive bacteria (Rao, 2012). Meropenem, biapenem, ertapenem, and doripenem are slightly more effective against Gram-negative organisms (Rao, 2012). Carbapenems can also be combined with other antimicrobials to treat serious infections (Papp-Wallace *et al.*, 2011). Combination therapy is a subject of intense interest, since the emergence of MDR pathogens often requires us to treat patients with more than one antibiotic (Papp-Wallace *et al.*, 2011). Some combinations demonstrate positive effects, such as extending the spectrum or working additively or synergistically (Papp-Wallace *et al.*, 2011). Adverse effects include increased resistance to one of the drugs used in the combination, as well as a lack of synergy and strain dependence (Papp-Wallace *et al.*, 2011).

Nephrotoxicity, neurotoxicity, and immunomodulation have been reported with the use of carbapenems, and thus predisposing factors should be considered when administering any carbapenem (Papp-Wallace *et al.*, 2011). In addition, the use of carbapenems can alter the intestinal microflora and select for carbapenem-resistant isolates (Rao, 2012).

2.2.1. Mechanisms of resistance against carbapenems

Mechanisms of resistance to carbapenems include production of β-lactamases, efflux pumps, and mutations that alter the expression and/or function of porins and PBPs. Combinations of these mechanisms can cause high levels of resistance to carbapenems in bacteria such as *K. pneumoniae*, *P. aeruginosa*, and *A. baumannii* (Rao, 2012). Carbapenem resistance in Gram-positive cocci is typically due to the result of substitutions in amino acid sequences of PBPs or acquisition/ production of a new carbapenem-

resistant PBP (Papp-Wallace *et al.*, 2011). Carbapenem resistance in Gram-negative bacteria is often due to production of β-lactamases; expression of efflux pumps, as well as porin loss and alterations in PBP (Papp-Wallace *et al.*, 2011). Production of β-lactamases appears to be the most widespread cause of carbapenem resistance (Rao, 2012). In addition to carbapenemases, resistances to carbapenems can also occur due to over production of certain AmpC beta-lactamases (Rao, 2012).

2.3. Carbapenemases

Carbapenem hydrolyzing enzymes (also known as carbapenemases) are broadly divided into two types based on the reactive site of the enzymes; serine carbapenemases and metallo-β-lactamases (Rao, 2012). Carbapenemases represent the most versatile family of β-lactamases, with a breadth of spectrum unrivaled by other β-lactam-hydrolyzing enzymes, many of these enzymes recognize almost all hydrolyzable β-lactams, and most are resilient against inhibition by all commercially viable β-lactamase inhibitors (Queenan and Bush, 2007).

2.3.1. Classification

Classification of β-lactamases can be defined according to two properties, functional and molecular (Queenan and Bush, 2007).

2.3.1.1. Functional classification

The earlier method of classification of β-lactamase enzyme involved biochemical analysis of enzyme, determination of isoelectric point, determination of substrate hydrolysis, enzyme kinetics and inhibition profiles (Rao, 2012). This functional classification currently divide β-lactamases into four functional groups (1-3). The group 2 has several subgroups that are differentiated according to group-specific substrate or inhibitor profile. In this classification, carbapenemases fall under group 2f, 2df and 3 (Rao, 2012).

2.3.1.2. Molecular classification

Ambler and others have classified β-lactamases according to the amino acid sequences into four groups (A-D). Although this classification correlates well with the functional scheme, it lacks in the details concerning the enzymatic activity of the β-lactamase (Rao, 2012). Molecular classes A and D contain beta-lactamases with serine in their active site while group B contains metallo-beta-lactamases with zinc in their active sites. Group B enzymes require one or two Zn cations for activity and are subdivided into three groups, B1, B2, and B3, based on sequence alignments and structural analysis. Subclasses B1 and B3 have two zinc ions whereas subclass B2 has only one zinc ion. B2 enzymes are preferentially carbapenemases whereas B1 and B3 enzymes have broad hydrolysis spectrum (Rao, 2012).

2.3.1.2.1. Molecular Class A enzymes

Class A serine carbapenemases of functional group 2f have appeared sporadically in clinical isolates since their first discovery over 20 years ago (Queenan and Bush, 2007). These β-lactamases have been detected in *Enterobacter cloacae*, *Serratia marcescens* and *Klebsiella spp.* as single isolates or in small outbreaks (Queenan and Bush, 2007). Characteristic properties of these enzymes include presence of an active-site serine at position 70 and presence of a disulfide bond between Cys69 and Cys238 (changes the overall shape of the active site) (Rao, 2012). All have the ability to hydrolyze a broad variety of β-lactams, including carbapenems, cephalosporins, penicillins, and aztreonam, and all are inhibited by clavulanate and tazobactam (Queenan and Bush, 2007). Three major families of class A serine carbapenemases include the NMC/IMI, SME and KPC enzymes (Queenan and Bush, 2007). A fourth member of this class, the GES β-lactamases were originally considered as ESBL but are now classified as serine carbapenemases (Rao, 2012). Early class A carbapenemases were mostly chromosomal (SME, IMI/NMC) but recently

plasmid mediated carbapenemases (KPC, GES) are on the rise (Rao, 2012). SME (for "*Serratia marcescens* enzyme") was first detected in England from two *S. marcescens* isolates that were collected in 1982 (Queenan and Bush, 2007). The IMI (for "imipenem-hydrolyzing β-lactamase") and NMC-A (for "not metalloenzyme carbapenemase") enzymes have been detected in rare clinical isolates of *E. cloacae* in the United States, France, and Argentina (Queenan and Bush, 2007). GES (for Guiana extended spectrum): this enzyme was first observed in a *K. pneumoniae* isolate from French Guiana in 1998 (Walther-Rasmussen and Høiby, 2007). The enzymes of the GES family differ from each other by 1-4 amino acid substitutions (Rao, 2012).

Two characteristics separate the KPC (for "*Klebsiella pneumoniae* carbapenemase") carbapenemases from the other functional group 2f enzymes (Queenan and Bush, 2007). First, the KPC enzymes are found on transferable plasmids; second, their substrate hydrolysis spectrum includes the aminothiazoleoxime cephalosporins, such as cefotaxime (Queenan and Bush, 2007). Currently there are 12 known KPC enzymes. Although predominantly seen in *K. pneumoniae* isolates, they have been observed in *Salmonella enterica*, *K. oxytoca*, *E. cloacae*, *E. coli*, and *P. aeruginosa* (Rao, 2012). The first case of *K. pneumoniae* expressing a carbapenemase was identified in North Carolina in 1996, and thus, this type of carbapenemase is called KPC (Paczosa and Mecsas, 2016). These enzymes confer resistance to all penicillins, cephalosporins, aztreonam and imipenem but remain susceptible to inhibition by clavulanic acid (Rao, 2012). Even though they can hydrolyze carbapenems, the resistance is not apparent and in many cases, the MIC values are less than the MIC breakpoints. This has resulted in under-detection of several KPC producers (Rao, 2012).

2.3.1.2.2. Molecular Class B enzymes

The MBLs are the most molecularly diverse carbapenemases and the greatest clinical threat (Walsh, 2010). This class of β-lactamases is characterized by the ability to hydrolyze carbapenems and by its resistance to the commercially available β-lactamase inhibitors but susceptibility to inhibition by metal ion chelators (EDTA) (Queenan and Bush, 2007). Inhibition by EDTA can be reversed by adding Zn2+ ions (Rao, 2012). These enzymes contains at least one Zn2+ ion in their active sites that is responsible for hydrolysis when interacted with β-lactam; B1 and B3 enzymes contain two Zn2+ ions where B2 enzymes contains only one Zn2+ ion (Rao, 2012; Queenan and Bush, 2007). The substrate spectrum is quite broad; in addition to the carbapenems, most of these enzymes hydrolyze cephalosporins and penicillins but lack the ability to hydrolyze aztreonam (Queenan and Bush, 2007).

The first metallo-β-lactamases were detected in environmental and opportunistic pathogenic bacteria (*Bacillus cereus* (BCI, BCII), *Aeromonas* spp (CphA) and *Stenotrophomonas maltophilia* (L1) as chromosomally encoded enzymes (Rao, 2012; Queenan and Bush, 2007). These bacteria also produced additional serine beta-lactamases and both the enzymes were inducible by exposure to β-lactams (Rao, 2012). Chromosomal MBL was also found in few strains of *Bacteroides fragilis* (CcrA) and are not easily transferred (Rao, 2012; Queenan and Bush, 2007).

Plasmid mediated MBLs have now attained significance because of its global spread (Rao, 2012). These include *bla*IMP, *bla*VIM, *bla*GIM and *bla*SIM (Rao, 2012). Most MBL genes (including *VIM*, *IMP*) are found as gene cassettes on class 1 integrons; few *IMP* genes are located on class 3 integrons (Walsh *et al*., 2005). However, *SPM-1* genes are not located on integrons or transposons (Rao, 2012); it was associated with common

regions that contain a new type of transposable structure with potential recombinase and promoter sequences (Queenan and Bush, 2007). Mechanism of carbapenem hydrolysis is complex and varies from one MBL to another (Rao, 2012). Across the groups, MBLs share only 25% amino acid homology, but all share the same unique αββα fold (Walsh *et al*., 2005).

IMP (for active on imipenem): Transferable carbapenem resistance was first detected in a *P. aeruginosa* isolate from Japan in 1990 (Walsh, 2010). They were subsequently reported in four *S. marcescens* isolates in Japan (Rao, 2012). This was followed by another report describing such a resistance in an isolate of *B. fragilis* (Rao, 2012). IMP-2 was observed in A. baumannii in Italy (Walsh *et al*., 2005). Despite there being 27 IMP-type derivatives they have never dominated the MBL global scene, with only sporadic outbreaks (Walsh, 2010). While these are more commonly seen in *P. aeruginosa* and *A. baumannii* isolates, they have been reported from most *Enterobacteriaceae* members (Rao, 2012).

VIM (for Verona integron encoded metallo-β-lactamase): This class 1 integron associated MBL was first observed in a *P. aeruginosa* isolate from Verona, Italy in 1997 (Rao, 2012), which was resistant to a series of β-lactams, including piperacillin, ceftazidime, imipenem, and aztreonam (Walsh *et al*., 2005). It is most closely related in BCII with only 39% amino acid homology (Rao, 2012). The VIM family currently consists of 14 members, with occurrences mostly in *P. aeruginosa* within multiple-integron cassette structures (Queenan and Bush, 2007). VIM-2 is the most dominant MBL across Europe and it has been detected in more than 23 species across 40 countries (Rao, 2012).

SPM-1 (for Sao Paulo metallo-β-lactamases) was first isolated in Brazil in 1997 from a *P. aeruginosa* clinical isolate that was resistant to all available antibiotics except colistin (Hong *et al.*, 2015). It was analyzed as part of

the SENTRY surveillance program and shown to contain a novel gene, designated *bla*SPM-1 (Walsh *et al*., 2005). The genetic context of *bla*SPM-1 is unique in that it is immediately associated with common region elements and not with transposons or integrons (Walsh *et al*., 2005).

BlaSPM-1 is a part of mobile pathogenicity island located on a plasmid (Rao, 2012). Generally, SPM-1 binds cephalosporins more tightly than penicillins (Walsh *et al*., 2005). Like IMP-1 and VIM-1, SPM-1 does not hydrolyze clavulanic acid or aztreonam particularly efficiently, which can act as competitive inhibitors (Walsh *et al*., 2005).

GIM-1 (for German imipenemase) was first isolated in 2002 from five *P. aeruginosa* isolates were recovered from different patients from a medical site in Dusseldorf, Germany, and shown to possess a novel class B β-lactamase (Walsh *et al*., 2005). GIM-1 had no clear preference for any substrate and did not hydrolyze azlocillin, aztreonam, and the serine-β-lactamase inhibitors (Rao, 2012). *BlaGIM-1* was found on class 1 integron in a 22-kb nontransferable plasmid (Rao, 2012).

SIM-1 (for Seoul imipenemase) was first isolated from *P. aeruginosa* and *A. baumannii* isolates during a large-scale screen of imipenem resistant isolates in Seoul (Rao, 2012). All SIM-1-producing isolates exhibited relatively low imipenem and meropenem MICs (8 to 16 μg/ml) and had a multidrug resistance phenotype (Rao, 2012).

NDM-1 (for New Delhi metallo-β-lactamase) was first reported in 2009 from a *K. pneumoniae* isolate obtained from a Swedish patient of Indian origin, who had received medical treatment in India (Rao, 2012). It is possibly the most worrying development since Fleming gave the world penicillin in 1929 (Walsh, 2010). NDM-1 shares very little identity with other MBLs, with the most similar MBLs being VIM-1/VIM-2, with which it has only 32.4% identity. Compared to VIM-2, NDM-1 displays tighter binding to most cephalosporins, in particular, cefuroxime, cefotaxime, and

cephalothin and penicillins (Dortet *et al*., 2014a). However, NDM-1 does not bind to the carbapenems as tightly as IMP-1 or VIM-2 (Rao, 2012). It is noteworthy that a quite systematic association with other antibiotic resistance determinants is observed in almost all NDM producers (*Enterobacteriaceae*, *Acinetobacter,* and *Pseudomonas*) (Dortet *et al*., 2014a). Those associated resistance determinants are AmpC cephalosporinases, clavulanic acid inhibited expanded-spectrum *β*-lactamases (ESBLs), other types of carbapenemases (OXA-48-, VIM-, and KPC-types), and resistance to aminoglycosides (16S RNA methylases), to quinolones (Qnr), to macrolides (esterases), to rifampicin (rifampicin-modifying enzymes), to chloramphenicol, and to sulfamethoxazole (Dortet *et al*., 2014a). Most other NDM-1 enzymes are susceptible to aztreonam, colistin and tigecycline (Rao, 2012). Currently, there are six known NDM types. Since its detection, NDM-1 producing *E. coli* and *K. pneumoniae* have been detected in several parts of the world (Rao, 2012).

2.3.1.2.3. Molecular Class D enzymes

Class D enzymes are OXA (for oxacillin hydrolyzing) enzymes, they commonly hydrolyse the isoxazolylpenicillin oxacillin much faster than classical penicillins (Walther-Rasmussen and Høiby, 2006). Currently there are 239 OXA enzymes, of which at least 9 are ESBLs and at least 37 are carbapenemases that are classified as subgroup 2df in the functional classification (Rao, 2012). These are poorly inhibited by clavulanic acid and EDTA and are known to have large amount of variability in amino acid sequences (Rao, 2012). In 1980 isolates of *Acinetobacter baumannii* that were resistant to the carbapenems emerged, manifested by plasmid-encoded β-lactamases (OXA-23, OXA-40, and OXA-58) categorized as OXA enzymes because of their sequence similarity to earlier OXA β-lactamases (Evans and Amyes, 2014). It was soon found that every *A. baumannii* strain possessed a chromosomally encoded OXA β-lactamase

(OXA-51-like), some of which could confer resistance to carbapenems when the genetic environment around the gene promoted its expression (Evans and Amyes, 2014). Based on sequence homology alone, class D carbapenemases can be divided into the following clusters: OXA-23 (includes OXA-27 and OXA-49); OXA-24 (includes OXA-25, OXA-26 and OXA-40) and OXA-58 (Walsh, 2010). Other minor groups, such as OXA-72 and OXA-143, are now emerging and becoming increasingly clinically important (Walsh, 2010). Among them, only several subgroups such as OXA-23, OXA-48, OXA-51 and OXA-58 are reported in *K. pneumoniae* (Lee *et al.*, 2016).

The most recent and worrying development is the rapid rise in OXA-48, particularly in *K. pneumoniae* (Walsh, 2010). Analysis of the enzyme kinetics of OXA-48 showed that the enzyme has a low level of hydrolytic activity against the carbapenems (Evans and Amyes, 2014). Ability to hydrolyze ceftazidime and aztreonam, which is not detectable in OXA-48 and an increased ability to hydrolyze cefotaxime and cefepime over OXA-48, making this enzyme more similar to an ESBL than to a carbapenem-hydrolyzing oxacillinase (Evans and Amyes, 2014). OXA-48 is not a strong carbapenemase and, rather like many carbapenemases evinced in *Enterobacteriaceae*, relies upon other synergistic mechanisms to mediate resistance against carbapenems (e.g. outer membrane mutations and efflux pumps) (Walsh, 2010).

2.3.2. Laboratory detection of carbapenemases

Detection of carbapenemase-producing (CP) organisms in the clinical microbiology laboratory is a matter of major importance for the choice of appropriate therapeutic schemes and the implementation of infection control measures (Miriagou *et al.*, 2010). However, it poses a number of difficulties, as it cannot be based simply on the resistance profile, and as the relevant methodology based on specific tests has not yet been well

standardized (Miriagou *et al.*, 2010). Screening the isolates against cephalosporins and carbapenems gives indication of carbapenemase presence (Rao, 2012). Phenotypic and molecular-based techniques are able to identify these carbapenemase producers, although with variable efficiencies (Nordmann *et al.*, 2012a). The detection of carriers still relies mostly on the use of screening culture media (Nordmann *et al.*, 2012a).

2.3.2.1. Screening tests

The prevention of spread of carbapenemase producers relies on early and accurate detection of carriers in hospital units or on admission/discharge either to the hospital or to a specific unit (Nordmann *et al.*, 2012a). Screening should include as a minimum 'at-risk' patients, such as those in intensive care units, transplant recipients and the immunocompromised, and those transferred from any foreign hospital (unknown prevalence of carbapenemase producer carriage) or from non-foreign hospitals but known to face a high risk of carriage of carbapenemase producers (Nordmann and Poirel, 2012).

2.3.2.1.1. Screen agars

The first marketed screening medium was the CHROMagar KPC medium, which contains a carbapenem (CHROMagar, Paris, France) (Nordmann and Poirel, 2012). It detects carbapenem-resistant bacteria only if they exhibit high-level resistance to carbapenems (Nordmann *et al.*, 2012a). Its main disadvantage therefore remains its lack of sensitivity since it does not detect carbapenemase producers exhibiting a low level of carbapenem resistance, as observed for several MBL or OXA-48 producers (Nordmann *et al.*, 2012a). The second screening medium also contains a carbapenem (CRE Brilliance, Thermo Fisher Scientific,UK) (Nordmann and Poirel, 2012). It detects KPC and MBL producers well, and most but not all OXA-48 producers (Nordmann and Poirel, 2012). Finally, a novel and patented medium (SUPERCARBA medium) containing cloxacillin, zinc and a

carbapenem molecule that has improved sensitivity and specificity for detecting all types of carbapenemase producer (including OXA-48 producers) (Nordmann *et al.*, 2012b).

2.3.2.1.2. Susceptibility breakpoints

Clinical and Laboratory Standards Institute (CLSI) recommends that zone diameter of 16–21 mm for ertapenem and 14–21 mm for meropenem may be considered indicative of carbapenemase production despite the fact that they are in the current susceptible interpretive categories (Rao, 2012). Similarly, a MIC of 2-4 µg/ml of ertapenem and 2-8 µg/ml for imipenem and meropenem should indicate possible carbapenemase production (Rao, 2012). Some isolates that produce carbapenemases are categorised as susceptible with these breakpoints and should be reported as tested, i.e. the presence or absence of a carbapenemase does not in itself influence the categorization of susceptibility (Doyle *et al.*, 2012). Nordmann and Poirel, (2012), actually disagree with CLSI guidelines, for the following reasons: (i) susceptibility to carbapenems is observed for several carbapenemase producers; and (ii) there is a paucity of clinical successes of carbapenem-containing regimens for treating infections due to carbapenemase producers that are susceptible to carbapenems in vitro. Based on their own experience, they propose that, as a minimum, detection of carbapenemase activity should be performed on enterobacterial isolates exhibiting MIC values of ertapenem ≥0.5 mg/L or of imipenem or meropenem ≥1 mg/L, and also on any enterobacterial isolate that exhibits even a slight decrease in susceptibility to carbapenems compared with a wild-type phenotype. Most serine carbapenemases are identified by reduced or full susceptibility to extended-spectrum cephalosporins, aztreonam and carbapenems but inhibition by clavulanic acid and tazobactam (Rao, 2012). MBLs are identified by reduced susceptibility to extended spectrum cephalosporin

and carbapenems but susceptibility to aztreonam and EDTA and this susceptibility is reversed by the addition of Mg2+ (Rao, 2012).

Ertapenem was a more sensitive indicator of KPC resistance than meropenem and imipenem independently of the method used (Anderson *et al.*, 2007). Disc diffusion lacks discrimination and MIC determination by microbroth dilution, agar dilution, E-test strips and automated systems such as Microscan Walkway, Vitek, Vitek2 and BD Pheonix Sensititre is preferred (Rao, 2012). Increased carbapenem MICs in *Enterobacteriaceae* may also result from high expression of AmpC or CTX-M ESBLs in combination with porin alterations (Rao, 2012). Actually, after this screening step, carbapenemase producers must then be identified with phenotypic and molecular techniques (Nordmann *et al.*, 2012a).

2.3.2.2. Phenotypic detection of carbapenemase activity

2.3.2.2.1. Modified Hodge test (MHT)

This test detects carbapenemase production in isolates of *Enterobacteriaceae* but does not differentiate between serine and metallo-beta-lactamases (Rao, 2012). It is based on the inactivation of a carbapenem by carbapenemase-producing strains that enables a carbapenem-susceptible indicator strain to extend growth toward a carbapenem-containing disk, along the streak of inoculum of the tested strain (Girlich *et al.*, 2011). The 1 in 10 dilution of 0.5 McFarland standard suspension of the susceptible indicator is inoculated on Mueller Hinton agar to produce a semi-confluent growth (Rao, 2012). A carbapenem disc (imipenem or ertapenem 10µg) is placed in the center of the plate (Rao, 2012). The MHT technique is highly sensitive for detecting class A, B, and D carbapenemases after addition of zinc in the culture medium (Girlich *et al.*, 2011). However, the limitations of the MHT in terms of clinical performance remain its lack of specificity and the delay in obtaining the results (24 to 48 h) after isolation of a bacterial colony (Girlich *et al.*, 2011).

2.3.2.2.2. Synergy test

Synergy test is based on in vitro inhibition of carbapenemase activity by addition of an inhibitor specific for a class of carbapenemases (Rao, 2012). For detection of class A carbapenemases, the inhibitor used is tazobactam, clavulanic acid or boronic acid (3-aminophenylboronic acid) (Rao, 2012; Nordmann and Poirel, 2012) and for detection of class B metallo-carbapenemases, ethylene diamine tetra-acetic acid (EDTA), 2-mercaptopropionic acid, sodium mercaptoacetic acid or dipicolinic acid can be used as an inhibitor (Rao, 2012).

2.3.2.2.3. Detection of carbapenemase activity

The carbapenemase activities of cell sonicates from several steps, including an 18 h culture (which can be shortened in some cases to 8 h) then a protein extraction step and measurement of imipenem hydrolysis using a UV spectrophotometer (Nordmann and Poirel, 2012). The assays are performed with or without EDTA (25 mM) to examine the inhibition of carbapenemase activity. This method does not significantly distinguish between the different carbapenemase types (Rao, 2012). Large amounts of extracts are used for isolates that produce very weak carbapenemases (Rao, 2012).

2.3.2.2.4. Carba NP test

The most important and recent development for the accurate identification of carbapenemase-producing *Enterobacteriaceae* is the Carba NP test (Nordmann and Poirel, 2012). It is based on *in vitro* hydrolysis of imipenem by a bacterial lysate, which is detected by the changes in pH values using the indicator phenol red (red to yellow/orange) (Tijet *et al.*, 2013). This test is 100% sensitive and specific, as are molecular techniques (Nordmann and Poirel, 2012). It detects not only all known carbapenemases (belonging to Ambler A, B and D classes) in *Enterobacteriaceae* but should also identify virtually any new emerging

carbapenemase, in contrast to molecular techniques (Nordmann and Poirel, 2012). Overall, the Carba NP method was easy to perform, inexpensive and, in most cases, easy to interpret (Tijet *et al.*, 2013). This technique will soon become a reference technique since it fulfils the clinical requirement of a rapid and low-cost identification method for carbapenemase-producing *Enterobacteriaceae* (Nordmann and Poirel, 2012).

2.3.2.3. Molecular detection of carbapenemases genes

Molecular techniques remain the gold standard for the precise identification of carbapenemase genes (Nordmann *et al.*, 2012a). 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) (Nordmann and Poirel, 2012). They are either single or multiplex PCR techniques (Nordmann *et al.*, 2012a). A PCR technique performed directly on colonies can give results within 4–6 h (or less when using real-time PCR technology) with excellent sensitivity and specificity (Nordmann and Poirel, 2012). The main disadvantages of the molecular based technologies are their cost, the requirement for trained microbiologists and the inability to detect novel unidentified genes (Nordmann *et al.*, 2012a). Sequencing of the genes is interesting mostly for research and epidemiological purposes (Nordmann and Poirel, 2012). Precise identification of the type of carbapenemase is not actually needed for treating patients or for preventing outbreaks (Nordmann *et al.*, 2012a). These molecular techniques may be mostly used in reference laboratories (Nordmann and Poirel, 2012).

The DNA microarray may be more useful for epidemiological purposes or for infection control studies in case of high numbers of isolates have to be rapidly characterized (Dortet *et al.*, 2014b). Additionally, this procedure may also detect potential new carbapenemases (Dortet *et al.*, 2014b).

Colony blot hybridizations using labeled probes have been used to efficiently screen large numbers of clinical isolates for carbapenemase genes (Rao, 2012). Southern blot hybridization too has been used to determine whether the carbapenemase gene resides on a plasmid or the chromosome (Rao, 2012).

2.3.3. Epidemiology of Carbapenemase-Producing *Klebsiella pneumoniae*

International travel is an important risk factor for colonization or infection with antibiotic-resistant organisms, the risk being highest among travelers to India, the Middle East and Africa (Rao, 2012). The epidemiology of *K. pneumoniae* producing KPCs varies geographically (Lee *et al.*, 2016). The endemic spread of these bacteria has been reported in the USA, China, Italy, Poland, Greece, Brazil, Argentina, Colombia and Taiwan (Lee *et al.*, 2016). Sporadic spread of KPC-producing *K. pneumoniae* has also been observed in many European countries including Spain, France, Germany, the Netherlands, the UK, Ireland, Belgium, Sweden, and Finland, and in several countries in the Asia-Pacific region, including India, South Korea and Australia (Lee *et al.*, 2016). Aside from KPC-type carbapenemases, other class A carbapenemases such as GES-2, GES-4, GES-5, GES-6, GES-11, GES-14, GES-18, SFC-1, SHV-38, NMC-A, SME-1 and IMI-type enzymes were rarely found in *K. pneumoniae* (Lee *et al*., 2016). NDM is one of the most clinically significant class B carbapenemases and it was considered endemic in the Indian subcontinent, including India, Pakistan, and Bangladesh (Lee *et al.*, 2016). The European epidemiology changed between 2013 and 2015 for NDM-producing *Enterobacteriaceae* with several countries reporting regional or inter-regional spread but with no endemic situation (Girmenia *et al.*, 2016). Single or sporadic hospital outbreaks were reported from many countries in France, Italy, Spain, Morocco and Tunisia in a patient transferred from Libya, and Egypt

(Girmenia *et al.*, 2016). Also in Turkey, South Africa, Singapore, Saudi Arabia, Oman, United Arab Emirates, China, Japan, Taiwan, South Korea and Australia (Lee *et al.*, 2016). Comparative analyses of the conserved NDM-1-encoding region among different plasmids from *K. pneumoniae* and *E. coli* suggested that the transposable elements and two unknown inverted repeat-associated elements flanking the NDM-1-encoding region aided the spreading of this resistance determinant (Lee *et al.*, 2016). Coexistence of NDMs and other carbapenemases in *K. pneumoniae* has also been reported worldwide, in Turkey (NDM- 1/OXA-48), Pakistan (NDM-1/KPC- 2), Switzerland (NDM-1/OXA-48), United Arab Emirates (NDM-1/OXA-48-like), Morocco (NDM-1/OXA-48) and the USA (NDM-1/OXA-232) (Lee *et al.*, 2016). Besides NDM-type carbapenemases, the IMP and VIM groups have also been detected worldwide in *K. pneumoniae*, but other carbapenemases, such as GIM-1, KHM-1 and SPM-1 have been not found in *K. pneumoniae* (Lee *et al.*, 2016). IMP producing *K. pneumoniae* is dominant in the Asian continent and Australia and has been only sporadically detected in Europe and the Mediterranean area (Girmenia *et al.*, 2016) but were rarely reported in other regions, such as America and Africa (Lee *et al.*, 2016). The VIM group is one of the most commonly reported carbapenemases worldwide when considering all bacteria species (the VIM groups have been mainly identified in *P. aeruginosa* (Lee *et al*., 2016). VIM-producing *K. pneumoniae* have spread in Europe and Asia but were rarely reported in other regions such as America and Africa (Lee *et al*., 2016). Single reports and local outbreaks of VIM- type producers have been reported in countries of the Mediterranean area, such as France, Spain, Morocco, Egypt, Algeria and Tunisia (Girmenia *et al.*, 2016). Class D OXA-48 was first identified in *K. pneumoniae* in Turkey in 2003 (Lee *et al*., 2016). Since 2003, the endemic spread of these bacteria has been reported in countries such as

Turkey, Morocco, Libya, Egypt, Tunisia, and India (Lee *et al*., 2016). The sporadic spread has been reported in France, Spain, Italy, Belgium, the Netherlands, the UK, Germany, Switzerland, Argentina, Lebanon, Kuwait, Saudi Arabia and Japan (Lee *et al*., 2016).

2.3.4. Treatment options against Carbapenemase-Producing *Klebsiella pneumoniae*

Carbapenem resistance in Gram-negative pathogens is dramatically limiting treatment options while for Gram-positives there are still reliable alternatives to carbapenems (e.g. glycopeptides, daptomycin) (Meletis, 2015). Based on the laboratory's antimicrobial susceptibility report, clinicians have to choose between the following schemes: (a) monotherapy using one of the possibly still active in vitro agents (these may be colistin, gentamycin, tigecycline and fosfomycin); (b) combination therapy without a carbapenem; (c) combination therapy with two or more drugs including at least one carbapenem, preferably when the carbapenem's MIC ≤ 4 mg/L (Meletis, 2015). KPC-producing *K. pneumoniae* are usually resistant to all β-lactam antibiotics, but temocillin can be active against some KPC-producing *K. pneumoniae*, particularly in the case of lower urinary tract infections (Lee *et al.*, 2016). Combination of tigecycline, colistin, and meropenem, was strongly effective in the treatment of KPC-producing *K. pneumoniae*, including colistin- resistant isolates (Lee *et al.*, 2016). Aztreonam is stable to metallo-carbapenemases, including IMP, VIM and NDM (Rao, 2012). However, in isolates that also co-produce AmpC or ESBL, aztreonam is ineffective (Rao, 2012). When double-and triple-antibiotic combinations of aztreonam, ciprofloxacin, colistin, daptomycin, fosfomycin, meropenem, rifampin, telavancin, tigecycline and vancomycin were used in patients infected with two NDM-producing *K. pneumoniae* strains susceptible to colistin, the combination of rifampin-meropenem-colistin was the most effective regimen against these strains

(Lee *et al.*, 2016). Ceftazidime, cefotaxime and aztreonam remain active against *Enterobacteriaceae* with OXA-48 unless these also have AmpC or an ESBL (Rao, 2012). The combination of fosfomycin with imipenem, meropenem and tigecycline was also synergistic against OXA48- positive *K. pneumoniae* strains (Lee *et al.*, 2016). A new compound, ceftazidime-avibactam (CAZ-AVI) has been made available recently (Meletis, 2015). This drug is a combination of ceftazidime with a novel beta-lactamase inhibitor able to inhibit ESBLs, AmpC and class A carbapenemases including KPCs but not MBLs (Meletis, 2015). Recently, combination of CAZ-AVI with ertapenem is a novel, safe, and effective antibiotic option for the treatment of serious infections with CRKP in critically ill patients who have limited or no alternative treatment options (Camargo *et al.*, 2015).

CHAPTER THREE

MATERIALS AND METHODS

CHAPTER THREE
MATERIALS AND METHODS

3.1. Study design

This study is a descriptive-cross sectional laboratory based study.

3.2. Study area

This study was conducted in Khartoum State. *K. pneumoniae* isolates were collected from the following hospitals; Royal Care International Hospital, Al-Baraha Hospital, Soba University Hospital, Al Amal National Hospital, East Nile Model Hospital, Omdurman Medical Military Hospital and Al Ribat Hospital. The study was performed at Sudan University of Science and Technology, College of Medical Laboratory Science.

3.3. Ethical considerations

The study was approved by College of Medical Laboratory Science, Sudan University of Science and Technology.

3.4. Study duration

The study was conducted during the period from March to November 2018.

3.5. Sample

The isolates of *K. pneumoniae* were obtained from laboratory of hospitals, which previously isolated clinical specimens urine, blood, wound swabs and sputum.

3.6. Sampling

3.6.1. Sample type

Non-probability convenience sampling technique.

3.6.2. Sample size

The total sample size was 60 isolates.

3.7. Data collection

The data were collected by non-self-constructing information list from patients.

3.8. Laboratory Methods

3.8.1. Samples preservation

The isolated organisms were stored in Research Lab in Sudan University of Science and Technology, in 20% Glycerol peptone media (Appendix 2.1).

3.8.2. Subculture

Few drops of glycerol peptone media containing organism were inoculated in MacConkey's agar (Appendix 2.2) and incubated overnight at 37°C aerobically.

3.8.3. Susceptibility testing

Kirby Bauer CLSI modified disc diffusion technique

Mueller Hinton agar (Appendix 2.3) was used for antimicrobial susceptibility testing. By using a sterile wireloop, 3-5 colonies of similar appearance of the tested organism were picked and emulsified in 3-4 ml of sterile physiological saline, and were matched with 0.5% McFarland standard (Appendix 2.4) in a good light. Then use sterile swab to inoculate a plate of Mueller Hinton agar, excess fluid was removed and swab was streaked evenly over the surface of the medium in three directions to ensure even distribution, and then allowed 3-5 minute to dry. Using sterile forceps, needle mounted in a holder or a multidisc dispenser, the appropriate antimicrobial discs were placed and distributed evenly on the inoculated plate. After incubation at 37˚C overnight, the zones diameters were recorded and interpreted according to CLSI guidelines.

Ceftazidime (30mcg), ciprofloxacin (5mcg), chloramphenicol (30mcg)، gentamicin (10mcg) and imipenem (10mcg) were used in this study with the control strain *Escherichia coli ATCC 25922*.

3.8.4. Identification of *K. pneumoniae* using PCR based method targeting 16S rRNA gene and detection of carbapenem resistance genes of *K. pneumoniae*

3.8.4.1. DNA extraction

DNA extracted using guanidine chloride method. All scraped colonies were washed with normal saline (NS), followed by the addition of 2mL lysis buffer, 5μL proteinase K, 1mL guanidine chloride, and 300μL ammonium acetate. Suspensions were incubated overnight at 37 °C; on the next day, 2mL of chilled chloroform was added. After centrifugation, the clear upper layer was collected in a new tube and 10 ml of cold absolute ethanol was added to enhance precipitation of DNA. The pellet was washed with 70% ethanol and allowed to dry. The pellet was suspended with nuclease-free water and quantified using gel electrophoresis. The DNA samples were stored at−20∘C until used for PCR (Sabeel *et al.*, 2017).

3.8.4.2. PCR technique

3.8.4.2.1. Primers

Primers targeting the 16S rRNA gene was used to detect *K. pneumoniae.* Another primer set were used to detect carbapenem resistant genes of *K. pneumoniae* (Table 3.1). The primers were dissolved according to manufacture guidelines to prepare 10pmol /µL.

Table 3.1 Primers sequences and amplicons size used for identification of *K. pneumoniae* using PCR based method targeting 16S rRNA gene and detection of carbapenem resistance genes of *K. pneumoniae*

Primer name	DNA sequence (5' to 3')	Amplicons size (bp)
16S rRNA	Forward: ATTTGAAGAGGTTGCAAACGAT Reverse: TTCACTCTGAATTTTCTTGTGTTC	130
KPC	Forward: CATTCAAGGGCTTTCTTGCTGC Reverse: ACGACGGCATAGTCATTTGC	498

NDM	Forward: GGTTTGGCGATCTGGTTTTC Reverse: CGGAATGGCTCATCACGATC	521
IMP	Forward: TTGACACTCCATTTACAG Reverse: GATTGAGAATTAAGCCACTCT	232
OXA-48	Forward: GCTTGATCGCCCTCGATT Reverse: GATTTGCTCCGTGGCCGAAA	281
SPM	Forward: AAAATCTGGGTACGCAAACG Reverse: ACATTATCCGCTGGAACAGG	624

3.8.4.2.2. Preparation of 10X Tris Base EDTA (TBE) buffer

Amount of 48.4g of Tris base was added to 55g of boric acid and 7.44g of EDTA and then dissolved into 1L of D.W.

3.8.4.2.3. Preparation of 1X TBE buffer

10ml of 10X were added to 90ml D.W and mixed.

3.8.4.2.4. Preparation of ethidium bromide

10mg of ethidium bromide were dissolved into 0.5ml D.W and kept into brown bottle.

3.8.4.2.5. Preparation of agarose gel

Amount of 1.5 g of agarose powder were dissolved into 100 ml of 1X TBE using microwave for 30s. The mixture had been cooled and then 2µl of ethidium bromide was added، mixed well and poured in a casting tray that had been taped up appropriately and equipped with spacers and stable comb to form wells. Any bubbles were removed and the gel allowed setting at room temperature, after solidification, the comb and the spacers were gently removed.

3.8.4.2.6. Master Mix

Maxime PCR PreMix kit (iNtRON Biotechnology, Seongnam, Korea).

3.8.4.2.7. Preparation of reaction mixtures

3.8.4.2.7.1. Reaction mixture of 16S rRNA gene

PCR was carried out in a 20 µl volume using the Maxime PCR PreMix kit (iNtRON Biotechnology, Seongnam, Korea), premix were dissolved by 13 µl of D.W, transferred into 0.5 ml PCR tube, 1 µl of forward primer, 1 µl of reverse primer and 5 µl of DNA were added.

3.8.4.2.7.2. Reaction mixture for carbapenem resistance genes

Multiplex PCR was carried out in a 20µl volume using the Maxime PCR PreMix kit (iNtRON Biotechnology, Seongnam, Korea), premix were dissolved by 16 µl of D.W, transferred in to 0.5 ml PCR tube, for each gene 0.4 µl of forward primer, 0.4 µl of reverse primer, 2 µl of DNA were added.

3.8.4.2.8. Protocols used for amplification

3.8.4.2.8.1. Protocol used for amplification of 16S rRNA gene

The PCR was done by using a thermocycler (Convergys, Germany) with the following conditions: initial activation at 95°C for 5 minutes, followed by 35 cycles at 94°C for 30s, 58°C for 90s, and 72°C for 90s, and a final extension at 72°C for 10 minutes.

3.8.4.2.8.2. Protocol used for amplification of *NDM*, *IMP* and *SPM* genes

The PCR was done by using a thermocycler (Convergys, Germany) with the following conditions: initial activation at 94°C for 2 minutes, followed by 35 cycles at 94°C for 20s, 56°C for 10s, and 72°C for 20s, and a final extension at 72°C for 5 minutes.

3.8.4.2.8.3. Protocol used for amplification of *KPC* and *OXA-48* genes

The PCR was done by using a thermocycler (Convergys, Germany) with the following conditions: initial activation at 94°C for 5 minutes, followed by 35 cycles at 94°C for 45s, 52°C for 1 minute, and 72°C for 1 minute, and a final extension at 72°C for 10 minutes.

3.8.4.2.9. Visualization of PCR product

The amplicons were separated at 50V for 1hr in a 1.5% (wt/vol) agarose gel containing ethidium bromide, bands were visualized under U.V transilluminator (UVitec–UK) to detect the specific amplified products by comparing with 100bp standard ladder (INtRON biotechnology. Korea).

3.9. Data analysis and presentation

The data obtained were analyzed and presented using Statistical Package for Social Science (SPSS) computer software version 16.0 for Windows. Significance of differences was determined using Chi-square test. Statistical significance was set at $P < 0.05$.

CHAPTER FOUR

RESULTS

CHAPTER FOUR

RESULTS

4.1. Gender and age groups

Sixty isolates of *K. pneumoniae* were collected from different hospitals in Khartoum State. Twenty-seven (45%) were females and thirty-three (55%) were males, they were divided into two age groups (adults and infants) (Figure 4.1).

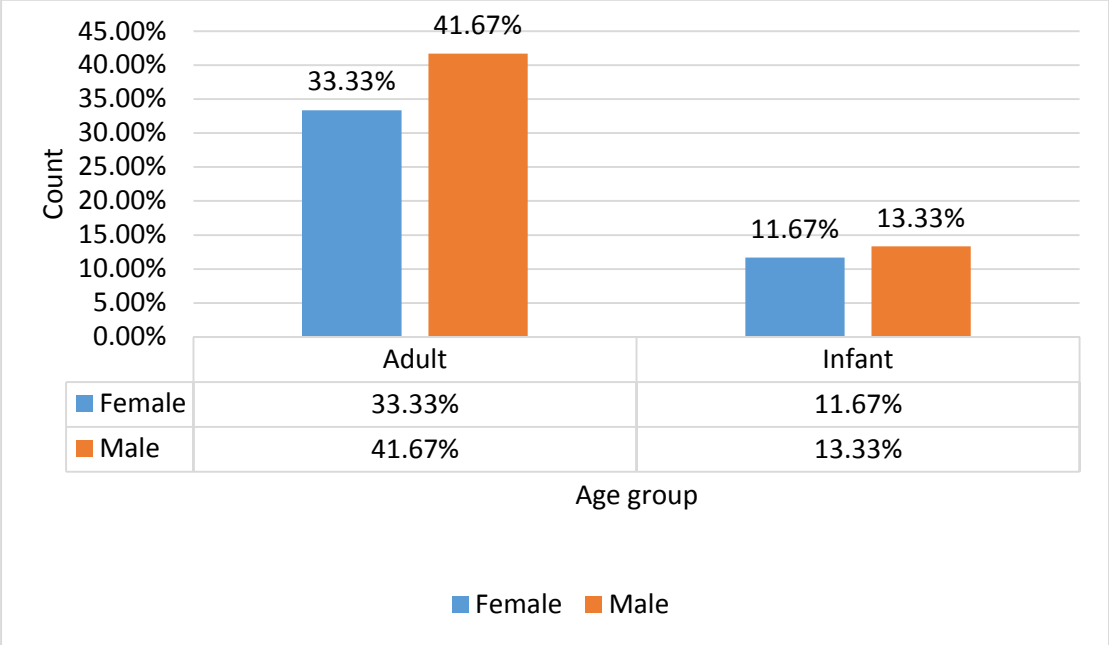

Figure 4.1 Distribution of patients according to gender and age groups

4.2. Distribution of specimen types

Out of sixty specimens, 37 (61. 7%) were urine, 14 (23. 3%) were blood، 5 (8. 3%) were wound swab and 4 (6. 7%) were sputum (Figure 4.2).

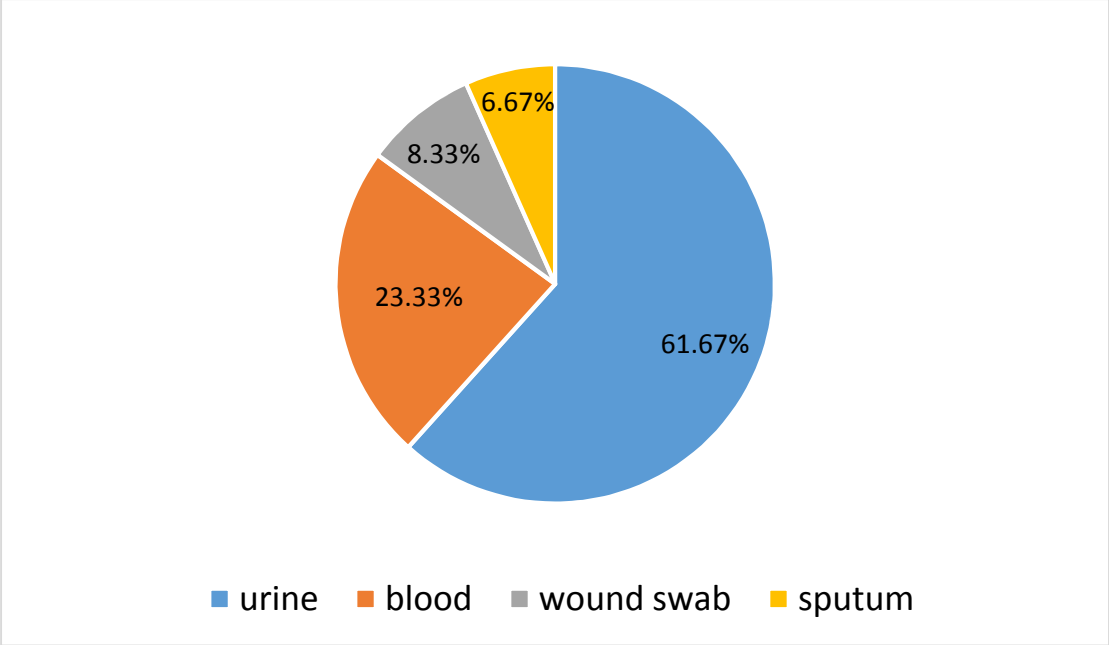

Figure 4.2 Distribution of bacteria according to the type of specimens

4.3. Growth of bacteria on MacConkey's Agar

Sixty isolates of *K. pneumoniae* were inoculated in MacConkey's agar medium. After overnight incubation at 37°C, the bacteria fermented lactose and produced smooth mucoid pink colonies (Appendix 1.1).

4.4. Results of Antibiotics susceptibility test

Out of sixty isolates, eighteen (30%) were sensitive to ceftazidime and 42(70%) were resistant. 42(70%) were sensitive to ciprofloxacin and 18(30%) were resistant. 37(61.7%) were sensitive to chloramphenicol and 23(38.3%) were resistant. 36(60%) were sensitive to gentamicin and 24(40%) were resistant. 55(91.7%) were sensitive to imipenem and 5(8.3%) were resistant (Table 4.1).

Multidrug resistant isolates were detected in 12 of urine isolates, 7 of blood, 2 of wound swab isolates and no sputum isolates were multidrug resistant. Three neonatal blood isolates and one adult wound swab were extensive drug resistant (resist all antibiotics even imipenem).

Table 4.1 Results of susceptibility test

Results / Antibiotics	Sensitive	Resistant
Ceftazidime	30%	70%
Ciprofloxacin	70%	30%
Chloramphenicol	61.7%	38.3%
Gentamicin	60%	40%
Imipenem	91.7%	8.3%

4.5. Identification of *K. pneumoniae* using PCR based method targeting 16S rRNA gene

All sixty isolates (100%) of *K. pneumoniae* identified by using PCR (Figure 4.3).

4.6. Detection of *K. pneumoniae* carbapenem resistance genes

All 60 *K. pneumoniae* isolates investigated for the presence of carbapenem resistance genes (*NDM*, *IMP*, *SPM*, *OXA-48* and *KPC*) using multiplex PCR. Eighty percent (48/60) were positive for one or more carbapenem resistance genes. 68.3% (41/60) were positive for *OXA-48* gene (Figure 4.4), 10% (6/60) were positive for *NDM* gene (Figure 4.5), 8.4% (5/60) were positive for *SPM* gene (Figure 4.6), 8.4% (5/60) were positive for *KPC* gene (Figure 4.7), and 3.3% (2/60) were positive for *IMP* gene (Figure 4.8).

One isolate possess 3 genes, nine isolates possess 2 genes and thirty-eight isolates possess 1 gene and the remaining twelve isolates free from these genes (Table 4.2).

Table 4.2 Frequency of carbapenem resistance genes out of *K. pneumoniae* isolates

No of genes Detected	Type of genes	No of isolated organisms	Percentage%
3 Genes	IMP+OXA-48+KPC	1	1/60 (1.7%)
2 Genes	NDM+OXA-48	4	9/60 (15%)
	SPM+OXA-48	2	
	SPM+KPC	1	
	OXA-48+KPC	2	
1 Gene	OXA-48	32	38/60 (63.3%)
	NDM	2	

	SPM	2	
	IMP	1	
	KPC	1	
Zero gene	No gene	12	12/60 (20%)

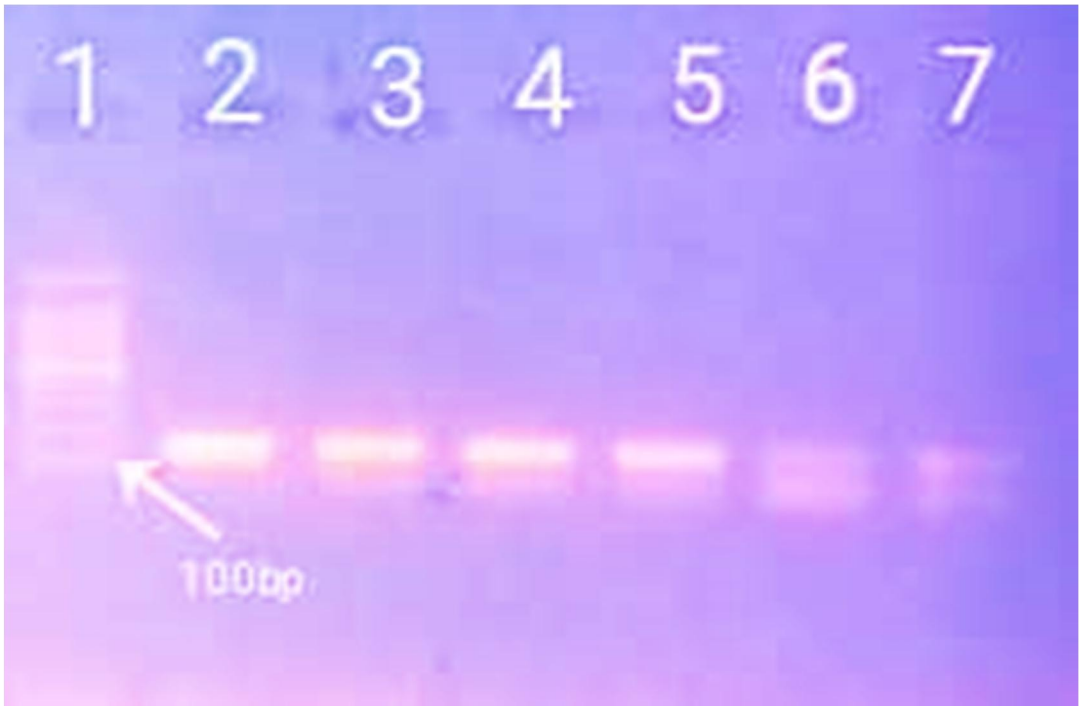

Figure 4.3. Amplified DNA of 16S rRNA gene. Lane 1 DNA laddar 100bp; Lane 2-7, typical band size of 130bp corresponding to the molecular size of 16S rRNA gene.

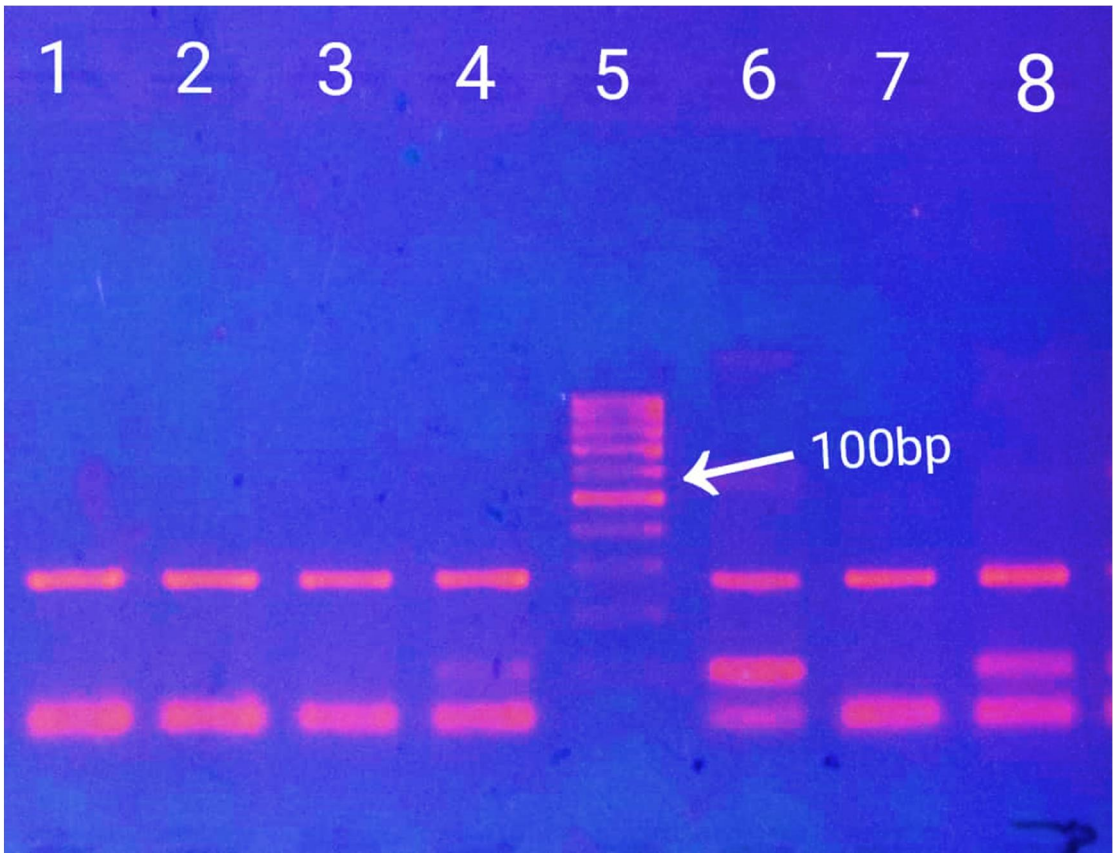

Figure 4.4 Amplified DNA of *OXA-48* gene. Lane 5, DNA ladder 100bp; Lane 1, 2, 3, 4, 6, 7 and 8, typical band size of 281bp corresponding to the molecular size of *OXA-48* gene.

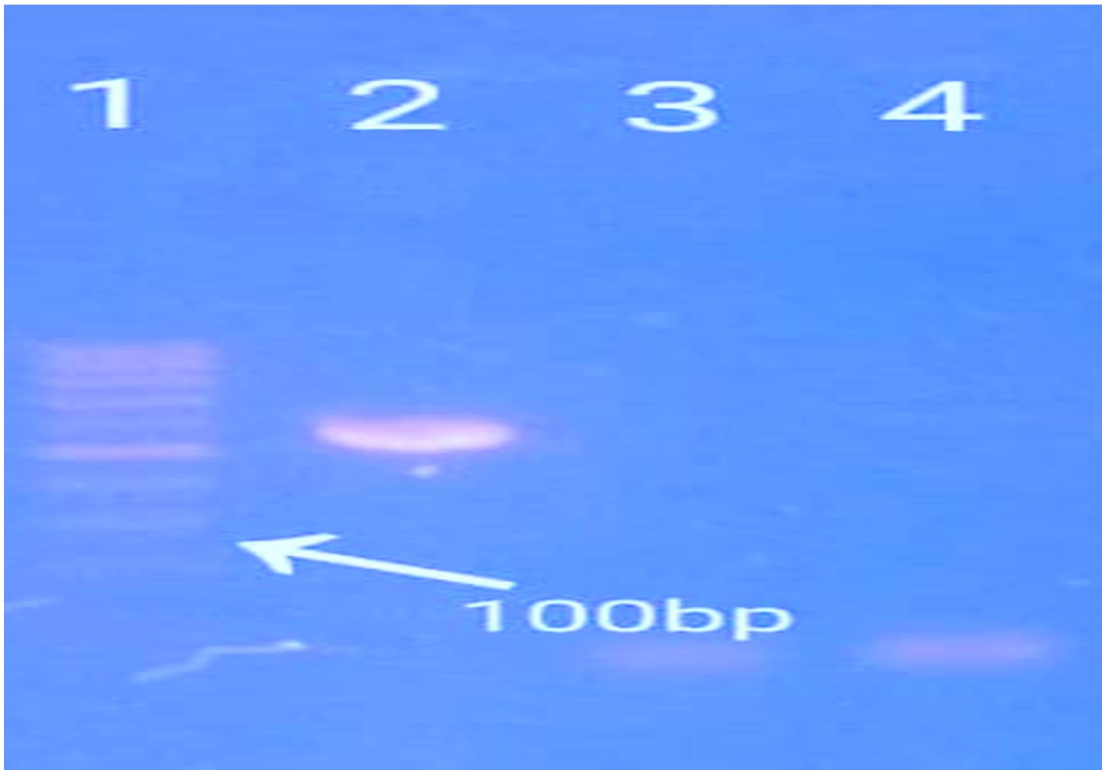

Figure 4.5. Amplified DNA of *NDM* gene. Lane 1, DNA ladder 100bp; Lane 2, typical band size of 521bp corresponding to the molecular size of *NDM* gene; Lane 3 and 4, negative samples.

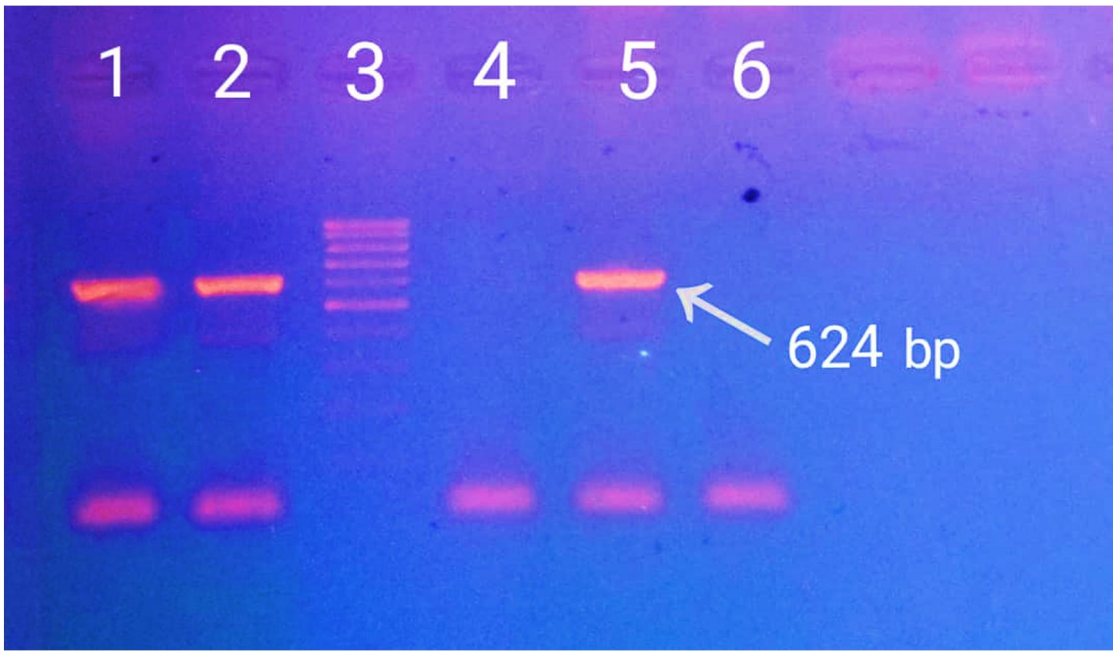

Figure 4.6. Amplified DNA of *SPM* gene. Lane 3, DNA ladder 100bp; Lane 1, 2 and 5, typical band size of 624bp corresponding to the molecular size of *SPM* gene; Lane 4 and 6, negative samples.

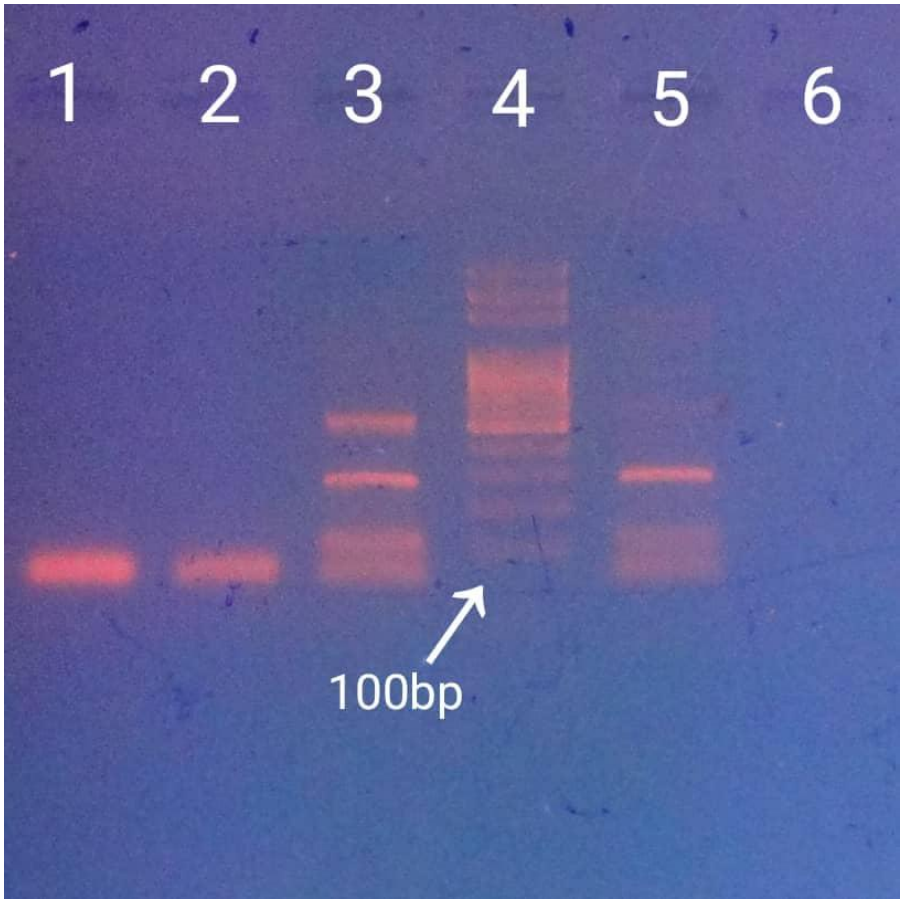

Figure 4.7. Amplified DNA of *KPC* and *OXA-48* genes. Lane 4, DNA ladder 100bp; Lane 3, typical bands size of 498bp and 281bp corresponding to the molecular size of *KPC* and *OXA-48* genes respectively; Lane 5, typical band size of 281bp corresponding to the molecular size of *OXA-48* gene; Lane 1,2 and 6, negative samples.

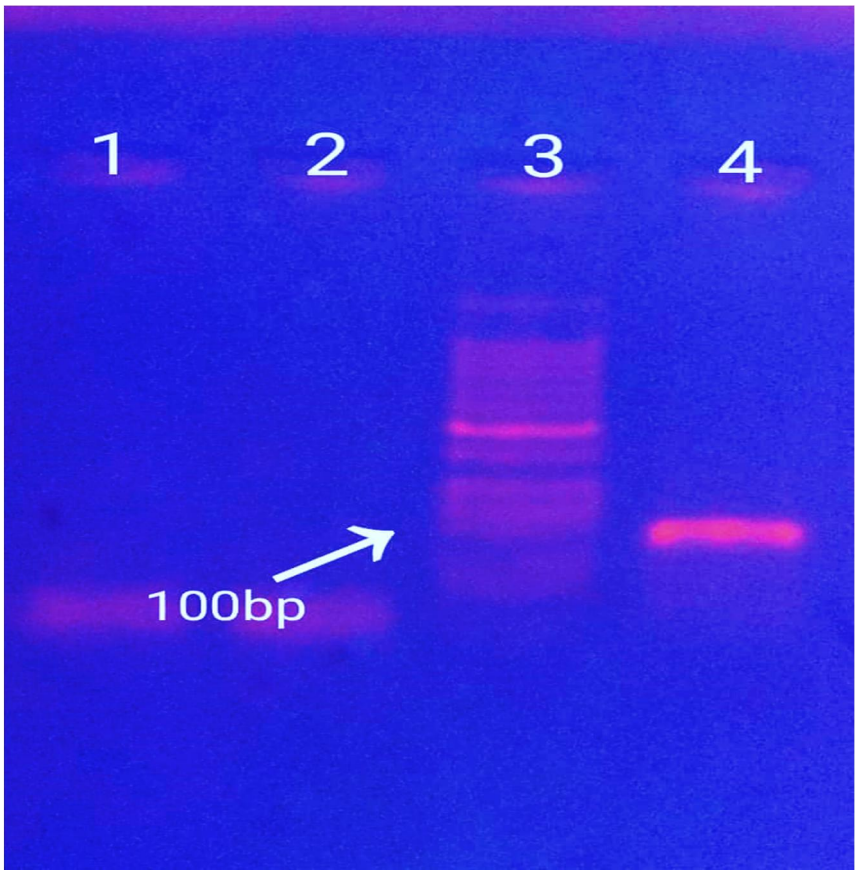

Figure 4.8. Amplified DNA of *IMP* gene. Lane 3, DNA ladder 100bp; Lane 4, typical band size of 232bp corresponding to the molecular size of *IMP* gene; Lane 1and 2, negative samples.

4.7. Association between carbapenem resistance genes and gender, clinical specimens, antibiotics resistance and age groups

There was association between the presence of carbapenem resistance genes with clinical specimens, antibiotics resistance and age groups. *KPC*

gene showed statistically significant association with both age groups and clinical specimens (*P* value= 0.003 and 0.020 respectively). *KPC* gene presents in 6.7% of infants and 1.7% of adults (Table 4.3), and in 1.7% of urine, 6.7% of blood and 0% in wound swab and sputum samples (Table 4.4). In addition, *IMP* gene showed statistically significant association with age groups (*P* value= 0.013). *IMP* gene presents in 3.3% of infants and 0% in adults (Table 4.5). *SPM* gene also showed statistically significant association with susceptibility to imipenem and chloramphenicol antibiotics (*P* value= 0.00 and 0.045 respectively). *SPM* gene presents in 3.3% of sensitive and 5% of resistant isolates to imipenem (Table 4.6), 1.7% of sensitive and 6.7% of resistant isolates to chloramphenicol (Table 4.7).

Table 4.3 Association between the presence of *KPC* gene and age groups

Age group		*KPC* gene		Total
		Present	Absent	
Adults	Count	1	44	45
	Total%	1.7%	73.3%	75%
Infants	Count	4	11	15
	Total%	6.7%	18.3%	25%
Total	Count	5	55	60
	Total%	8.4%	91.6%	100%
P value= 0.003				

Table 4.4 Association between the presence of *KPC* gene and clinical specimens

Clinical specimens	*KPC* gene		Total
	Present	Absent	

Urine	Count	1	36	37
	Total%	1.7%	60%	61.7%
Blood	Count	4	10	14
	Total%	6.7%	16.5%	23.2%
Wound swab	Count	0	5	5
	Total%	0%	8.4%	8.4%
Sputum	Count	0	4	4
	Total%	0%	6.7%	6.7%
Total	Count	5	55	60
	Total%	8.4%	91.6%	100%
P value= 0.020				

Table 4.5 Association between the presence of *IMP* gene and age groups

Age group		*IMP* gene		Total
		Present	Absent	
Adults	Count	0	45	45
	Total%	0%	75%	75%
Infants	Count	2	13	15
	Total%	3.3%	21.7%	25%
Total	Count	2	58	60
	Total%	3.3%	96.7%	100%
P value= 0.013				

Table 4.6 Association between the presence of *SPM* gene and resistance to imipenem antibiotic

Imipenem antibiotic		*SPM* gene		Total
		Present	Absent	
Sensitive	Count	2	53	55
	Total%	3.3%	88.4%	91.7%
Resistant	Count	3	2	5
	Total%	5%	3.3%	8.3%
Total	Count	5	55	60
	Total%	8.3%	91.7%	100%
P value= 0.00				

Table 4.7 Association between the presence of *SPM* gene and resistance to chloramphenicol antibiotic

Chloramphenicol antibiotic		*SPM* gene		Total
		Present	Absent	
Sensitive	Count	1	36	37
	Total%	1.7%	60%	61.7%
Resistant	Count	4	19	23
	Total%	6.7%	31.6%	38.3%
Total	Count	5	55	60
	Total%	8.4%	91.6%	100%
P value= 0.045				

CHAPTER FIVE
DISCUSSION

CHAPTER FIVE
DISCUSSION

5.1. Discussion

In this study, 60 *K. pneumoniae* isolates were collected from different hospitals in Khartoum State and were cultured on MacConkey's agar. All isolates were identified using PCR.

Multiplex PCR was used to detect five carbapenem resistance genes (*NDM*, *IMP*, *SPM*, *OXA-48* and *KPC*). In this study, 80% of *K. pneumoniae* isolates were positive for one or more genes which in agreement with previous study from Sudan, the percentage was (72%) revealed by Ali and Omer, (2017), from South Africa (71%) detected by Singh-Moodley and Perovic, (2016) and in KSA (67.6%) observed by Al Tamimi *et al.*, (2016). In addition, this result disagree with the study from Tanzania by Mushi *et al.*, (2014) which detected carbapenem resistance genes only in (11%) of *K. pneumoniae* isolates. The higher percentage of those genes in this study may be due to high antibiotic use, self-medication by patients and lack of implementation of antibiotic policies in our hospitals (Elhag, 2014).

In the current study, most of isolates harboring carbapenem resistance genes were phenotypically susceptible to imipenem and most of them were positive for *OXA-48* gene. This confirm what Walsh, (2010) said that this gene is not strong and relies upon other synergistic mechanisms to mediate resistance against carbapenems. In addition to imipenem other antibiotics were analyzed in this study. Majority of the isolates showed varying degrees of resistance to the other antibiotics (ciprofloxacin, gentamicin and ceftazidime). Resistance to these antibiotics may also be due to the presence of ESBLs and other mechanisms like efflux pumps and porin mutations (Singh-Moodley and Perovic, 2016), which were not covered in this study. The former statement may be supported for example by the low

ceftazidime susceptibility rate (30 %) in our isolates, suggesting that ESBLs enzymes may be very prevalent in our sample population.

In the current study, the most prevalent gene among the 60 *K. pneumoniae* isolates was *OXA-48* gene (68.3%). *NDM, KPC* and *IMP* genes were also present but on a smaller scale (10%, $n = 6$; 6.7%, $n = 4$; 3.3%, $n = 2$ respectively). This is consistent with previous findings by Candan and Aksöz, (2015) in Turkey, which detected *OXA-48* gene (58%) and *NDM* gene (2%), Al Tamimi *et al.*, (2016) detected *OXA-48* gene (64.7%) and *NDM* gene (5.8%) and Shibl *et al.*, (2013) detected *OXA-48* gene (78%) and *NDM* gene (20%) both from Saudi Arabia, *KPC* and *IMP* genes were not detected in these studies. The percentages of those genes differ from previous studies in Sudan by Ali and Omer, (2017) which detected *OXA-48* gene (35.4%), *NDM* gene (57.3%) and *KPC* gene (59.4%), by Dahab *et al.*, (2017) which detected only *OXA-48* gene in (4.0%) of *K. pneumoniae* isolates and by Satir *et al.*, (2016) detected *IMP* gene in (73%) and *KPC* gene in (50%) of *K. pneumoniae* isolates. These variations in results may be because, they only detect resistant genes in carbapenem-resistant isolates or due to differences in method of detection as the first one used real-time PCR or differences in the sample size of tested organism and that was seen with the other two studies.

Up to our Knowledge, this is the first study in the world detected *K. pneumoniae* harbored *SPM* gene at 8.4% (5/60). Lee *et al.*, (2016) state that among class B carbapenemases only NDM, IMP and VIM have been detected in *K. pneumoniae* while other carbapenemases, such as SPM have been not found in *K. pneumoniae*. In addition, *SPM* gene showed statistically significant association with resistance to imipenem and chloramphenicol antibiotics. Also three positive *SPM* isolates were extensively drug resistant (resist all antibiotics), which agree with the study from Brazil by Franco et al., (2010), which revealed that all SPM-

producing *Pseudomonas aeruginosa* strains showed 100% resistance to imipenem, ceftazidime, gentamycin and ciprofloxacin. This may indicate that *SPM* have high ability to cause resistance to these antibiotics even imipenem.

Of 48 *K. pneumoniae* isolates detected of having carbapenem resistance genes, 10 had multiple genes. This finding agree with Ali and Omer, (2017) and Satir et al., (2016) which showed multiplicity of genes in their isolates. The presence of multiple resistance genes in one isolate have not been commonly detected in a large number of studies probably due to the limited number of genes studied since most of the studies research on one or two genes (Satir *et al.*, 2016).

Regarding *KPC* and *IMP* gene, 5 (8.3%) and 2 (3.3%) were positive respectively. *KPC* gene showed significant association with both clinical specimens and age groups as detected in four blood samples collected from infant males. While *IMP* gene showed significant association only with age groups as detected in two isolates collected from infants. This may be due to organisms harboring these genes have high ability to cause systemic infections particularly in immunocompromised patients (Chen *et al.*, 2012).

5.2. Conclusion

The findings of the present study disclosed a high percentage and multiplicity of carbapenem resistance genes among *K. pneumoniae* isolates in Khartoum State. An important finding of this study, shown for the first time, *K. pneumoniae* carried *SPM* gene and showed association with resistance to imipenem. In addition, this study demonstrates a simple and useful PCR-based approach for identification of *K. pneumoniae*.

5.3. Recommendations

1. Sequencing of detected carbapenem resistance genes as well as further studies with large number of isolates required to validate the results of this study.
2. It is of great importance to make a unique and precise guideline for routine microbiology laboratories in order to detect carbapenemase-producing isolates.
3. Adhering to extensive infection control measures in community and hospital-acquired infections to disrupt the further spread of carbapenemase-producing isolates among hospitalized patients in Sudan.

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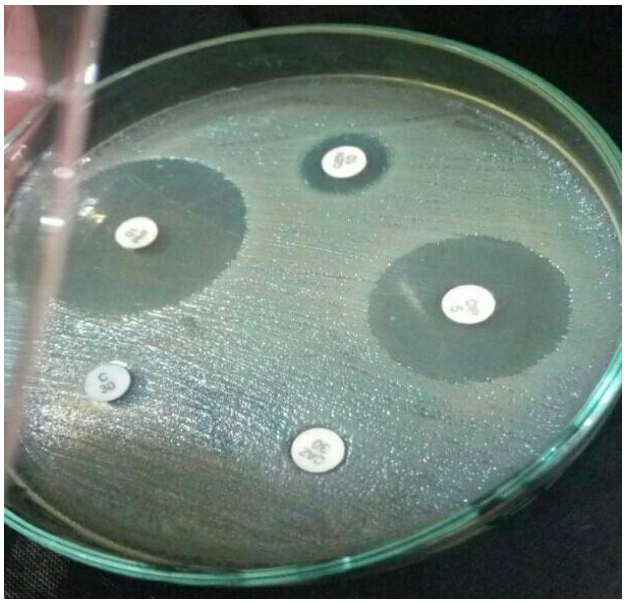
APPENDICES

APPENDIX 1

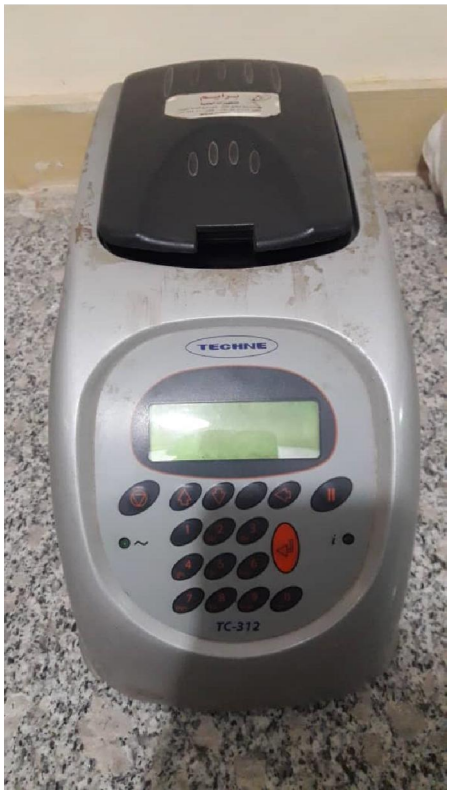
COLORED PLATES

Colored plate 1.1 Appearance of mucoid pink colonies of *K. pneumoniae* on MacConkey's agar



Colored plate 1.2 Susceptibility test results: Using ceftazidime (CAZ) 30 mcg, ciprofloxacin (CIP) 5mcg, chloramphenicol (C) 30 mcg, gentamicin (GEN) 10 mcg and imipenem (IPM) 10 mcg on Muller Hinton agar.

Colored plate 1.3 Microwave



Colored plate 1.4 Thermocycler

Colored plate 1.5 Electrophoresis system

Colored plate 1.6 Transilluminator

APPENDIX 2

REAGENTS AND CULTURE MEDIA

Appendix (2.1): Glycerol peptone media

Peptone water (HiMedia Laboratories Pvt. Ltd. Mumbai, India)

Ingredient	g/L
Peptic digest of animal tissue	10.0g
Sodium chloride	5.0g

Final pH (at 25˚C) 7.4 ± 0.2

Preparation

Suspend 15.0 grams in 80 ml D.W then add 20ml of glycerol. Mix well and dispense into tubes with or without inverted Durham's tubes and sterilize by autoclaving at 15lbs pressure (121˚C) for 15 minutes.

Appendix (2.2): MacConkey's media (HiMedia Laboratories Pvt. Ltd. Mumbai, India)

Ingredient	g/L
Peptone	20g
Lactose	10g
SodiumTaurocholate	5g
Neutral red	0.04g
Agar	20g

Final pH (at 25˚C) 7.4 ± 0.2

Preparation

Suspend 55.04g in 1000ml D.W. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15lbs pressure (121°C) for 15 minutes. Cool to 45-50°C, mix well and pour into sterile Petri plates.

Appendix (2.3): Mueller Hinton Agar (HiMedia Laboratories Pvt .Ltd. Mumbai, India)

Ingredient	g/L
Casein acid hydrolysate	17g

Meat infusion solids	2g
Starch, soluble	1.5g
Agar	17g

Final pH (at 25°C) 7.3 ± 0.1

Preparation

Suspend 38.0 grams in1000 ml D.W. Heat until boiling to dissolve the medium completely and sterilize by autoclaving at 15lbs pressure (121˚C) for 15 minutes. Cool to 45 - 50°C, mix well and pour into sterile Petriplates.

Appendix (2.4): 0.5% McFarland standard

Prepare a 1% v/v solution of sulphuric acid by adding 1ml of concentrated sulphuric acid to 99ml of water. Mix well; prepare a 1% w/v solution of barium chloride by dissolving 0.5 g of dehydrate barium chloride (BaCl 2. 2H2O) in 50 ml of D.W. Add 0.6 ml of the barium chloride solution to 99.4 ml of the sulphuric acid solution, and mix. Transfer a small volume of the turbid solution to a capped tube or screw cap bottle of the same type as used for preparing the test and control inoculam. When stored in a well-sealed container in the dark at room temperature (20–28°C), the standard can be kept for up to 6 months.