University of Kerbala
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Molecular detection of some carbapenemases in *Klebsiella pneumoniae* isolated from different clinical sources

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بسم الله الرحمن الرحيم

(وَالرَّاسِخُونَ فِي الْعِلْمِ يَقُولُونَ آمَنَّا بِهِ كُلٌّ مِنْ عِنْدِ رَبِّنَا ۗ وَمَا يَذَّكَّرُ إِلَّا أُولُو الْأَلْبَابِ)

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ال عمران اية ٧

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Dedication

I'd like to dedicate my work To the spirit of my father, who used to accompany and inspire me, and to my beloved mother, who with tenderness in her palms begged God to guide me to my beloved husband, without whom I would not be in this place, To my sons, Malak, Mustafa, Mahdi, who have always supported me and prepared the appropriate conditions to complete this work. To my dear brother, Dr. Ahmed, for his support and assistance , and to my brothers, Dr. Zaid and Dr. Ali.

Summary

This study included a diagnosis of *Klebsiella pneumoniae* responsible for many infections after being isolated from different clinical sources such as blood, sputum, burns, wounds, the pharynx, the ear, urine, and vagina, and also the investigation of virulence factors these bacteria cross detecting the following genes: *bla*$_{KPC}$, *bla*$_{IMP}$, *bla*$_{VIM}$, *bla*$_{NDM}$, and *bla*$_{OXA48}$, which encode for beta-lactam-degrading.

The results yielded in Sixty-eight isolates being obtained out of 275 samples that were collected from patients who were reviewed and inpatients in hospitals and outpatient clinics in the Holy Karbala Governorate, of both sexes and of different ages, for the period between the first of September and the end of December 2022. It was diagnosed through phenotypic examination on culture imedia blood agars and MacConkey agars, and by using Vitek-2 system.

The sensitivity of the bacterial isolates obtained in this study was conducted against 12 types of antibiotics that are used in the treatment of *K. pneumoniae* infections, which included Meropenem, Imipenem, Cefipeme, Cefotaxime, Ceftazidime, Gentamicin, Aztreonam, Levofloxacin, Ciprofloxacin, Trimethoprim-sulfamethoxazole, Amikacin, Piperacillin, and Tazabactam. The results showed that the highest rate of resistance was to antibiotics Imipenem 80% and followed by Cefotaxime 74%, while she was the lowest rate of resistance to Levofloxacin and Piperacillin/Tazabactam 37%. As was also evident from the results of this examination showed that 40 isolates were multi-resistant (multi-drug resistant).

This study also included a phenotypic examination of the multi-resistance was also carried out using the Combined Disc Synergy Test to detect the presence of metal beta-lactam enzymes that contain zinc ione and the result was positive for 33 isolates and negative for seven of them. The results of the molecular detection of our current study confirmed the presence of the gene in the highest percentage (60%) in *K. pneumoniae* bacteria isolates, followed by the *bla*$_{OXA48}$ gene by 45%, then the

*bla**NDM*** gene by 30%, While it became clear that the ***blaIMP*** gene was present in a low percentage 12.5%, and the lowest percentage of the *bla**KPC*** gene by (2.5%) for 33 isolates out of 40 isolates, while it turns out that seven isolates devoid of any gene.

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Abbreviations

AMR	Antimicrobial Resistance
BLs	Beta-Lactamase
CDST	Combined Disc Synergy Test
CLSI	Clinical and Laboratory Standards Institute
CPS	capsular polysaccharide synthesis
CRE	Carbapenem-Resistant Enterobacteriaceae
CRKP	Carbapenem-Resistant *Klebsiella pneumoniae*
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic acid
ESBL	Extended-Spectrum β-Lactamase
ID-GNB	Identification Gram-Negative Bacteria
IMI	Imipenem-Hydrolyzing β-Lactamase
IMP	Imipenemase, β-Lactamase Active on Imipenem
IP/IPI	imipenem/imipenem-inhibitor
K	Capsular Antigen
LPS	Lipopolysaccharide
MBCs	minimum bactericidal concentrations
MBL	Metallo-β-Lactamase
MBL	Mannose-Binding Lectine
MDR	Multi-Drug Resistance
MFC	Minimum fungal concentration
MHA	Multi-Hinton Agar
MHT	Modified Hodge Test
MIC	Minimum Inhibitory Concentration
NDM	New Delhi Imipenemase

OmpA	outer membrane protein A
OMPs	Outer Membrane Proteins
OXA	Oxacillinase β-Lactamase Active on Oxacillin
PBPs	Penicillin-Binding Proteins
PCR	Polymerase Chain Reaction
PDR	Pan Drug Resistance
QS	Quorum Sensing
rmpA	regulator of mucoid phenotype A
TBE	Tris-Borate- Ethylenediaminetetraacetic Acid
UTI	Urinary Tract Infection
VIM	Verona Integron-Encoded Metallo-β-Lactamase
XDR	Extensive-Drug Resistance
Zn	Zinc

Chapter One Introduction

Introduction

The genus *Klebsiella* belongs to the Enterobacteriaceae family. A species of this genus is *Klebsiella pneumoniae*, which is considered a pathogen, opportunistic means sometimes a type of natural flora is endemic to the intestine and other places in the body, and it seizes the opportunity of her presence here to take advantage of the weakness of the body in certain special cases during infection with other diseases. So, it can be isolated from different infections (Wyres *et al.*, 2020).

Klebsiella pneumoniae is a pathogenic Gram-negative bacterium, in MacConkey agar medium the polysaccharide capsule aligned with the bacterial outer membrane appear a mucoid phenotype that ferments lactose. Interest in these bacteria has increased due to the raise in the percentage of infections caused by them, and due to its high resistance to antibiotics, including broad-spectrum antibiotics. This bacterium is considered one of the most antibiotic-resistant strains of Enterococcus and it is a good host for resistant plasmids (Martin and Bachman, 2018).

Klebsiella pneumoniae has many resistance and virulence loci that code for siderophores, capsules, lipopolysaccharides, pili efflux pumps, enzymes, and adhesins that may contribute to its successful adaptation to the hospital environment. This made it the main pathogen responsible for injuries occurring in hospitals (Wang *et al.*, 2020).

Klebsiella pneumonia's emergence of antimicrobial resistance isolates are a significant global issue in human medicine because they enhance the likelihood that antibiotics may fail to cure people. Community-acquired and nosocomial infections brought on by multidrug-resistant bacteria are challenging to treat with current treatments (Kalelkar *et al.*,2022).

Patients with hematological and immunological deficiencies are at greater risk for *K. pneumoniae* colonization and infection. The dramatic increase in the occurrence of multidrug-resistant (MDR) and extremely drug-resistant (XDR) infections belonging to the Enterobacteriaceae family is a huge economic issue since these organisms are widespread natural inhabitants of human and animal microbiomes, such as *K. pneumoniae* (Effah *et al*., 2020).

Carbapenems are the final therapeutic choice for treating infections caused by MDR Enterobacteriaceae. In spite of this, the overuse of carbapenems has resulted in the evolution of several resistance mechanisms and diminished their efficacy (Ramadan *et al.,* 2022). *K. pneumonia's* resistance to carbapenems represents a major challenge to the global delivery of health services (Ssekatawa *et al.,* 2021).The virulence factors of *K. pneumoniae* are encoded by genes across its (core)chromosomal gene loci and accessory genomes. The accessory genome is essential for *K. pneumoniae* antibiotic resistance, such as carbapenemases, β-lactamases, and extended-spectrum β-lactamases (ESBL) (Martin and Bachman, 2018).

Carbapenem-resistant Gram-negative bacteria are widespread, and the nature of their resistance loci varies considerably among clinical isolates. In Iraq, no substantial investigations have been conducted on the nature and spread of carbapenemases in carbapenem-resistant *K. pneumoniae*.

The current study aims to the investigation of *K. pneumoniae* pathogenicity through the following objectives:

1. Isolation of *K. pneumoniae* from different clinical specimens and identification using Vitek II system.
2. Study susceptibility testing among *K. pneumoniae* isolates (according to CLSI 2022 instructions).
3. Detection of Carbapenemase enzymes phenotypically.

4. Detection of Carbapenemase genes in *K. pneumoniae* isolates by Molecular methods.

5. Detection of the relationship between antibiotic resistance and the presence of the Carbapenemase genes in *K. pneumoniae* isolates.

Chapter Two

Literatures Review

Chapter Two: Literature Review

2.1 Enterobacteriaceae

Enterobacteriaceae is a diverse family of Gram-negative, facultatively anaerobic, rod-shaped, nonsporulating proteobacteria. Most Enterobacteriaceae feature pili (fimbriae) of type 1, which aid bacterial motility. Several species adhere to epithelial cells and have flagella that make them mobile. Important biochemical properties of Enterobacteriaceae include the conversion of nitrate to nitrite, the fermentation of sugars (various species ferment particular sugars), the presence of catalase, and the absence of oxidase. Enterobacteriaceae thrive between 25°C and 37°C and occupy a wide range of environmental, animal, and human habitats, including water, soil, plants, and the mammalian gut (Barco *et al*., 2014).

Enterobacteriaceae are the causative agents of both community and hospital-acquired infections. They spread easily between humans (via hand carriage, contaminated food and water) and acquire genetic material via horizontal gene transfer, which is primarily mediated by plasmids and transposons (Urban-Chmiel *et al*., 2022).

They are an abundant part of the normal human gut flora and also a common cause of both health care and community-associated infections in humans (Kachrimanidou & Tsintarakis . 2020). Most clinically important Enterobacteriaceae can be put into two groups: opportunistic pathogens like *Enterobacter*, *Citrobacter*, and *Klebsiella*, and overt pathogens like *Escherichia coli* and *Salmonella*(Forbes *et al*., 2014).

2.2 *Klebsiella*

Klebsiella genus is a member of Enterobacteriaceae family Gram-negative, facultatively anaerobic, rod-shaped, nonsporulating, immobile that included other perceived pathogens, for instance, *Shigella spp, Escherichia*

coli, Yersinia spp and *Salmonella spp*. In nature, *Klebsiella spp* could be ubiquitous including plants, animals as the same as humans immobile, which accepted to be a causal operator of various types of contaminations in humans including diseases of the respiratory system, bloodstream infections and the urinary system contaminations (Podschun & Ullmann., 1998).

Klebsiella is so-called after the German microbiologist Edwin Klebs (1834–1913) (Righini *et al*., 2020). Rendering to Bergey's Manual of Systematic Bacteriology, Second Edition (UniProt.2015) (NCBI taxonomy accessed in 2018). It is classified as rendering to the following:

Domain: *Eubacteria*

Phylum: *Proteobacteria*

Class: *Gamma Proteobacteria*

Order: *Enterobacteriales*

Family: *Enterobacteriaceae*

Genus: *Klebsiella*

The genus Klebsiella is comprised of four species: *Klebsiella pneumoniae (K. pneumoniae), Klebsiella oxytoca (K. oxytoca), Klebsiella terrigena (K. terrigena),* and *Klebsiella planticola (K. planticola),* with *K. pneumoniae* consisting of three subspecies (Vasaikar *et al*., 2017).

Klebsiella pneumoniae is characterized into three different phylogroups (KpI, KpII, KpIII) dependent on the few numbers of their genes sequenc, such phylogroups have been re-designated into three species involving *K. pneumoniae* /KpI; *K. quasipneumoniae* /KpII and *K. variicola* / KpIII that can infect human (Brisse *et al*.,2006; Maatallah *et al*., 2014).

In relation to species naming and composition, the genus *Klebsiella* has several unresolved taxonomic difficulties. With the recent introduction of *Klebsiella quasipneumoniae* and *Klebsiella michiganensis* to the genus, there are presently 15 species that have been reliably published: *Klebsiella granulomatis, K. pneumoniae, Klebsiella mobilis, Klebsiella michiganensis,* and *K.ornithinolytica, K.oxytoca, K.planticola, K.singaporensis, K.terrigena, K. trevisanii,* and *K. variicola*) However, the fact is that two previous species, *K. ozaenae* and *K. rhinoscleromatis*, have been reclassified as subspecies of *K. pneumoniae*, and three species have been reassigned to the genus Raoutella (*K. planticola, K. ornithinolytica, and K. terrigena)*. Initially, the medicinal significance of the genus *Klebsiella* was split into three disease-related species: *K. pneumoniae, K. ozaenae, and K. rhinoscleromatis* (Janda *et al.*, 2015).

They are also important opportunistic pathogens, particularly among the immunocompromised; pathogenicity factors of *Klebsiella spp*. include adhesins, siderophores, capsular polysaccharides (CPLs cell surface lipopolysaccharides (LPSs), and toxins, each of which plays a specific role in mode of infectivity, cells of *Klebsiella spp*. May adhere and attack upper respiratory tract epithelial cells, cells in gastrointestinal tract, endothelial cells, or uroepithelial cells, followed by colonization of mucosal membranes, common underlying conditions include alcoholism diabetes mellitus, chronic liver disease (cirrhosis), chronic renal failure, cancer transplants, burns, and/or use of catheters (Janda, and Abbott, 2006).

2.3 Klebsiella pneumoniae

Klebsiella was named after German scientist Edwin Klebs (1834–1913). It is also known as Friedlander's bacillus, after Carl Friedländer, a German pathologist who hypothesized that this bacterium was the cause of tuberculosis etiological factor for pneumonia, particularly in

immunocompromised patients folks with chronic ailments or alcoholics (Friedlander) *Klebsiella pneumoniae*-caused community-acquired pneumonia may be referred to as Friedländer's bacillus (Zander and Fraver,2016).

In the late 19th century *K.pneumoniae* was first isolated and was initially termed as Friedlander's bacterium. (Gonzalez-Ferrer *et al.*, 2021), *Klebsiella pneumoniae* is a ubiquitous Gram-negative encapsulated bacterium that resides in the mucosal surfaces of mammals and the environment (soil, water, etc.). In humans, *K. pneumoniae* colonizes the gastrointestinal tract and less frequently the nasopharynx, whence it gains entry to the circulation and other tissues causing infection. In the pre-antibiotic era, *K. pneumoniae* was an important cause of community-acquired pneumonia, especially in alcoholics and diabetics. In the antibiotic era, *K. pneumoniae* has become the main cause of healthcare-associated infections in hospitals (piperaki *et al.,* 2017). Although he was not the first to isolate *K. pneumoniae*, naming a genus after him honored his work with *Corynebacterium diptheriae*. Around the same time, Hans Christian Gram (1853–1938) created a microbiological technique known as the Gram stain in 1884 to differentiate between *K. pneumoniae* and *S. pneumoniae* (UniProt., 2015).

Klebsiella pneumoniae is an opportunistic pathogen associated with both community-acquired and nosocomial infections, including pneumonia, urinary tract infections, septicemia and wound infections, with the increasingly multidrug-resistant (MDR) *K. pneumoniae* being a major public health concern (Cao *et al.*, 2014). This bacterium is involved in surgical-wound infection, hospital acquired pneumonia, bacteremia, ventilator-associated pneumonia in addition to urinary tract transmissions (Mancini *el at.*, 2018).

This pathogen has an extensive accessory genome consisting of plasmids and chromosomal gene loci (Wyres *et al*.,2020). This extra genome distinguishes *K. pneumoniae* from two closely related species, *Klebsiella variicola* and *Klebsiella quasipneumoniae*, by separating opportunistic, hypervirulent, and multidrug-resistant *K. pneumoniae* strains (Martin and Bachman, 2018). According to the popular view, these bacteria acquire multidrug resistance by horizontal transfer of antimicrobial resistance genes mediated by mobile genetic components such as integrons. Multiple nosocomial epidemics caused by *K. pneumoniae* with multiple treatment resistance have been observed globally (Ares *et al.*, 2016). *Klebsiell* species-caused endemic, epidemic, and hospital-acquired infections are main causes of death and illness (Pokra *et al.*, 2016).

Since the mid-1980s, *K. pneumoniae* has been among the most regularly seen nosocomial pathogens. This bacterium has been identified as the source of extremely persistent community-acquired illnesses. (Vasaikar *et al.*, 2017). This organism accounts for about one-third of all Gram-negative infections for instance, urinary tract infections, cystitis, pneumonia, surgical wound infections, endocarditis and septicemia (Navon-Venezia *et al.*, 2017). *Klebsiella pneumonia's* antimicrobial resistance is a clear and present risk throughout Asia, necessitating vigilant monitoring to combat this scourge. It is crucial that public health agencies track and report changes in antimicrobial-resistant isolates (Effah *et al.*, 2020).

2.4 Virulence factors of *Klebsiella pneumoniae*

K. pneumoniae has many resistance and virulence loci that code for a varied spectrum of siderophores, efflux pumps, beta-lactamases, and adhesins that may contribute to its successful adaptation to the hospital environment (Ramos *et al.*, 2014). The main virulence factors and

pathogenicity of *K. pneumonia* are capsular polysaccharide (CPS) antigens (O-and K-antigens), lipopolysaccharide, siderophores (endotoxins), and pili (Ares *et al*., 2016).

Most *K. pneumoniae* do not exist, but they possess a wide range of virulence factors. It is known arsenal of virulence multiple types of fimbriae, O-antigen, and a few siderophores (Aerobactin, salmochelin, enterobactin and yersiniabactin) and yersiniabactin in the extracellular capsule. It has attracted much interest because it improves tolerance, makes bacteria resistant to drug treatment, and conceals surface antigens (Rendueles ., 2020).

2.4.1 Capsule

Capsule polysaccharide (CPS) is recognized as one of the most important virulence factors of *K. pneumoniae* (Hegerle *et al.,* 2018), (CPS) around bacteria is a crucial virulence factor of *K. pneumoniae*, participating in the resistance to phagocytosis and serum bactericidal activity (Liao *et al.,* 2021). *Klebsiella pneumoniae* has CPS antigen that assists with resistance and prevents immune response (Hasegawa *et al.,* 2014). Gene clusters CPS (capsular polysaccharide synthesis), rmpA (regulator of mucoid phenotype A), and (O-specific) make up the genetic structure of the capsule (direct by swbs gene cluster) polysaccharide (gene linked with mucoviscosity) (Shakib *el at.,* 2018). It is a complex acidic polysaccharide comprised of repetitive subunits of 4-6 sugars and acids, such as thick hydrophilic polysaccharides such as glucose, galactose, and lactose, mannose, fructose, and rhamnose, and often acids such as uronic acid, glucouronic acid, galacturonic acid, and pyruvic acid. (Jasim *et al.,* 2020). It is responsible for the glossy, mucoid appearance of colonies on agar plates. CPS may shield bacteria against phagocytosis and serum factor-induced death. In addition to its antiphagocytic action, CPS promotes bacterial colonization and biofilm development at the sites of infection (Jian-Li *et al.,* 2017).

Member of the *Klebsiella* genus typically express 2 types of antigens on their cell surface. The first is a lipopolysaccharide (O antigen); the other is a capsular polysaccharide (K antigen). Both of these antigens contribute to pathogenicity (Kappler& Hennet., 2020). There are 77 capsular types of K-antigen that were diagnosed in different chemical and antigenic type. The structural variability of these antigens forms the basis for classification into various serotypes (Arato *et al.,* 2021).

2.4.2 Lipopolysaccharide

Lipopolysaccharide is a significant and vital ingredient in the pathogenicity of bacteria, particularly *K. pneumoniae* since it is one of the outermost components of bacteria. It aids in phagocytosis resistance and is distinguished by its capacity to activate the complement factor (Jasim *et al.*, 2020). Lipid A component has toxic properties that often cause fever in the infected host, resulting in a potent immune response (Liobet *et al.*, 2015).

2.4.3 Fimbria

The fimbrial adhesive initiates biofilm development in *K. pneumoniae*. In addition, fimbriae allow bacteria to bind to the surface of epithelial cells, so aiding in disease development (Li *et al.,* 2014). The attachment of *K. pneumoniae* to mammalian tissue is mediated by type 1 and type 3 bacterial pili (Huynh *et al.,* 2017).

2.4.4 Siderophore production

Siderophores (from the Greek: "iron carriers") is relatively low-molecular-weight, ferric ion-specific chelating compounds released by bacteria in response to iron deficiency for acquisition. Iron is extracted from insoluble forms by mineralization and sequestration. Siderophores are proteins that bind iron and have a molecular weight between 400 and 1500 Da. The function of these molecules is to scavenge iron from the

environment and make the practically necessary mineral accessible to microbial cells (Kannahi and Senbagam, 2014). Siderophores are tiny, iron-chelating molecules with high affinity that are essential to the pathogenicity of several Gram-negative bacteria. *K. pneumoniae* secretes siderophores that are necessary for bacterial growth and reproduction (Khasheii *et al.,* 2021).

2.4.5 Quorum Sensing (QS)

Quorum sensing (QS) is a technique through which bacteria communicate by secreting auto inducers, which are chemical signaling molecules. It is used by several bacterial species to modulate gene expression through this intercellular communication mechanism, called quorum sensing (QS) (Huang *et al.*, 2019).

2.4.6 Efflux Pumps

There are several instances of chromosomally carried two-component systems in *K. pneumoniae* that seem to regulate the synthesis and effectiveness of efflux pumps; the activity of these pumps is crucial in determining resistance to a broad spectrum of antimicrobials (Clegg &Murphy, 2016).Efflux pumps are known to promote resistance to host-derived antimicrobial peptides in other bacteria, which are a crucial component of the host's innate immune system. It is probable that this mechanism plays a similar role in the virulence of *K. pneumoniae* (Martin and Bachman, 2018). The majority of efflux pumps are consistent between strains, and most of them can identify more than one substrate (Henderson *et al.,* 2021).

2.4.7 Outer Membrane Proteins (OMPs)

The outer-membrane proteins (OMPs) of Enterobacteriaceae are medically significant; their members play crucial roles in the permeability of antimicrobial drugs, substrates, and interactions with the host immune

system(Vergalli *et al.*,2020).Several OMPs, including outer membrane protein (OmpA), murein lipoprotein (LppA), and peptidoglycan-associated lipoprotein (Pal), which are encoded by genes with the same names, have been identified as being essential for *K. pneumoniae* pathogenicity (Paczosa and Mecsas, 2016).

2.4.8 Porins

Antimicrobial agents must first penetrate the outer membrane barrier in order to reach their targets, and hydrophilic and charged β-lactams presumably diffuse through the porin channels (Sugawara *et al.*, 2016).The deregulation of these porins seems to give an advantage to these bacteria in the face of antibiotic selection, with the porins perhaps serving as a route that permits the entrance of antibiotics into the bacterium. In terms of virulence, however, Porin downregulation may be detrimental (Paczosa and Mecsas, 2016). Carbapenem-resistant *K. pneumoniae* (CRK) can arise through mutations that diminish envelope permeability, such as porin deficiency. Nevertheless, most CRK clinical isolates contain several resistance pathways, including permeability deficits (Laws *et al.*, 2019).

2.4.9 Allantoin Metabolism

Allantoin is a nucleic acid destruction product that certain bacteria may utilize as a nitrogen source. Through the metabolism of allantoin, bacteria may acquire nitrogen and carbon from their environment. In a search for *K. pneumoniae* genes, an operon including allantoin-metabolizing genes whose transcription was unregulated in HV *K. pneumoniae* strains relative to classical strains was found (Paczosa and Mecsas, 2016).

2.5 *Klebsiella pneumoniae* Genome

This natural structure creates a framework for comprehending the epidemiology and development of clinically significant genetic variants, as

shown by genomic studies of the *K. pneumoniae* population variation. This knowledge is essential for the successful design and interpretation of experimental research aiming at unraveling mechanisms of antimicrobial resistance (AMR), pathogenicity, and/or virulence in *K. pneumoniae*, as well as for the creation of effective control methods (Wyres & Holt, 2018).

Plasmids play a crucial role in the transmission of antimicrobial resistance (AMR), pathogenicity, and other accessory genes between bacterial cells. The bulk of horizontally acquired AMR genes in *K. pneumoniae* are carried large plasmids. Conjugative (self-transmissible) plasmids belonging to a restricted number of incompatibility groups may host AMR genes in *K.pneumoniae*. Small (mobilizable but not self-transmissible) plasmids can also harbor AMR genes in *K. pneumoniae* (Ramirez *et al.*, 2019).

Extended spectrum beta-lactamase (ESBL) genes can be found both chromosomally or on transferable genetic elements such as plasmids, they are predominantly found in Enterobacteriaceae but are not uncommon among non-fermentative Gram-negative rods such as *P.aeruginosa* or *Acinetobacter spp* (Hammoudi Halat & Ayoub Moubareck ., 2020).

Resistance to carbapenems (imipenem and meropenem) can partly be attributed to the presence of some carbapenemase resistant-mediated genes such as *bla*$_{OXA}$, *bla*$_{NDM}$ and *bla*$_{KPC}$ .The *bla*$_{OXA}$ gene has been found in plasmid and integron locations in *K.pneumoniae* and it has frequently been found to be associated with genes encoding extended-spectrum beta-lactamases (ESBLs) (Ferreira *et al.*, 2019).

2.6 Antibacterial Resistance of *Klebsiella pneumoniae*

The resistance mechanisms of *K. pneumoniae* to different antibiotic classes includes the creation of antibiotic-inactivating enzymes, a variation in antibiotic target locations, a change in cell membrane permeability,

efflux pump systems, and a change in metabolic pathways (Verma *et al.*, 2015). Among these mechanisms, efflux pump systems and enzymatic degradation play a major role in the spread of multidrug-resistant *K. pneumoniae*. This pathogen was shown to acquire antibiotic resistance as a result of the creation of novel enzymes that degrade antimicrobials more efficiently than most bacteria (Venkatachalam *et al.*, 2014).

Resistance to the complement system is another significant virulence factor for *K. pneumoniae*, as the complement system plays an important role in humoral immunity against microbial pathogens. *K. pneumoniae* is resistance to the complement system because it has different surface antigen ,mediating its escape from host complements such as CPS, LPS (Doorduijn *et al.*, 2016).

Extended-spectrum β-lactamase (ESBL)-producing *K.pneumoniae* shown resistance to β-lactam antibiotics such as penicillins, cephalosporins, and monobactams. *K. pneumoniae* containing ESBL enzymes commonly demonstrates resistance to another class of antibiotics, such as aminoglycosides, fluoroquinolones, and chloramphenicol (Riwu *et al.*,2020).

Carbapenemases were considered as the last resort drugs for the treatment of multi-drug resistant (MDR) *K. pneumoniae*. This was before the first description of *K. pneumoniae* Carbapenemase (KPC) production in isolates from North Carolina since then, carbapenem-resistant *K. pneumoniae* (CRKP) have been frequently identified in multiple nosocomial settings throughout the world. CRKP organisms are resistant to all β-lactams and often to other important therapeutic agents(Annavajhala *et al.*, 2019).

The most frequently encountered enzymes are the Ambler class A KPCs, class B metallo-β-lactamases (VIM, IMP and NDM-1) and the class D

OXA-type enzymes (OXA-48-like). Additionally, the upregulation of efflux pumps or loss of porins and the upregulation of ESBLs or AmpC β-lactamases can lead to carbapenem resistance (Paczosa & Mecsas,2016). Because Carbapenemase genes are carried on mobile genetic elements , these genes can be spread horizontally to bacteria, thus contributing to the reservoir of resistance in both environmental and clinical Enterobacteriaceae (Lutgring *et al.,* 2016).

2.7 multi-resistance to antibacterial agent's Multidrug resistance (MDR)

Refers to acquired resistance to at least one antimicrobial agent from three or more antimicrobial classes (Sweeney *et al.,* 2018). The spread of carbapenem-resistant *K. pneumoniae* isolates, both locally and internationally, creates a therapy problem due to less effective antibiotics for therapy, leading to an increase in the evolution of extensively drug-resistant (XDR) and pan drug-resistant (PDR) Gram-negative bacteria (Alkofide *et al.,* 2020).

Extensively drug-resistant (XDR) is defined as an isolate that is resistant to at least one agent in all but one or two available classes and pan drug-resistant (PDR) is defined as an isolate that is resistant to all agents in all available classes (Sweeney *et al.*,2018).XDR for acquired nonsusceptibility to at least one antimicrobial agent in all antimicrobial classes, with the exception of two or fewer antimicrobial classes, and PDR for acquired nonsusceptibility to all antimicrobial agents from all classes (Bedos *et al.,* 2021).

2.8 The β-Lactam Antibiotics

The β-lactam antibiotics inhibit the cell-wall biosynthesis, there are a group of antibacterial comprising four major groups: penicillins, cephalosporins, monobactams and carbapenems (Dik *et al.,* 2018).They consisted of a β-lactam ring, which consists of three carbon atoms and one

nitrogen atom and is linked to a thiazolidine ring. All β-lactams antibiotics have a similar molecular structure (a beta-lactam ring) (Zango *et al.,* 2019), in the carbapenems the β-lactam ring is joined with a hydroxyethyl side chain, deficient of an oxygen or sulphur atom in the bicyclic nucleus (Ali *et al.,* 2018).The β-lactams inhibit the bacterial cell wall, peptidoglycan, and manufacturing by obstructing the exact function of the penicillin binding protein, which is also referred to as transpeptidase (Ramakrishna *et al.,* 2014).

The Beta-lactam antibiotics function by disrupting the structure of the peptidoglycan layer of the bacterial cell wall. The peptidoglycan layer is critical to the construction of the cell wall, particularly in Gram-positive species. Penicillin binding proteins assist the last transpeptidation step in the production of peptidoglycan (PBPs). Beta-lactam antibiotics differ in their affinity for PBPs (Pandey & Cascella, 2019).

Resistance to B-lactam antibiotics may be attributed to the antibiotics' inability to reach their sites of action via porin channels in the outer cell wall. Several minor hydrophilic antibiotics, including β-lactams, tetracycline, chloramphenicol, and fluoroquinolones, diffuse through aqueous channels in the outer membrane formed by porin proteins (Omp) (Ghai & Ghai, 2018).

2.9 Carbapenems

Carbapenems are a group of beta-lactam antibiotics used to treat infections caused by extended-spectrum beta-lactamase (ESBL)-producing bacteria (Safavi *et al.*,2020). Carbapenems, among the beta-lactams, are the most effective against Gram-positive and Gram-negative bacteria presenting a broad spectrum of antibacterial activity (Meletis *et al.*,2016).

Carbapenems are the main antimicrobial agents of choice for treating infections caused by extended-spectrum beta-lactamases (ESBLs) and Amp

producing Gram-negative bacteria (Shahbazi *et al.*,2018). Carbapenems are beta-lactams antibiotics such as other antibiotic penicillins (cephalosporins and monobactams), it has five-membered ring, but the sulfur at C-1 in the five-membered ring is replaced by a carbon atom and a double bond between C-2 and C-3 has been introduced (Silhavy *et al.*,2010).

Carbapenems were regarded as the most effective treatment against multidrug-resistant *K. pneumoniae* and were often used as such medications of last resort (Pyakurel *et al.,* 2021). For carbapenems, the characteristic setting of the side chain in the trans position instead of the cis position, commonly found in other β-lactams made them insensitive to the effects of β-lactamases (Aurilio *et al.,* 2022). Carbapenems are1980s-era molecules with the broadest range of antibacterial action among all beta-lactams, including numerous extended-spectrum beta-lactamases (ESBLs),there are four carbapenem members clinically used: imipenem, meropenem, doripenem, and ertapenem (Tarnberg *et al.*,2012).

2.10 Mechanisms of resistance to carbapenems

Mechanisms of carbapenem resistance may include the formation of Carbapenemases (β-lactamases with the capacity to hydrolyze carbapenems), efflux pumps, and changes in the expression and/or activity of porins and penicillin-binding proteins (PBPs). The most prominent mechanism of carbapenem resistance by CRKP is the production of various Carbapenemases (Aurilio *et al.,* 2022).

The Ambler classification of Carbapenemases in *K. pneumoniae* identifies three major types: class A, or serine-lactamases (*KPC*); class B, *IMP*, *NDM* and *VIM* type Carbapenemases in CRKP; and class D, or oxacillinase-hydrolyzing Carbapenemases (OXA) (Pourgholi *et al.,* 2022).

2.10.1 Decreased Permeability

Resistance to β-lactam antibiotics may be attributed to the antibiotics' inability to reach their sites of action via porin channels in the outer cell wall. Several minor hydrophilic antibiotics, such as β-lactams, tetracycline, and chloramphenicol and fluorochinolones diffuse via aqueous channels generated by proteins (Omp) called porins in the outer membrane (Ghai & Ghai, 2018).

2.10.2 Overexpression of Efflux Pump

Active efflux pumps serve as another mechanism of resistance, removing the antibiotic from its site of action before it can act, multidrug efflux pumps are incorporated into bacteria and their purpose is to transport antibiotics outside the outer membrane of bacteria. This mechanism is promoted by plasmids and others mobile transporters. Several minor hydrophilic antibiotics, including β-lactams, tetracycline, chloramphenicol, and fluoroquinolones, diffuse through aqueous channels in the outer membrane formed by porin proteins (Omp) (Aurilio *et al.,* 2022).

This active efflux system is comprised of a complex of specialized proteins that form a bridge between the cytoplasmic membrane and the outer membrane. A carrier protein in the cytoplasmic membrane, capable of capturing molecules located in the membrane or the cytoplasm, is linked to an "accessory protein," connected in turn with an outer membrane protein channel (Ali *et al.,* 2018).

2.10.3 Mutation and Transformation in Antibiotic Target Structures

The mutation of the antibiotic target is another mechanism that might lead to antibiotic resistance. A succession of gene mutations may develop antibiotic resistance to streptomycin, quinolones, rifampin, and other antibiotic classes encoding the target protein, the drug transporter, and/or the drug activation protein. The mechanism of mutation may occur in a functional target with a

decreasing affinity for the antibiotics, which do not work completely and behave with reduced efficiency (Kamoshida *et al.,* 2020).

2.10.4 Modification of Antibiotics by the Hydrolysis of the Molecule

The modification of antibiotics by hydrolysis is a major mechanism of antibiotic resistance that has been relevant since the first use of antibiotics, with the discovery of penicillinase (a β-lactamase) in 1940. Afterwards, the β-lactam-hydrolyzing enzymes have broadened their activity from penicillinases, followed by cephalosporinases, then extended to spectrum β-lactamase ESBLs and, most recently, to the mannose-binding MBLs for lectines and other Carbapenemases (Codjoe& Donkor, 2017).

2.11 Beta-lactamases

Lactamases are bacterial enzymes that hydrolyze the β-lactam ring of β-lactam antibiotics, making them inactive. β- Lactamases are classified into four molecular types based on mechanism, conserved residues, and sequence homology. To hydrolyze the β-lactam bond, β-lactamases of Classes A, C, and D employ a conserved serine-based mechanism. Metallo-β-lactamases of class B use a Zn^{2+} based mechanism to catalyze the hydrolysis of the β-lactam link. For the sake of this review, modifications to class A and class C β-lactamases are explored using Ambler and colleagues' standardized numbering method and structural alignment-based numbering of class C β-lactamases, respectively (Papp-Wallace *et al.,* 2020).

Two classification systems for these enzymes are currently in use. The first is Ambler molecular classification which is based on the conserved residues and protein sequence into four such as classes A, B, C, and D enzymes. These enzymes utilize serine for β-lactam hydrolysis and class β-metallo enzymes that require divalent zinc ions (metal ion) for substrate hydrolysis (Ali *et al.,* 2018).

Beta-lactamases are classified into four main groups based on their amino acid sequences (classes A, B, C and D). The production of all four classes of beta-lactamases (A, B, C and D) is generally chromosomally encoded. Class A includes extended spectrum beta-lactamases (ESBLs) and *Klebsiella pneumoniae* Carbapenemase enzymes; class B enzymes are the metallo-B-lactamases (MBLs) that have a broad substrate range, being able to inhibit all beta-lactam antibiotics except the monobactams (Aurilio *et al.*,2022), the hydrolysis activity of MBLs depends on the interaction of the beta-lactam molecule with Zn^{2+} ion(s) in their active site. Consequently, their activity is inhibited by chelators of divalent cations, such as EDTA (Dortet *et al.*, 2014).

Class C enzymes are the cephalosporinases, broadly disseminated enzymes usually resistant to (cephamycins, cefoxitin and cefotetan), penicillins and cephalosporins; and class D enzymes are oxacillinases (Bush & Bradford., 2020).

The increase in ESBL-producing bacteria has enlarged the clinical use of carbapenems as well as increasing the number of carbapenem hydrolyzing activity. These enzymes known as Carbapenemases, are a large variety and were identified for the first time in Enterobacteriaceae and were divided into the Ambler four classes of beta-lactamases, class A , B, C and D, and all are characterized by having a part in common of serine in the target (Queenan *et al.*, 2010 ; Aurilio *et al*.,2022).

Lactamases are classified by various schemes, the most useful of which describe both their functional and structural profile. Enzymes in groups 1 and 2 (Ambler class C or A and D respectively) contain an active site serine, but group 1 (primarily AmpC enzymes) members demonstrate preferential hydrolysis of cephalosporins whereas group 2 enzymes (including ESBLs, *Klebsiella pneumoniae* Carbapenemase [KPC], and OXA enzymes) are active on a wider range of substrates, extending as broadly as the extended-

spectrum cephalosporins and monobactams (ESBL) and the carbapenems (KPC and OXA-48-like). Metallo-beta-lactamases (MBLs) comprise group 3 (Ambler class B); these include IMP (active on imipenem), VIM (Verona integron-encoded MBL), and NDM (New Delhi MBL), among others (Lutgring & Limbago, 2016).

MBLs are characterized by the requirement for zinc ions in their active site which can be useful diagnostically, as chelators like EDTA inhibit MBL activity by binding zinc. MBLs demonstrate broad-spectrum-lactamase activity, including Carbapenemase activity, but are not active against monobactams. This particular characteristic is not often clinically useful, as most MBL producers also produce other beta-lactamases that result in monobactam resistance (Bush & Jacoby, 2010).

2.12 Carbapenemases

These enzymes are of a large variety, and they were identified for the first time in Enterobacteriaceae. They were divided into the Ambler four classes of beta-lactamases, classes A, B, C, and D, and all were characterized by having a part in common with serine in the target. Their function is to inactivate beta-lactam antimicrobials, and among these are mostly carbapenems. (Queenan *et al.,* 2010).The serine Carbapenemase KPC was first identified in *K. pneumoniae* in 1996; these enzymes use Zn (II) as a critical cofactor for the breakdown of the beta-lactam ring, rendering these antimicrobial agents ineffective (Alizadeh *et al.,* 2021).

2.12.1 Class A Carbapenemases

Class A Carbapenemases are chromosomally encoded (SME, NmcA, SFC-1, BIC-1, PenA, FPH-1, SHV-38), plasmid-encoded (KPC, GES, FRI-1), or both (IMI). Among these, the best known KPC (*Klebsiella pneumoniae Carbapenemase*) was spread all around the world and has been isolated in most of the clinical enterobacterial species such as *P.aeruginosa* and *A.*

baumannii. Generally, class A Carbapenemases reduce susceptibility to imipenem for the bacteria sensitive to it and allow the hydrolysis of a broad variety of beta-lactams, including carbapenems (Aurilio *et al.*, 2022).

2.12.2 Class B Carbapenemases

The classes B of the Ambler group belonging to the (MBLs) are clinically the most relevant Carbapenemases. These are divided into three subclasses: B1, B2, and B3, but the largest number of clinically relevant MBLs belong to the B1 subclass, including the most frequently Verona integron-encoded MBL (VIM), Imipenemase (IMP) and New Delhi MBL (NDM). Those MBLs are generally located within different integron structures, which are connected with mobile plasmids or transposons facilitating the transfer of resistance genes between bacteria (Aurilio *et al.*, 2022).

MBLs can hydrolyze all beta-lactams except for aztreonam and are not inhibited by the inhibitors. Their suppression in vitro is accomplished by metala chelators, such as ethylenediaminetetraacetic acid (EDTA), since they contain zinc (Meletis *et al.*,2016)

2.12.3 Class D Carbapenemases

Class D Carbapenemases (OXA-type beta-lactamases) are a class of enzymes found in human strains of *A. baumannii* and *K.pneumoniae* (Aurilio *et al.*, 2022). These Carbapenemases, especially OXA-48 and similar variants, are clinically significant because they make it challenging to treat infections (Boyd *et al.*, 2022).

Beta-lactamase inhibitors (e.g., amoxicillin-clavulanic acid) inhibit class A β-lactamases but not class D Carbapenemases in clinical treatment. To make carbapenem antimicrobials effective, it is crucial to discover inhibitors of *A. baumannii* class D Carbapenemases in the near future (Aurilio *et al.*, 2022).

2.12.4 Class C Carbapenemases

Carbapenemases are not regarded as class C enzymes. However, it has been shown that they have a limited carbapenem hydrolysis potential, and their overproduction may lead to carbapenem resistance and adamaged outer membrane increased permeability or efflux pump (Meletis., 2016).

2.13 Carbapenemase variants (Genes):

The most effective Carbapenemases, in terms of carbapenem hydrolysis and geographical spread are *KPC, VIM, IMP, NDM* and *OXA-48* types (Poirel *et al.,* 2012):

2.13.1 KPC (*Klebsiella pneumoniae Carbapenemase*)

Klebsiella pneumoniae Carbapenemases (KPCs) is regarded as one of the most significant enzymes involved in the degradation of β-lactam antibiotics, since it works to break down the β-lactam ring and impede the activity of these antibiotics, particularly class A antibiotics (Martin & Bachman., 2018).

They were discovered for the first time in the late 1990sa in the New York City region of the United States (Yigit *et al.,* 2001).

2.13.2 (Oxacillin-hydrolyzing carbapenemases) OXA gene *(blaOXA48)*

The OXAs are characterized by an important genetic diversity and a great heterogeneity in terms of β-lactam hydrolysis spectrum. The acquired OXAs possess either a narrow spectrum or an expanded spectrum of hydrolysis, including carbapenems in several instances. Acquired class D β-lactamase genes are mostly associated to class 1 integron or to insertion sequences (Peirano & Pitout., 2019).

Six varieties like OXA-48 have been found so far, with OXA-48 being the most prevalent. A few amino acid changes or deletions distinguish them (one to five amino acids). The enzymes hydrolyze penicillins to a high degree and

carbapenems to a low degree, while sparing broad-spectrum cephalosporins and they are resistant to β-lactamase inhibitors, since its discovery in 2004 (Poirel *et al.*, 2012 ; Mmatli *et al.*,2020).

The *bla*$_{OXA-48}$-type genes are invariably plasmid-borne, and insertion sequences associated with their acquisition and expression have been found. The present propagation of the *bla*$_{OXA-48}$ gene is mostly attributable to the transmission of a 62-kb IncL/M-type plasmid that does not include an extra resistance gene.OXA-48-type carbapenemases have been isolated mostly from North African, Middle Eastern, Turkish, and Indian (Poirel *et al.*,2012).

Acquisitions of *bla*$_{OXA48}$ like genes have only been detected in Enterobacteriaceae but never in other Gram-negatives like *A. baumannii* or *P. aeruginosa*. One of the key challenges for regulating the spread of OXA48-like producers is the lack of phenotypic testing that would facilitate their identification (Nordmann *et al* .,2011).

2.13.3 New Delhi metallo-beta-lactamase (NDM) gene (*blaNDM)*

Members of the NDM family are among the most prevalent carbapenemases since it can hydrolyze all beta-lactam antibiotics except monobactam (Aztreonam)(Rawson *et al.*, 2022), which are often transmitted by horizontal plasmid transfer, *bla*$_{NDM}$ including *bla*$_{NDM-1}$, *bla*$_{NDM-5}$ and *bla*$_{NDM-7}$are distributed in different sequences types (ST) (Safavi *et al.*,2020).Since its identification in 2008 in India, NDM has shown a remarkable worldwide expansion. NDM-producing microorganisms have been identified in a number of countries spread from healthcare facilities and environmental niches in the Indian subcontinent to Europe, North America, the Far East, and Australia through patient transfer or colonized travelers (Meletis *et al.*,2016).

Like all other MBLs, NDM-1 efficiently hydrolyzes a broad range of lactams, including penicillins, cephalosporins, and carbapenems, sparing monobactams such as aztreonam. They are located mostly on conjugative plasmids belonging to several incompatibility groups (Dortet *et al.*,2014).

The *bla*$_{NDM}$ gene may be encoded on plasmids or transposons with the potential to transmit horizontally across gram-negative species, and it may be associated with a variety of insertion sequence types. The distinct character of mobile genetic components associated with acquired metallo-beta-lactamase genes may explain their propensity to spread. The majority of integrons with gene cassettes include resistance determinants for other antibiotic classes, such as aminoglycosides (Porretta *et al.*, 2020). Beta-lactam antibiotics act by disrupting the structure of the peptidoglycan layer of cell wall of the bacteria. The peptidoglycan layer is essential for cell wall structure especially in Gram-positive organisms. In the synthesis of the peptidoglycan, the final transpeptidation step is facilitated by penicillin binding proteins (PBPs). Beta-lactam antibiotics vary in terms of PBPs affinity (Pandey and Cascella, 2022). Penicillins are the first antibiotics to be commercialised in the 1940's. They are derived from Penicillium fungi and divided in sub groups depending on their antimicrobial spectrum and stability against penicillinases. The main feature of the penicillins is the four membered beta-lactam ring structural that essential for their antibacterial activity.

2.13.4 (IMP) Imipenemase gene *(blaIMP)*

In 1988, Japan was the first country to identify the *blaIMP-1* gene. The isolates conferred carbapenem (e.g., imipenem) and extended-spectrum cephalosporin (e.g., ceftazidime) resistance (Pongchaikul & Mongkolsuk ,.2022). In 1991, *bla*$_{IMP-1}$ was the first MBL to be identified. Since then, over 55 distinct *bla*$_{IMP}$ alleles have been characterized. The order of these enzymes varies, but they have almost the same impact on their resistance

potency. The majority of identified variations are found in *P. aeruginosa* and other gram-negative organisms (Subedi *et al.*, 2018).

2.13.5 Verona Integron Metallo Beta-lactamase (VIM) gene (*blaVIM*):

The Verona Integron-encoded Metallo-β-lactamase(VIM) is among the most common MBLs causing human infection. In healthcare facilities in countries such as Greece, Italy, and Spain, this carbapenemase has emerged as a major danger during the last decade. The presence of the *bla*$_{VIM}$ gene on gene cassettes of class 1 integrons, which are often found on mobile genetic elements like transposons and plasmids, helps explain the gene's widespread distribution , the presence of the *bla*$_{VIM}$ gene on gene cassettes of class 1 integrons, which are often found on mobile genetic elements like transposons and plasmids, helps explain the gene's widespread distribution (Kohler *et al.*,2020).

The addition of zinc^{2+} restored carbapenem-hydrolyzing activity that had been reduced by ethylenediaminetetraacetic acid (EDTA). 1996 witnessed the first identification of *bla*$_{VIM-2}$ in a blood culture from the south of France. Monobactams (Aztreonam) are monocyclic compound active against gram negative multidrug-resistant including *P. aeruginosa*, *Acinetobacter spp*., and *Burkholderia spp*, by binding to PBPs of bacteria and damage the cell wall synthesis. It is stable towards metallo-beta-lactamases and also has some inhibitory activity towards class C beta-lactamases (Gangadharappa *et al* .,2020).

Carbapenems are compounds started to use in the 1980's, it has the widest spectrum of antibacterial activity of all the beta-lactams, including many of the extended spectrum beta-lactamases (ESBLs) (Balsalobre *et al.*, 2019). Similar to other antibiotics like penicillin, carbapenems inhibit the bacterial cell wall synthesis by binding to PBP of gram positive and gram-negative bacteria, leading to cell elongation and lysis. Resistance to

carbapenems by imipenem is most commonly due to reduced permeability, while meropenem resistant has a combination of porin loss and increase the rate of efflux pump. These antibiotics show less resistance as compared to other types of beta-lactams because of their stability to be hydrolysed by many ESBLs (Vázquez-Ucha *et al.,* 2020).

Chapter Three

Materials

and

Methods

Chapter Three: Materials and Methods

3.1. Materials

3.1.1. Equipment's and Apparatus

Equipment's and apparatus that used in this study are listed below table 3.1.

Table 3.1. Laboratory equipments and apparatus

Name of apparatus	Company	Origin
Autoclave	HIRAYAMA	Japan
Bact AlERT 3D	BioMerieux	France
Balance	METTLER TOLEDO	SWITZEL
Biological safety Cabinet	BIOAIR	Italy
Centrifuge	HITTICH	Germany
Electrophoresis Equipment	CLEAVER SCIENTIFIC Ltd.	UK
Gel Documentation Imaging	QUANTUM	France
Hotplate	STUART	UK
Incubator	NIIVE	Turkey
Magnetic stirrer	UIKA RH basic 2	Germany
Micro – centrifuge	HITTICH	Germany
Microscope	NIKN	CHINA
Microwave oven	Gallenhamb	England
Oven	Gallenhamb	England

Prime Thermal Cycler (PCR)	Analytik jena	Germany
Sensitive Balance	SARTORIUS	Germany
UV Gel Documentation	Analytik jena	Germany
Vitek 2 compact	BioMerieux	France
Vortex	HEIDLPH	Germany

3.1.2 Biological and Chemicals Materials

The chemicals and biological materials that used in this study are listed in table 3.2 .

Table 3.2 Chemicals and biological materials

Chemicals	Company	origin
Absolute ethanol	Himedia	India
Agarose	Condalab	Spain
Antibiotic disk	Liofilchem	Liofilchem
DNA Ladder 100–1500 pb	Promega	USA
Glycerol	Merck	England
Gram stain: crystal violet, Iodine acetone and safranin	RBIO	Switzerland
Green Star^{tm} Nucleic Acid Staining Solution	Promega	USA
Normal Saline (Sterile)	PSI	Saudi Arabia
Nuclease Free Water	Promega	USA
Tris-Borate-EDTA (TBE)	Himedia	India

3.1.3.Culture Media

All cultures media that are used in this study are listed in table 3.3.

Table 3.3 : Culture Media

Medium	Company	rigin
Blood Agar	HIMEDIA	India
Brain heart infusion broth	HIMEDIA	India
MacConkey Agar	HIMEDIA	India
Muller Hinton Agar	HIMEDIA	India

3.1.4. Antibiotic Agents

All antibiotics that used in this study for susceptibility test and phenotypic detection in table 3.4.

Table 3.4. Antibiotic Agent and their Concentration

Antibiotic Group	Antibiotic Disk	Concentration
Aminoglycoside	**Amikacin**	**30 µg**
	Gentamicin	**30 µg**
beta-Lactam	**Piperacillin-tazobatom**	**100 µg/10µg**
	Ceftazidime	**30 µg**
Cephems(Cephalosporins)	**Cefepime**	**30 µg**
	Cefotaxime	**30 µg**
Carbapenem	**Imipenem**	**10 µg**
	Imipenem / EDTA	**10 µg/750 µg**
	Meropenem	**10 µg**
Fluoroquiolone	**Levofloxacin**	**5 µg**

	Ciprofloxacin	**5 µg**
Monobactam	**Aztreonam**	**30 µg**
Sulfonamide (folate pathway)	**Trimethoprim-sulfamethoxazole**	**25 µg**

3.1.5 Kits

The Kits that used in this study are listed in table 3.5.

Table 3.5 Laboratory Kits

Kits	Company	origin
DNA Extraction Kit	FAVORGEN	Austria
Taq Master Mix	PROMEGA	USA
VITEK 2 Kit	BioMerieux	France

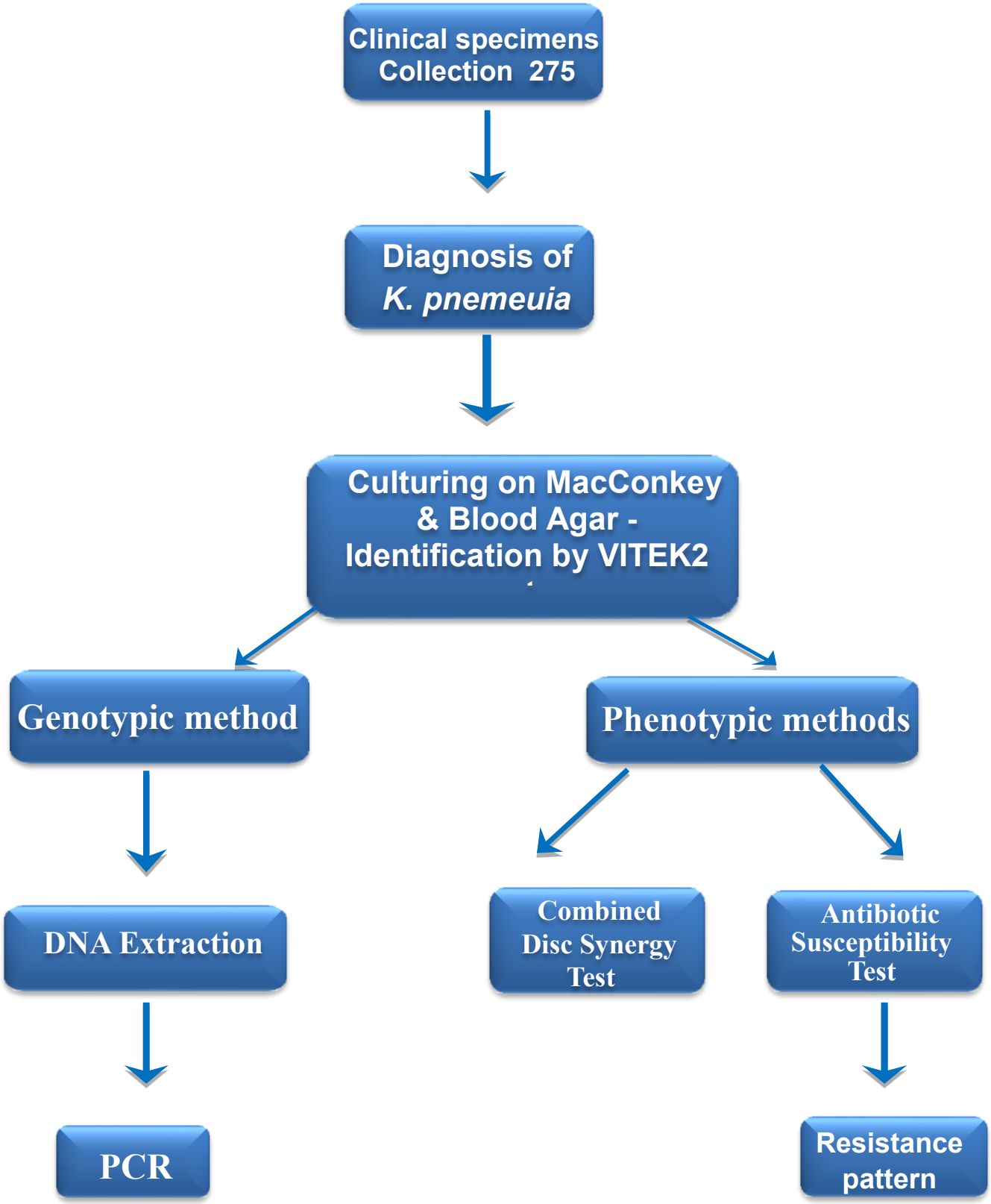
3.1.6. Primer Pairs

The primers pairs sequence product size and their PCR conditions that used in this study are listed in table 3.6.

Table 3.6 Sequences products size and references of primers used for PCR amplification of genes.

Primer	Sequence 5 to 3	Product size	Anneali (Step 3)	Reference
***blaIMP-* F**	GGAATAGAGTGGCTTAAYTC	232bp	57℃ 30sec	**(Pourgholi *et al* ., 2022)**
blaIMP-R	TCGGTTTAAYAAAACAACCACC			
***blaVIM-* F**	GATGGTGTTTGGTCGCATA	390bp	57℃ 30sec	**(Pourgholi *et al* ., 2022)**
***blaVIM*-R**	CGAATGCGCAGCACCAG			
***blaNDM-* F**	GGTTTGGCGATCTGGTTTTC	621bp	52℃ 30sec	**(Pourgholi *et al* ., 2022)**
blaNDM-R	CGGAATGGCTCATCACGATC			
blaKPC-F	CGTCTAGTTCTGCTGTCTTG	798bp	55℃ 30sec	**(Pourgholi *et al* ., 2022)**
blaKPC-R	CTTGTCATCCTTGTTAGGCG			
blaOXA 48-F	CTTGATCGCCCTCGATT	281bp	57℃ 30sec	**(Dallenne *et al*., 2010; Gurung *et al* ., 2020)**
blaOXA48 -R	GATTTGCTCCGTGGCCGAAA			

3.1.7 Study design



3.2. Method

3.2.1. Specimens Collection

During the period from September 2022 to December 2022, two hundred and seventy-five (275) specimens (blood, burn, urine, sputum, wound, high vagina swab, pharynx swab and ear swab) were collected from various clinical sources.

The specimens were collected from hospitals in Kerbala (Imam Hussein Medical Education City, Imam Hassan Al-Mojtaba Medical City, Imam Hassan Center for glands and diabetes, obstetrics, and Gynecology Hospital) and around outpatient clinics in Kerbala.

Table 3.7. Distribution of Specimens

Source of Samples	Male	Female	Total (Percentage)
Burns swab	7	3	10 (4.09)%
Wound swab	17	13	30 (11.88)%
Ear swab	6	5	11(4.5)%
Pharynx swab	6	8	14 (5.09)%
High vagina swab	-	30	30 (10.95)%
Blood	6	4	10(4.09)%
Urine	19	81	100(36.36)%
Sputum	46	24	70(25.45)%
Total	**107**	**168**	**275**

2.2. Isolation of *Klebsiella pneumoniae*:

All collected specimens were grown separately on MacConkey agar and then incubated for 24 hours at 37°C. After incubation and depending on their

colonies morphology (shape, size, color and texture) isolates suspected to belonging to *Klebsiella* genus were taken for identification (Patel *et al.*, 2017).

3.2.3. Preparations of Culture Media

All media used for culture in this study (MacConkey agar, Blood agar , Brain Heart Infusion broth, Mueller – Hinton Agar (MH)) were prepared using their outline methods according to the manufactures instructions as described below:

3.2.3.1. MacConkey agar

The medium was prepared by dissolving 51.53 gm of its powder to 1 litre of distilled water and sterilized by autoclave at 121°C for 15 minutes. The medium is poured into sterile Petri dishes after being cooled down and then left to solidify at room temperature. This medium is utilized for detection the family Enterobacteriaceae (Shakib *et al.*, 2018).

3.2.3.2. Brain Heart Infusion Broth

It was prepared by dissolving 37 gm of the medium powder in (1) L of distilled water and autoclaved at 121°C for 15 minutes, after that its left to be cooled at room temperature to be poured then in plane tubes. This media is used for activation of bacteria or to keep it for long time in freezer after adding glycerol of 15 % concentration (Shakib *et al.*, 2018).

3.2.3.3 Blood agar

Blood agar base media 40 gm /1 L of D.W was suspended, then heated until the materials completely dissolved and sterilized by autoclaved at 121℃. After cooling to 45°C, 5% v/v sterile blood was added to the medium, mixed well and poured into sterile petri dishes (Shakib *et al.*, 2018).

3.2.3.4 Blood Culture

After collecting blood samples from patients with cardiac catheterization,

within a period not exceeding an hour, they were transferred to the laboratory and placed in a device BACT/ALERT 3D from 1–7 days of daily examination, and the positive result was planted on blood acres by drawing a small amount of the bacterial culture by means of a medical syringe after sterilizing the bottle cap with alcohol (pathological vial), and after the appropriate incubation period and the appearance of growth in the medium, the diagnosis of the developing bacteria was made (Ransom *et al* .,2019).

3.2.3.5 Mueller - Hinton Agar

The medium was made in accordance with the manufacturer's instructions by dissolving (38) gm of it in (1)L of distilled water, autoclaving it at 121°C for 15 minutes, and then pouring it into Petri plates at 40–45°C. This medium was used in antimicrobial susceptibility and inhibitory activity testing (Shakib *el at.*, 2018).

3.2.4 Preparation of Solutions

To prepare the following solutions:

3.2.4.1 McFarland Turbidity Standard

Prepare this solution according to the method described by (Shakib *el at.*, 2018)

It consists of two solutions:

A. Barium chloride solution (1.175%) : 1.175 gm of barium chloride were dissolved in a quantity of distilled water, and after completing the dissolution, the volume was added to 100 ml with distilled water.

B. Sulfuric acid solution (1%) :1 ml of concentrated sulfuric acid was added to 99 ml of distilled water.

Prepare McFarland's solution (tube No. 0.5) by adding 0.5 ml of solution A to 99.5 ml of solution B and mixing the solution well.

3.2.4.2. Tris-Borate-EDTA (TBE) Buffer

Tris-Borate-EDTA (TBE) is the most commonly utilized buffer for RNA and

DNA gel electrophoresis. The 10x solution was prepared by dissolving (10^8) g of Tris base, 55 gm of boric acid, and 40 ml of 0.5 □ EDTA in 1000 ml of distilled water. However, the final concentration of working stock solution is prepared by adding 100 ml of 10× TBE buffer to 900 ml of sterile distilled water and stored at room temperature (Chen *et al.*, 2011).

3.2.5. Identification methods of *Klebsiella pneumoniae* isolates

3.2.5.1 Morphological Test:

Morphology examination is carried by observation the characteristics of colonies of bacteria that grow on MacConkey agar Blood agar containing 5% blood in terms of shape, color, pigment forming, odor, texture, hemolysis and fermentation of lactose. In addition, the isolates were examined under a microscope by producing a bacterial smear on a slide and using Gram stain to identify the cell's shape and color (Patel *et al.*, 2017).

3.2.5.2 VITEK 2 system

The VITEK 2 Compact instrument, a computer, and a printer are all part of the system. Analysis and data-management software are included with the VITEK 2 Compact system by using Identification Gram-Negative Bacteria (ID-GNB) card. The manufacturer described the results as 96% to 100% excellent identification, 93% to 95% very good identification, 89% to 92% good identification, 85% to 88% acceptable identification, and no identification in other isolates. The colonies were identified by biochemical identification which was performed by Vitek-2 automated identification system that provides 64 chemical tests, the incubation period from 18-24 hours. In this study, the VITEK 2 system was utilized in this investigation to confirm *Klebsiella* identification at the species level and to eliminate variability in biochemical test (Hamam *et al.*, 2019).

3.2.6 Identification of Antibiotic susceptibility of *Klebsiella pneumoniae*

Identification of drugs susceptibility of *Klebsiella pneumoniae* isolates involved

phenotypic methods and genotypic methods, as described below:

3.2.6.1 Phenotypic methods

3.2.6.1.1 Antibiotic Susceptibility Testing

Minimum inhibitory concentrations (MICs) were determined using the broth microdilution method set by the Clinical Laboratory Standards Institute (CLSI 2022). Determination of the minimum bactericidal concentrations (MBCs) was performed by subculturing 10 µl from each well without visible microbial growth. After 24 hours of incubation the bacterial three colonies or less were scored as the MBC/MFC as described by the (CLSI, 2022) for starting inoculant of 1×10^5 CFU/ml (Pourgholi *et al.*, 2022).

The susceptibility of *Klebsiella pneumoniae* to 12 antibiotics agents for 7 classes Carbapenem antibiotics including Meropeneme and Imipenem, Cephems group including Cefipeme and Cefotaxime, beta lactam antibiotics group including Ceftazidime and piperacllin/tazobactam, Aminoglycoside including Amikacin, Gentamicin, Monobactam including Aztreonam, Fluoroquinolone including Levofloxacin and Ciprofloxacin, Sulfonamide(folate pathway) including Trimethoprim-sulfamethoxazole ,it was determined by the Kirby-Bauer disk diffusion method according to (CLSI , 2022) instructions(Pourgholi *et al.*, 2022). Antibiotics used for this test are listed in table (3.4). Activation of isolates were performed using MacConkey Agar plate culture for 24 hours at 37°C and the growth was transferred to a tube containing 3 ml of normal saline. The turbidity was adjusted to (0.5) McFarland tube equal to 1.5×10^8 CFU/ml standard, and then spread on Muller Hinton agar by a sterile cotton swab was used to inoculate the dried surface of the Mueller-Hinton agar plates by streaking across the whole agar surface three times. Antibiotic disks were placed onto (MHA) inoculated with bacteria and incubated for 24 hours at 37°C and then inhibition zone diameter was recorded and interpretation as a sensitive, intermediate or resist (CLSI, 2022). In this study, carbapenem resistant *K. pneumoniae* isolates were defined as carbapenem non-susceptible (intermediate or resistant) based on

the CLSI (2022). MDR was defined as acquired resistance to at least one agent in three or more antibiotic classes (Sweeney *et al.,* 2018). Extensively drug-resistant bacteria XDR are those resistant to at least one antibiotic in all but one or two classes. PDR is defined as antibiotic resistance to all antibiotic classes (Alkofide *et al.,* 2020).

3.2.6.1.2 Combined Disc Synergy Test

Combined disc synergy test for detection of metallo β-lactamases (MBLs) using the Imipenem-EDTA was performed as described by (Chowdhury *et al*., 2016). After growing bacteria on MacConkey agar for 24 hours at 37°C, the inoculum density was adjusted to match 0.5 McFarland turbidity standards with sterile normal saline and then inoculated in Muller-Hinton agar. Imipenem (10 g) and imipenem-EDTA disks were put at a 15 mm edge-to-edge spacing and incubated at 37 °C for 18 hours. A carbapenem-nonsusceptible isolate with a 5 mm increase in the inhibition zone with carbapenem compared to carbapenem-EDTA disk alone was classified as an MBL generator (Thapa *et al*., 2017).

3.2.6.2 Genotypic method

The DNA is extracted from the bacterial cells, and then the genomic DNA is subjected to amplification using the primers listed in Table 3.6. To detect the genes responsible for the production of the enzyme carbapenase.

3.2.6.2.1 DNA Extraction from *Klebsiella pneumonia*

The Genomic DNA Kit was used for DNA extraction (Genomic DNA purification Kit FAVORGEN) by the following steps:

1. When the wash buffer is first opened, added ethanol (96-100%).

2. A suitable number of bacterial cells (up to 1 x 10^9) were transferred into a 1.5-ml micro centrifuge tube (not provided) and centrifuged for 1 minute at 14.000 rpm or 18.000 xg.

3. FATG Buffer 200µl was added and the pellet was resuspended by vortexing or

pipetting. Incubated at room temperature for 5 minutes.

4. Cell Lysis by following the Cultured Cell Protocol.

5. The sample was vortexed for 5 seconds after adding 200 µl of FABG Buffer.

6. The sample lysate was incubated for 10 minutes at 70 °C, or until it was clear.

The tube was inverted every 3 minutes during the incubation period.

7. Elution buffer was required to be preheated (for step 5 DNA elution) in a 70°C water bath.

8. Ethanol (96~100) 200 µl of DNA binding solution were added to the sample and vortexed for 10 seconds. To precipitate forms, pipette the sample to thoroughly mix it.

9. The FABG column was placed in a collection tube . The sample was carefully transferred to the FABG Column. For 1 minute, centrifuge at 14,000 rpm or 18.000 xg. Discard the collection tube and place the FABG column in a new collection tube.

10. After that (Column Washing) was added 400µl of W1 Buffer to the FABG Column and centrifuge for 30 sec at speed 14.000 rpm or 18.000xg.

Discard the flow-through and place the FABG Column back to the Collection Tube.

11. The column was centrifuged for 3 minutes at 14.000 rpm or 18.000 xg.

12. The electrophoresis was migrated to ensure the presence of eluted DNA in the extract.

13. The micro centrifuge tube which contains eluted DNA was stored at 4ºC.

3.2.6.2.2 Primers Preparation

The primers were delivered in a lyophilized condition, according to the

instruction leaflet of the primer supplier (Humanizing Genomics Macrogen, Inc., South Korea); therefore, they were dissolved in 250µl of nuclease-free water and it took a 30 minute wait to make stock of primers. Before opening the cap, the tube was placed on the decline, and the appropriate amount of nuclease-free water was added and vortexed thoroughly to get the stock. The stock was kept at a temperature of -20 °C.

3.2.6.2.3 Polymerase Chain Reaction (PCR) Protocol

Conventional PCR were performed to amplify the target DNA using particular primer pairs. Single plex was used in this study, 12.5 µl of master mix, 2 µl of each primer, 5 µl of template DNA and 3.5 µl of Nuclease free water mixed in 25 µl of total reaction volume. The PCR cycle conditions described in table 3.8.

Table 3.8. The PCR cycle conditions of genes amplification used in this study.

Gene	Cycle	Temperature ˚C	Time
KPC	1	95ºC	5min
		95ºC	30sec
		55°C	30sec
		72°C cycles	1min
	1	72°C	5min
		4°C	
NDM	1	95ºC	5min
		95ºC	30sec
		52ºC	30sec
		72°C cycles	1min
	1	72°C	5min

		4℃	
VIM *IMP* *OXA48*	1	95ºC	5min
		95ºC	30sec
		57ºC	30sec
		72℃ cycles	1min
	1	72℃	5min
		4℃	

3.2.8.2 Agarose Gel Electrophoresis

Agarose was prepared at final concentration 0.5 gm agarose and was added to 50 ml 1X TBE (50 ml of 10X TBE) + 450 ml D.W). Buffer was boiled for two min. Then left to cool at 50 °C. Afterward (2.5µl) of Safe-Rad was added to agarose solution and was poured on preparing tray. A suitable comb was placed near one edge of electrophoresis tray. The comb was removed after hardening of agarose leaving wells. The gel was placed into the gel chamber that was filled with 1X TBE buffer (50ml of 10X TBE) + 950 ml of D.W); the gel was completely covered with the buffer. After that, the wells were loaded with 10 µl of PCR products, while 10 µl DNA ladder (100bp) was loaded in the first well to serve as marker during electrophoresis run, the power supply was set at 100V for 80 min. The agarose gel was removed from the tank and the gel was exposed to UV transilluminator (320 nm/360w) then photographed by digital camera. All gel electrophoresis criteria were accomplished using the method provided by Bartlett and Stirling (1998).

Chapter Four

Results

and

Discussion

Chapter Four: Results and Discussion

4.1 Isolation and Identification of *Klebsiella pneumoniae*:

A sixty-eight isolates of *K. pneumoniae* from the total of 275 specimens were obtained from patients in different hospitals of Kerbala province; Imam Hussein Medical Education City, Imam Hassan Al-Mojtaba Medical City, Imam Hassan Center for glands and diabetes, Martyr Thamer Hussein Center for Chest Diseases and Tuberculosis, Obstetrics and Gynecology Hospital and around Outpatient clinics during the period from September 2022 to December 2022. The sixty-eight isolates of *K. pneumoniae* were collected from different clinical specimens such as 22/68 (32.35%) isolates from sputum, 16/68 (23.52%) isolates from urine, 8/68 (11.76%) isolates from wound, 5/68 (7.35%) isolates from burns, 6/68 (8.82%) isolates from blood, 6/68 (8.82%) isolates from higher vagina swab (HVS), 3/68 (4.41%) isolates from pharynx , 2/68 (2.94%) isolates from ear swab as shown in figure 4-1.

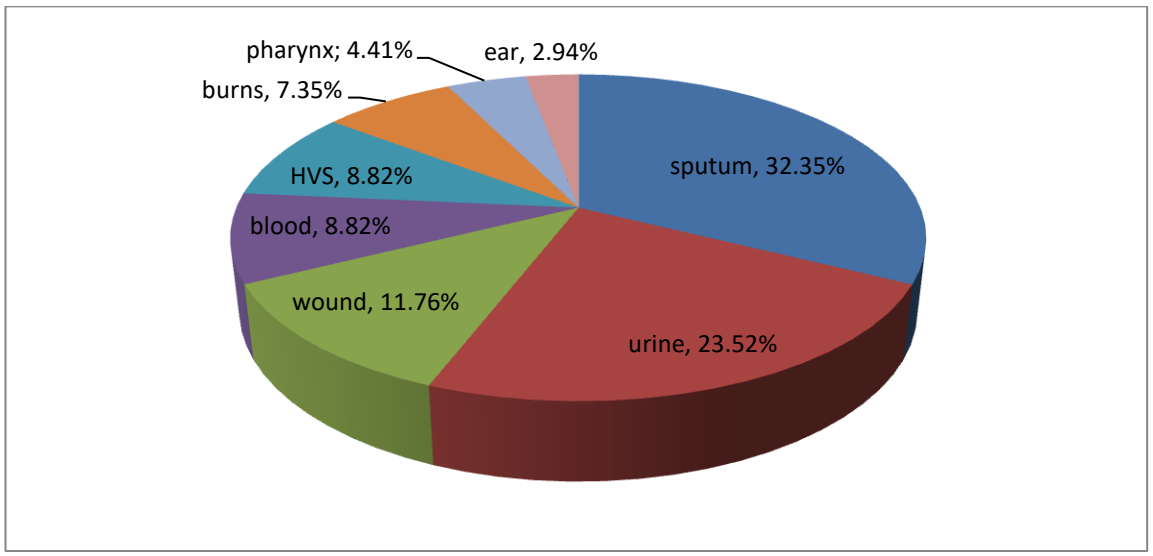


Figure 4-1: Distribution of *Klebsiella pneumoniae* isolates among different clinical specimens.

Klebsiella pneumoniae is a nosocomial pathogen that causes a broad spectrum of diseases and is increasingly resistant to antibiotics. It is becoming well known for its resistance to the majority of commonly used last-line antibiotics. It is particularly troubling in hospitals, where it causes a variety of acute infections (Effah *et al.,* 2020). The Females had a higher isolation rate than males (35:33) in this study that agreement with prior study in Kirkuk by (Hmood *et al.*,2021).

Figures 4-1 demonstrate that a greater distributional analysis of *K. pneumoniae* strains revealed that 32.35% of the isolates were collected from sputum, followed by urine. This ratio is agreement with the results of (Pyakurel *et al* .,2021) in Kathmandu, Nepal.

According to (Ashurst & Dawson .,2018) *K. pneumoniae* colonizes human mucosal surfaces and the most prevalent cause of hospital-acquired pneumonia . (Ranjbar *et al.*,2016), reported that *K. pneumoniae* is the second most prevalent cause of UTIs in hospital settings, which supports the result of the current study.

Initial identification of *K. pneumoniae* isolates based on morphological characteristics of the colonies on MacConkey agar and blood agar. *K. pneumoniae* isolates appeared mucoid, large, and pink on MacConkey agar due to lactose fermenting, while on blood agar they appeared as large, white, mucoid colonies without hemolysis as shown in figure 4-2. On blood agar, however, some isolates of *K. pneumonia* were found to be capable of producing hemolysis (Gharrah *et al.,* 2017). The microscopic examination of all isolates revealed gram-negative bacteria as short bacilli and as a single, short chain, or double chain of *K. pneumoniae (*Nithya *et al*.,2010; Mahon & Lehman 2022). According to the results, all *Klebsiella spp*. Isolates were identified as *Klebsiella pneumoniae,* and the survey validated the prevalence of *K. pneumoniae* in Kerbala hospitals.

(A) (B)

Figure 4-2: *Klebsiella pneumoniae* colonies on (A) MacConkey agar (B) Blood agar

4. 2 Antibiotic Susceptibilities of the *Klebsiella pneumoniae* Isolates

All isolates of *K. pneumoniae* which used in this study were subjected to susceptibility test by using 12 different antibiotic discs for 7 classes (anti β-lactam group, which includes a wide range of antibiotics, mainly includes Penicillins, Monobactams, Carbapenems, Cephalosporins) Fluoroquiolone, Aminoglycoside, Sulfonamide (according to CLSI 2022 instructions), as shown in figure 4-3; Antimicrobial susceptibility test of *K. pneumoniae* isolates.

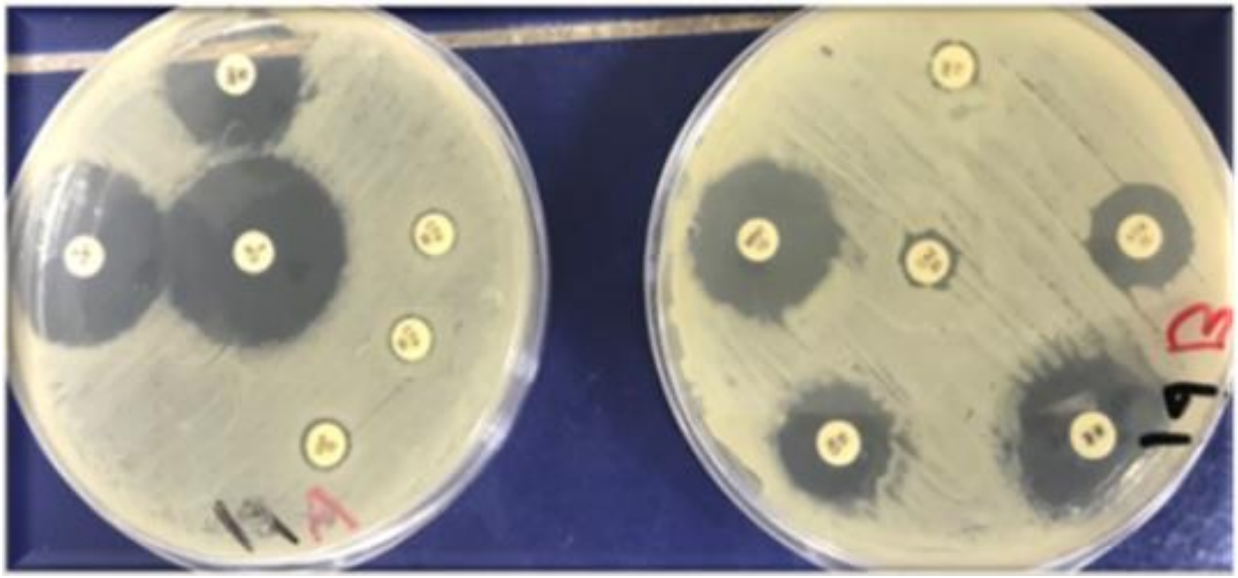


Figure 4-3: Antibiotic susceptibility test of *Klebsiella pneumoniae* isolates.

Table 4.1: Antibiotic susceptibility test for *Klebsiella pneumoniae* isolates.

Antibiotics	**Sensitive**	**Intermediate**	**Resistance**	**Total**
Cefotaxime 30µg	18 (26%)	-	50 (74%)	68
Levofloxacin 5 µg	39(57%)	4(5.8%)	25 (37%)	68
Piperacillin/Tazabactam 100/10 µg	29 (43%)	14(21%)	25 (37%)	68
Cefipeme 30 µg	20 (29%)	10(15%)	38(56%)	68
Trimethoprim/sulfamethoxazole 25 µg	37(55%)	2(3%)	29 (43%)	68
Ceftazidime 30 µg	18(26%)	5(6%)	45(66%)	68
Meropeneme 10 µg	32(47%)	8(12%)	28 (41%)	68
Imipenem 10 µg	5 (7%)	3(4%)	60(88%)	68
Amikacin 30 µg	33(49%)	5(6%)	30 (42%)	68
Gentamicin 10 µg	27 (40%)	6(9%)	35 (51%)	68
Ciprofloxacin 5 µg	30 (44%)	1(1%)	37(55%)	68
Aztreonam 30 µg	28 (41%)	1(1%)	39(57%)	68

Through the results of antibiotic susceptibility tests shown in the table 4-1 above that *K. pneumoniae* isolates to β-lactam group were highly resistant to Carbapenem antibiotics including Imipenem 60(88%), Meropenem 28(41%), and Cephems (Cephalosporins) group including Cefotaxime 50(74%), was agreement with the study performed by (Mirzaie and Ranjbar, 2021) Cefipeme 38(56%), Cefepime is the most common fourth-generation cephalosporin in hospital protocols. It is more stable to hydrolysis by ESBLs than the third generation cephalosporins (Teo *et al.*, 2012).

Penicillins group including Ceftazidime 45(66%), piperacllin/tazobactam 25(37%) , Monobactams group including Aztreonam 39(57%), Ciprofloxacin 37(55%). This agrees with what the study in Tehran (Pourgholi *et al*.,2022) which indicated that the number of carbapenemase-resistant strains showed an increasing trend over the last five years. These results contradict the results of similar studies in Isfahan, Iran, such as (Alizadeh *et al* .,2021).

The group of these antibiotics contains the β-lactam ring in its composition , bacterial resistance to these antibiotics can be achieved by the first three mechanisms: It is represented by the secretion of enzymes β-lactamase that degrade the beta-lactam ring present in these antibiotics. Second: reducing the bacteria's permeability to antibodies and thus preventing it from entering the cell. As for the third, it depends on changing the specific target of the antibiotic present in the cell, which makes it impossible to bind to the target intended for it, thus not killing the bacteria (Kumar &Varella.,2013). Some studies have found that β-lactam resistance can be activated genes in *K. pneumoniae* plays an important role in improving acquired β-lactamase-mediated β-lactam resistance (Wang *et al* ., 2020).

Gentamicin 35(51%), Amikacin 30(42%), which in agreement with (Jassim *et al*., 2022) , these antibiotics belong to the group of aminoglycosides that resist bacteria through several mechanisms. The first includes inhibiting the antibiotic through an enzyme that works to change the structure of the antibiotic by transferring an active group to the antibiotic, such as an acyl group , phosphoryl, Thiol and ribosyl group by changing the structure of the antibiotic due to the nucleotide transferase enzyme. The second is represented by modifying the target of the antibiotic through a process Methylation on 16srRNA and the third is by changing the permeability of the bacterial wall to antibiotics, thus preventing them from entering the bacteria (Kumar &Varella.,2013).

The findings also, have indicated that *K. pneumoniae* isolates were

intermediate to piperacllin-tazobactam 14(21%), Cefipeme 10(15%), meropenem 8(12%) and imipenem 3(4%), Gentamicin 6(9%), Amikacin and Ceftazidime 5(6%), Trimethoprim-sulfamethoxazole 2(3%), Ciprofloxacin and Aztreonam 1(1 %) .

The results of antibiotic susceptibility tests indicated that *K.pneumoniae* isolates were highly sensitive to Sulfonamide (folate pathway) group including Trimethoprim-sulfamethoxazole 37(55%), the use of Trimethoprim and sulfonamides together has a synergistic effect. Both drugs have an effect on bacterial folic acid production. Sulfonamides inhibit dihydropteroate synthetase , the enzyme responsible for the synthesis of dihydrofolate from para-aminobenzoic acid. Trimethoprim then inhibits the enzyme dihydrofolate reductase, which catalyzes the production of tetrahydrofolate from dihydrofolate in the next stage of the process. Permeability barrier-mediated resistance is effective against both sulfonamides and Trimethoprim (Eliopoulos & Huovinen.,2001) .

Fluoroquiolone group including Levofloxacin 35(51%) Ciprofloxacin 30(44%), this corresponds to the study in Najaf by (Al-Hasnawi ,2020), The effect of these antibiotics is bactericidal because of the mechanism of action of fluoroquinolone antibiotics is clear by stopping the process of DNA replication by inhibiting the DNA gyrase enzyme, which leads to stopping the growth and reproduction of bacteria(Kumar &Varella.,2013).

Amikacin 33(49%) Gentamicin 27(40%), meropenem 32(47%), Piperacllin-tazobactam 29(43%), (Monobactams) Aztreonam 28(41%), Cefipeme 20(29%) Cefotaxime and Ceftazidime 18(26%) as shown in table 4.1 and figure 4.3.

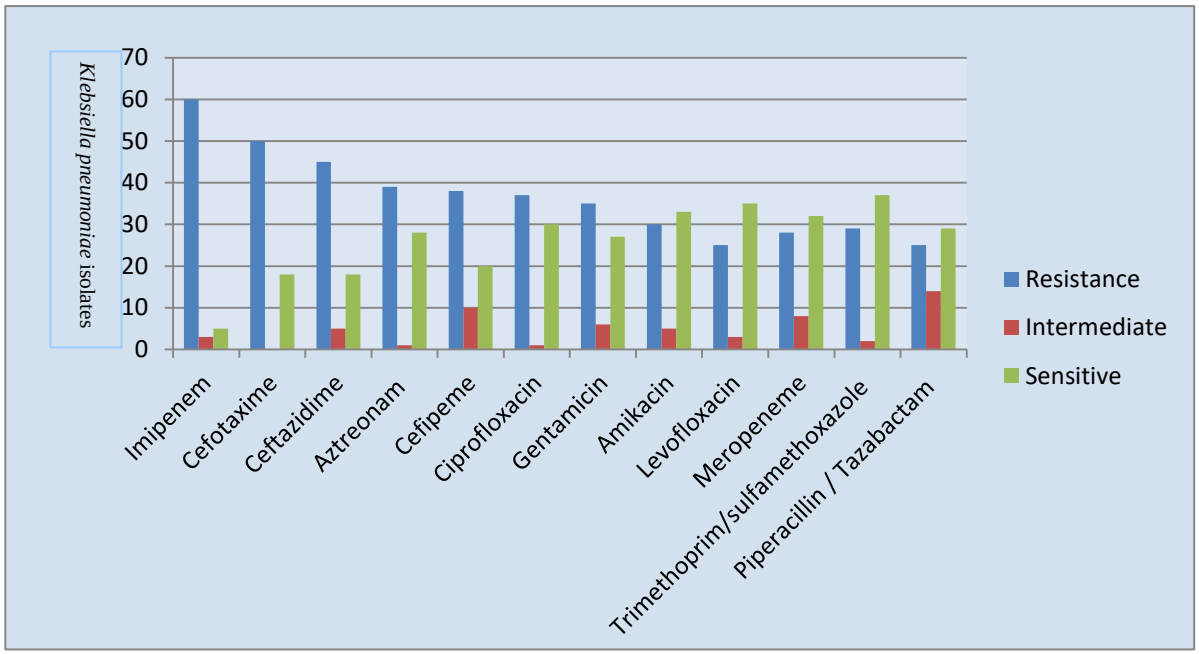


Figure 4.4: Antibiotic susceptibility test of *Klebsiella pneumoniae* isolates.

In modern medicine, one of the most significant discovery and development of antibiotics were because antibiotic-resistant bacteria. Regrettably, the emergence of resistance is endangering the efficacy of several antibiotic treatments (Manage & Liyanage,2019). It is noted from the resistance ratios in figure 4-4 that the sensitivity ratios of these bacteria towards antibiotics, the activity of modern groups and generations is considered low, and this is what puts us in a dangerous areabiotic. This may be due to several factors, including the widespread and indiscriminate use of antibiotics in our society does not pay attention to the seriousness of this issue, and the lack of health awareness among people. Also the variation of antimicrobial resistance in *K. pneumoniae* has been mostly attributed to genes found on conjugant plasmids and interpreted by conjugation, transformation, or transduction processes (Partridge *et al.*,2018; Cai *et al.*,2022).

4.3 Antibiotic susceptibility patterns

Among sixty-eight *K. pneumoniae* isolates 40/68 (59%) were diagnosed as multi-drug resistant (MDR) *K. pneumoniae*, 28 /68(41%) non MDR as shown in figure 4-5.

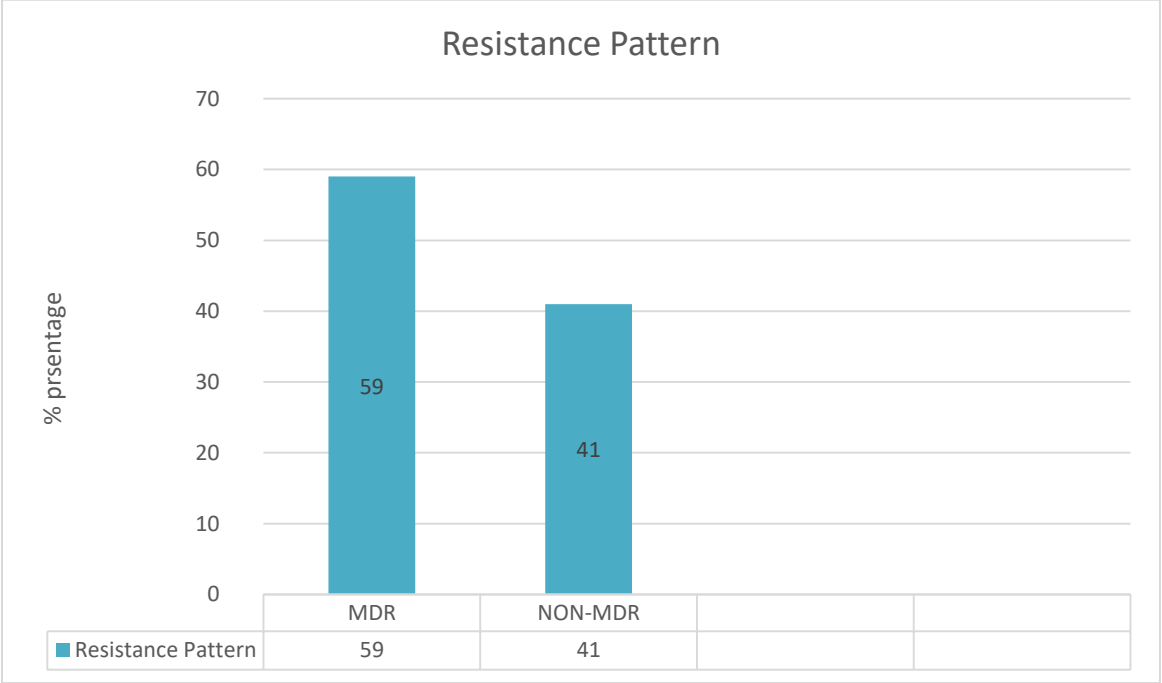


Figure 4-5: Resistance patterns among *Klebsiella pneumoniae* isolates.

MDR can be defined as acquired resistance to at least one antimicrobial agent from three or more antimicrobial classes (Sweeney *et al.*, 2018). In this study, 40 isolates of *K. pneumoniae* considered MDR were resistant to 3 antibiotics, up to 12 antibiotics for 7 classes, as shown in table 4-2.

Table 4-2: Phenotypic resistance patterns of MDR *Klebsiella pneumoniae*

No. of classes	Classes of Antibiotic	isolates	%
3	Cephems /Carbapenem /Fluoroquinolone	1	10%
	Cephems /Carbapenem / beta lactam	2	
	Cephems / beta lactam / Sulfonamide	1	
4	Monobactam / Cephems / beta lactam / Fluoroquinolone	1	15%
	Monobactam / Carbapenem/ Aminoglycoside /Fluoroquinolone	1	
	Monobactam /Carbapenem/ Beta lactam/ Cephems	5	
5	Beta lactam/Monobactam/Carbapenem/ Sulfonamide /Fluoroquinolone	1	5%
	Beta lactam / Sulfonamide/ Monobactam/ Cephems/Fluoroquinolone	1	
6	Cephems/ Monobactam /Carbapenem/Fluoroquinolone/Aminoglycoside / Beta lactam	4	20%
	Monobactam /Carbapenem/Beta lactam /Fluoroquinolone/Aminoglycoside / Sulfonamide	1	
	Cephems /Carbapenem /Beta lactam /Fluoroquinolone /Aminoglycoside / Sulfonamide.	2	
7	Monobactam /Carbapenem/ Beta lactam /Fluoroquinolone/Aminoglycoside /Sulfonamide / Cephems.	20	50%
Total		40	

The results also illustrate that resistance to 7 classes was the most prevalent, with 20/40 (50%) considered extensively drug-resistant (XDR), followed by 4, 6 classes of MDR 7/40 (17.5 %), 3 classes of MDR 4/40 (10%), and 5 classes of MDR 2/40 (5%).

According to the criteria provided by (Poulikakos *et al*.,2014)for multiple antibiotic-resistant bacteria, the current investigation found that all *K. pneumoniae* isolates were multidrug resistant to at least one antibiotic in three or more of the 7 antimicrobial classes tested in this study. Multi-resistant isolates

were categorized as MDR, perhaps XDR, or possibly PDR based on this criterion. The reason may be attributed to the spread of multidrug-resistant *K. pneumoniae* are efflux pump systems and enzymatic degradation.

4.4 Phenotypic Detection of Carbapenemases enzymes:

The Combined Disc Synergy Test (CDST) was used to detect carbapenemases by detecting zinc metal, which is present in some carbapenemase enzymes that producers class B MBLs that are inhibited by metal chelators EDTA. Among 40 isolates of *K. pneumoniae*, the results showed that 33 (82.5%) of the isolates had ≥5 mm difference in inhibition zone diameter between the imipenem disk and the imipenem-EDTA disk. As a result, which is shown in (figure A 4-6), these isolates were considered potential MBL producers, whereas 7 (17.5%) of the isolates *K. pneumoniae* were negative result, as shown in (figure B 4-6).

When comparing the results of the phenotype assay Test (CDST), which showed a negative result for seven of the same isolates devoid of carbapenemase genes(class B MBLs) 10, 14, 19,27, 46, 67,68.

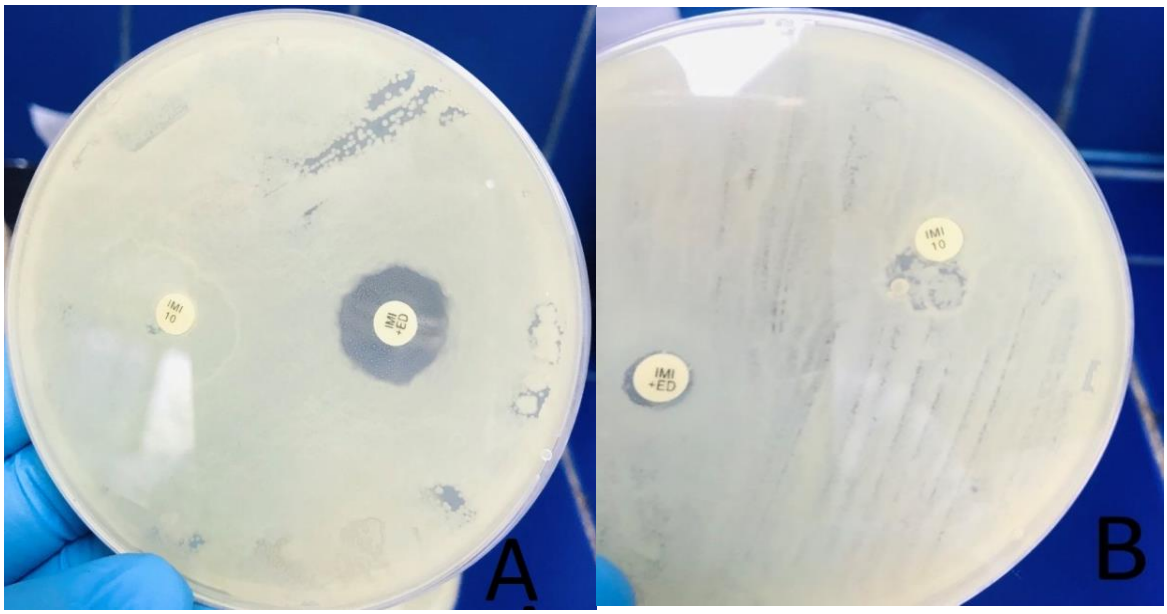


Figure 4-6: The Combined Disc Synergy Test (CDST) of *Klebsiella pneumoniae* (A) positive result, (B) negative result .

4.5 Resistance Pattern of Carbapenem Resistance *Klebsiella pneumoniae* Isolates.

Carbapenem resistance *K. pneumoniae* was described as carbapenem non-susceptible (resistant or intermediate) according to the CLSI (2022) standards. In the current study, carbapenem non-susceptibility was defined as resistance or intermediate susceptibility to one or more of the two carbapenem antibiotics tested: imipenem and meropenem. Data on antibiotic susceptibility for 68 *K. pneumoniae* isolates revealed that 60/68 (88.2%) were resistant carbapenems. 25 (41.6%) of the 60 carbapenem-resistant isolates tested positive for imipenem and meropenem resistance together. Meropenem resistance was detected in 25/60 (41.6%) of the carbapenem-resistant isolates. 26/60 isolates (43.3%) were classified as sensitive, while 8/60 (13.3%) had intermediate meropenem results. In addition, 57/60 (95%) of the carbapenem-resistant isolates showed resistance to imipenem, and 2/60 (3.3%) isolates were sensitive to this antibiotic as shown in figure 4-7 .

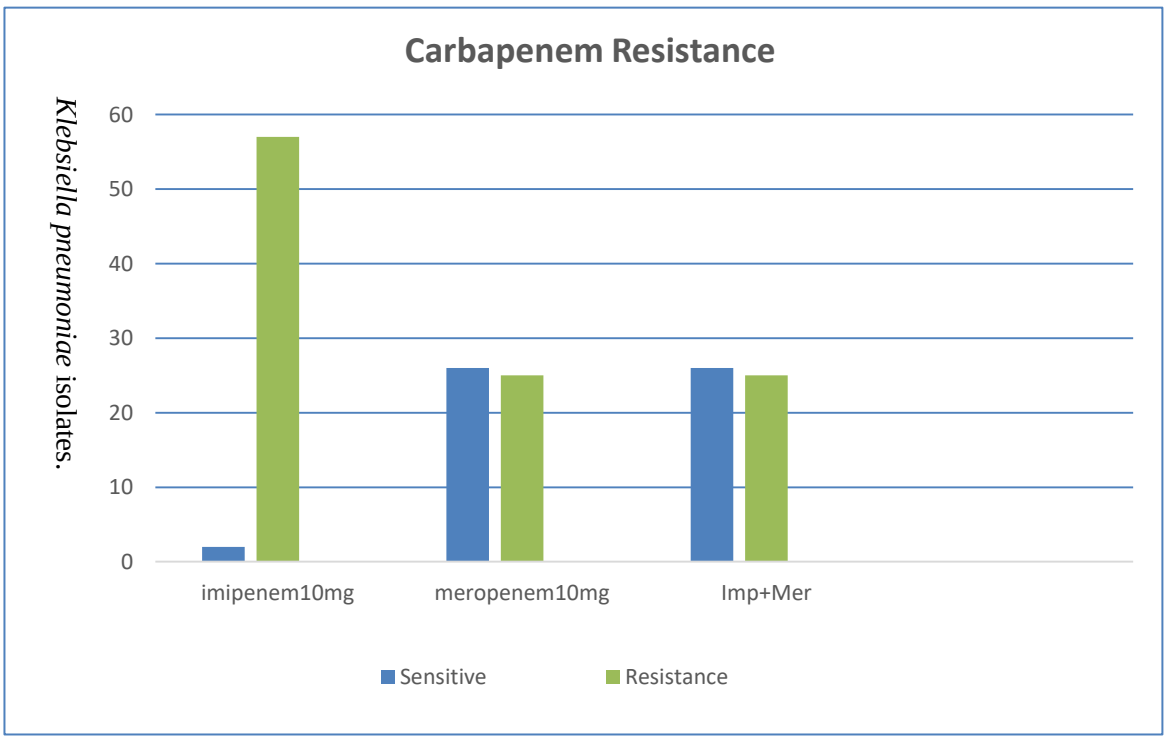


Figure 4-7: Carbapenem resistance of *Klebsiella pneumoniae* isolates

4.6 Genotypic Detection of Carbapenemases enzyme

Polymerase chain reaction (PCR) is a laboratory method for nucleic acid identification that was introduced in 1985. This reaction has played an important role in molecular diagnostics, enabling the realization of a number of applications that were previously impossible (Ellis & Zambon., 2002) .

The PCR technique occupies great importance in diagnosis due to its high speed and sensitivity in detecting microbial pathogens in clinical samples, especially when there are some microorganisms that are difficult to culture in the laboratory or require a long cultivation period (Yamamoto., 2002).

Carbapenemases enzymes were investigated via detection of Carbapenemases variants genes by PCR using specific primers for each Carbapenemases gene; *blaKPC, blaIMP, blaVIM, blaNDM* and *blaOXA48* as mentioned in table 3-6 in chapter three. For 40 MDR K. pneumoniae, carbapenemase genes were amplified.

4.7 Agarose gel Electrophoresis of Carbapenemases Genes:

Results of Agarose gel electrophoresis was illustrated in figures (4-10,11,12,13,14) for *bla KPC, bla IMP, bla VIM, bla NDM,* and *bla OXA 48* genes respectively.

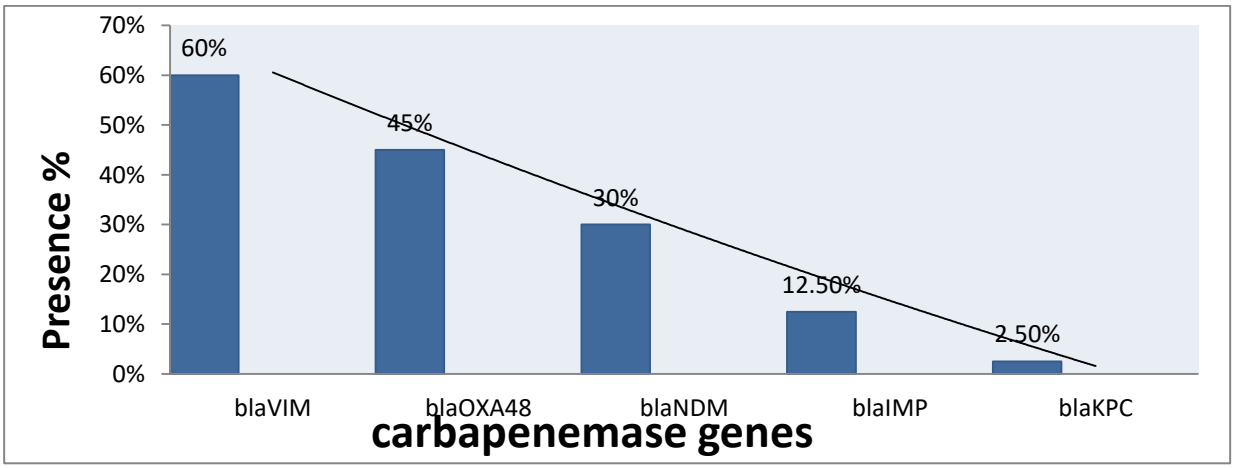


Figure 4-8:The prevalence of carbapenemase genes among *Klebsiella pneumoniae* isolates.

4.7.1 Gene *blaKPC* detection

K. pneumoniae isolates expressing *KPC*-type carbapenemases are among the most troublesome multiple antibiotic-resistant infections to emerge in recent years, attributable to their XDR phenotypes and propensity for rapid diffusion in hospitals, with a significant effect on mortality (Zurfuh *et al.*, 2016).

KPCs inactivate all beta-lactam antibiotics and are only slightly inhibited by beta-lactamase inhibitors such as clavulanic acid tazobactam and boronic acid. They were discovered for the first time in the late 1990sa in the New York City region of the United States (Yigit *et al.*, 2001).

The *blaKPC* gen has a lower prevalence 1/40 (2.5%) as shown in figure 4-8, 9 this result approach with results (Pourgholi *et al*., 2022) and 1/22 (4.5%) isolate by (Al-Hasnawi ., 2020) in Najaf. According to study ((Ramadan *et al*.,2022) found that the prevalence of KPC-producing *K. pneumoniae* was relatively low, this is agreement with result ratio the current investigation.

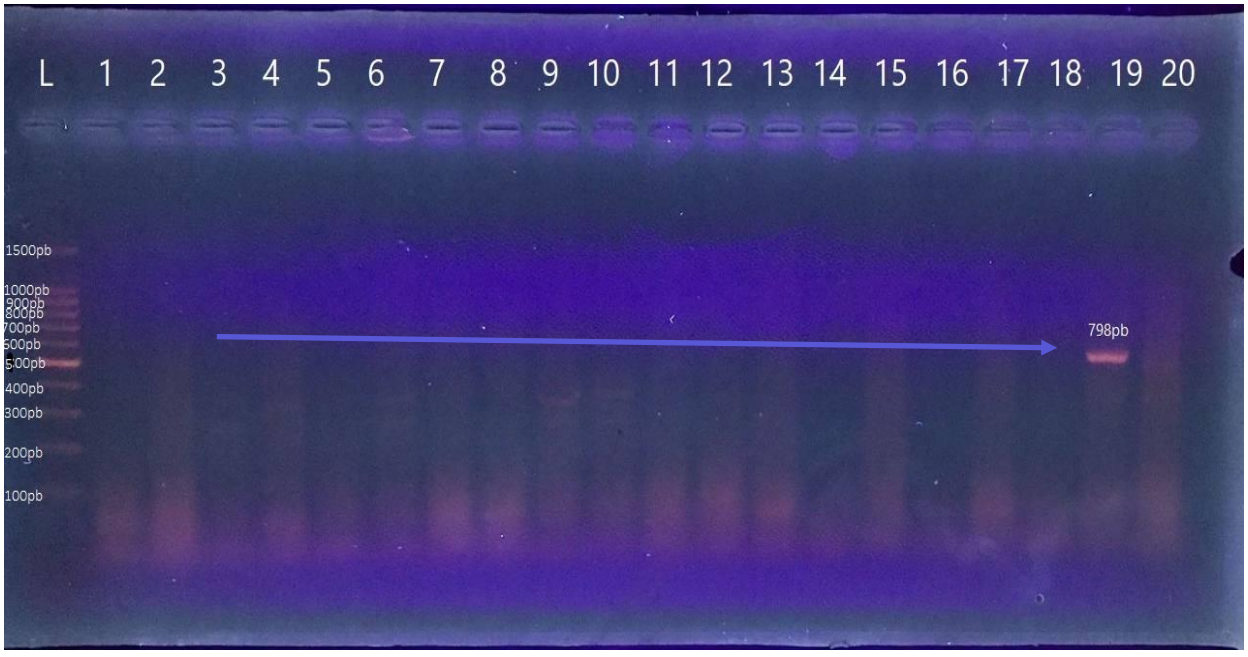
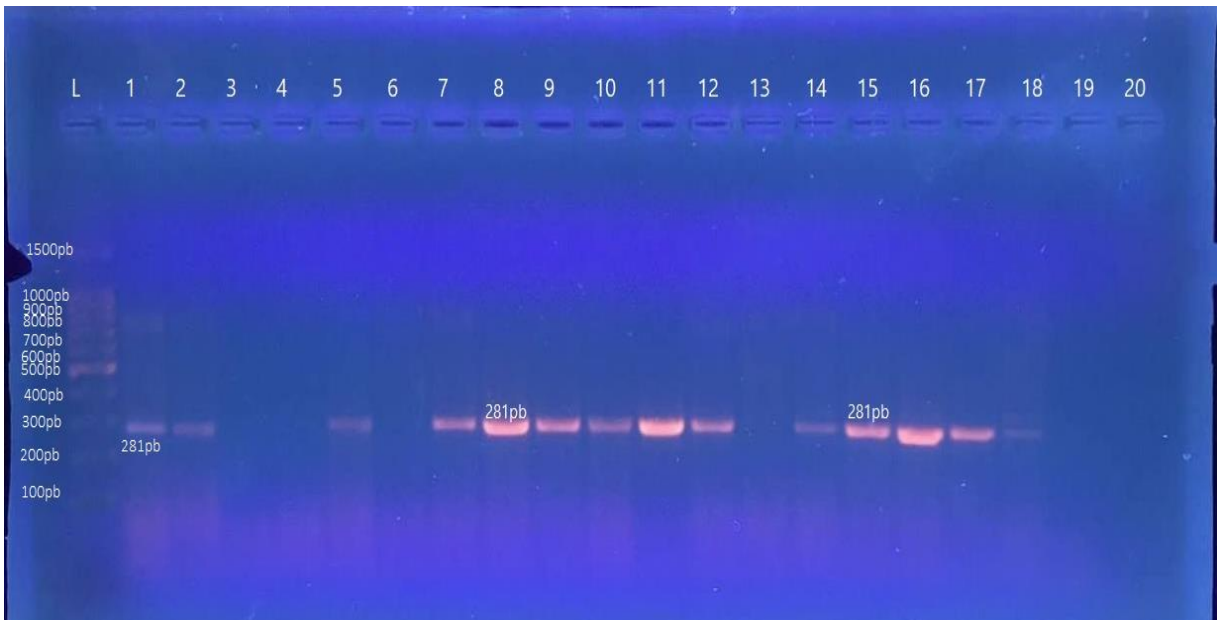


Figure 4-9: Electrophoresis of PCR product of carbapenemase gene (*blaKPC* , 798bp) from *Klebsiella pneumoniae* isolates. Using 1.5% agarose , 75 volts for 90 min.

4.7.2 Gene bla *OXA-48* detection

The prevalence of OXA-48-type carbapenem-hydrolyzing class D β-lactamases in enterobacterial species is rising (Poirel *et al.*, 2012). The *bla*OXA-48 gene is usually encoded on a self-conjugative plasmid and may spread to other species of *Enterobacteriaceae*, but most cases have been reported in *K. pneumoniae* (Cuzon *et al.*, 2011). OXA-48 producing strains have been extensively reported as sources of nosocomial outbreaks in Turkey (Nordmann & Poirel, 2013). Since 2003, the endemic spread of these bacteria has been reported in many countries such as Kuwait (Poirel *et al.*, 2012). According to the results presented here the frequently detected carbapemases in study population was OXA-48, 18 (45%) of the carbapenem resistant *K. pneumoniae* isolates were *bla OXA-48* positive (1, 2, 5, 7, 8, 9, 10,11,12,14,15,16,17, 18, 24, 25, 32 and 33), as shown in the figure 4-8,10 that agreement with result ratio (41%) by (Al-Hasnawi.,2020) in Najaf. The prevalence of OXA-48 carbapenemase among carbapenemase producing *K. pneumoniae* in Arabian Peninsula was particularly high, 32.5-56% (Sonnevend *et al.*, 2015).



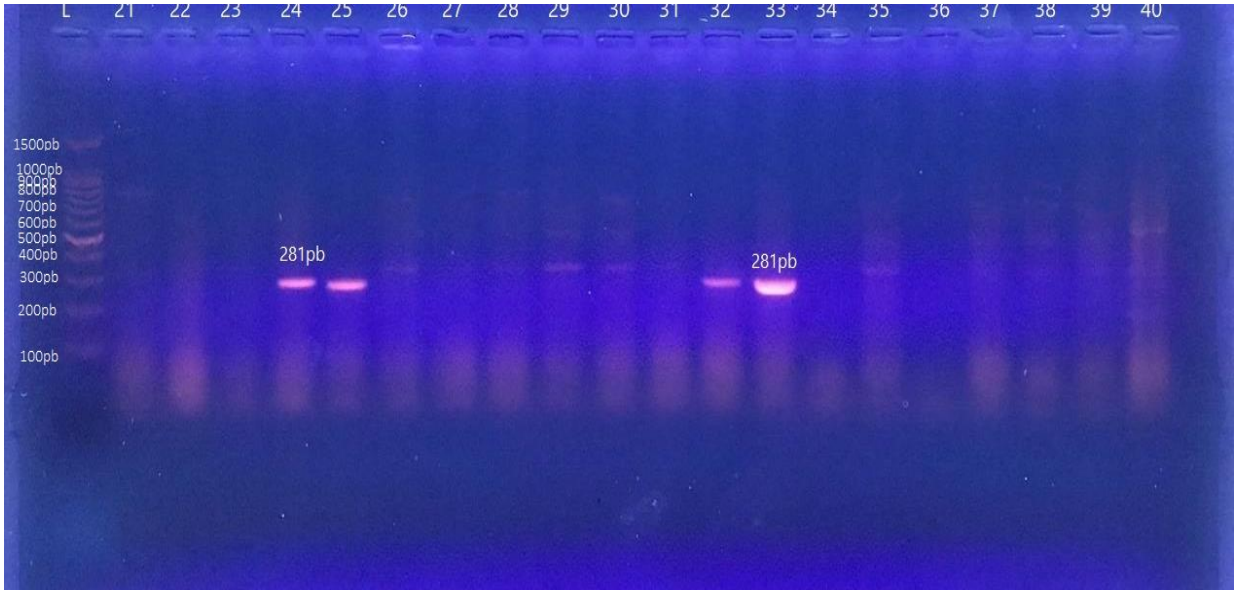


Figure 4-10: Electrophoresis of PCR product of carbapenemase gene *(blaOXA-48* , 281bp) from *Klebsiella pneumoniae* isolates. Using 1.5% agarose , 75 volts for 90 min.

4.7.3 Gene *blaIMP* detection

Multidrug resistance, particularly in lactamase-harbouring pathogens, is a significant global public health issue causing high mortality, morbidity, and economic costs β-lactamase, produced by both grammeme-positive and grammeme-negative bacteria, inactivates (41%) β-lactam antibiotics by hydrolyzing the amide bond. Carbapenemase, particularly IMP-type MBLs, is a global public health concern, IMP-type MBLs were encoded by *blaIMP* gene (Pongchaikul & Mongkolsuk 2022). The *blaIMP* emerged and spread during the early 1990s in Japan then was found in other countries (Urmi *et al* ., 2020). The results of current study in Kerbela the *blaIMP* gene was founded in 5/40 (12.5%) isolates as shown in the figure 4-8,11. This corresponds to a study in Clinical Centers in Isfahan, Iran (Pongchaikul & Mongkolsuk .,2022) and rapprochement to study (Urmi *et al* ., 2020) , this study disagreement with the results in Kenya (Michodigni *et al*.,2021).

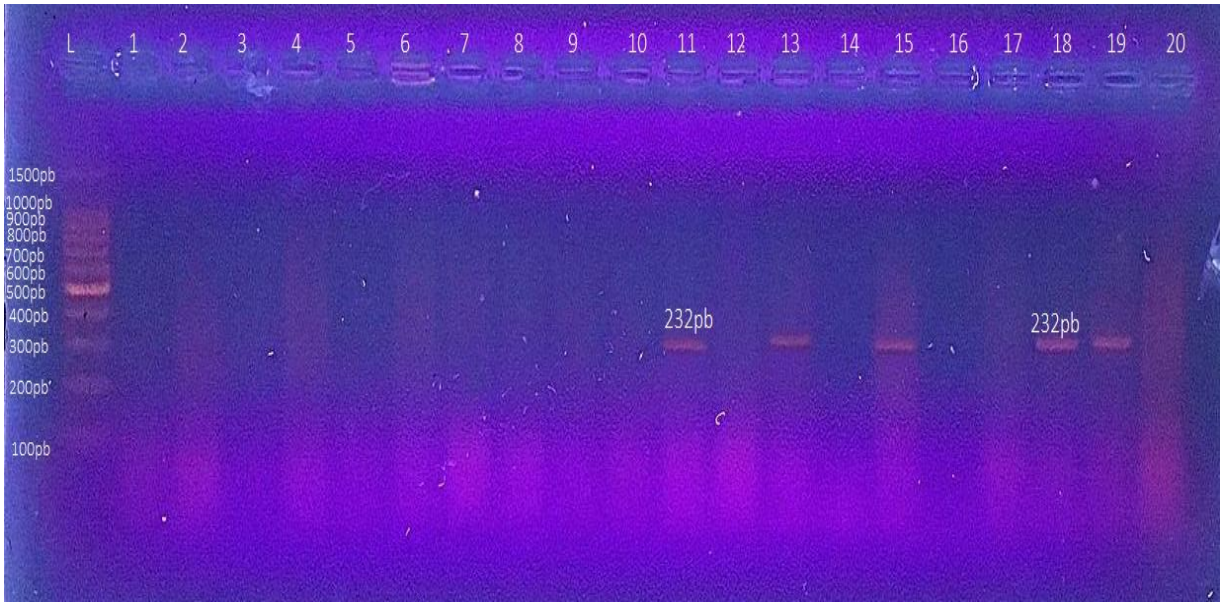


Figure 4-11: Electrophoresis of PCR product of carbapenemase gene *(blaIMP*, 232bp) from *Klebsiella pneumoniae* isolates. Using 2% agarose,75 volts for 90 min.

4.7.4 Gene *blaNAD* detection

The emergence of one of the most newly described and the most clinically significant carbapenemase, namely, the New Delhi metallo-lactamase (NDM) was become a major public health threat that presents a challenge for treatment of serious infections throughout the world (Rahman *et al.*, 2018). The *blaNDM* gene may be encoded on plasmids or transposons with the potential to transmit horizontally across gram-negative species, and it may be associated with a variety of insertion sequence types. The distinct character of mobile genetic components associated with acquired metallo-beta-lactamase genes may explain their propensity to spread (Porretta *et al.*, 2020). According to study, (Ramadan *et al*.,2022; Al-Musawi, 2018) found that is agreement with result ratio the current investigation *blaOXA-48* gene in18/40 (45%) isolates and *blaNAD* gene in 12/40(30%) isolates as shown in the figure 4-8,12 while disagreement with the results 4 (18.2%) (Al-Hasnawi.,2020) in Najaf. This agreement to a study in Clinical Centers in Isfahan, Iran (Pongchaikul & Mongkolsuk .,2022).

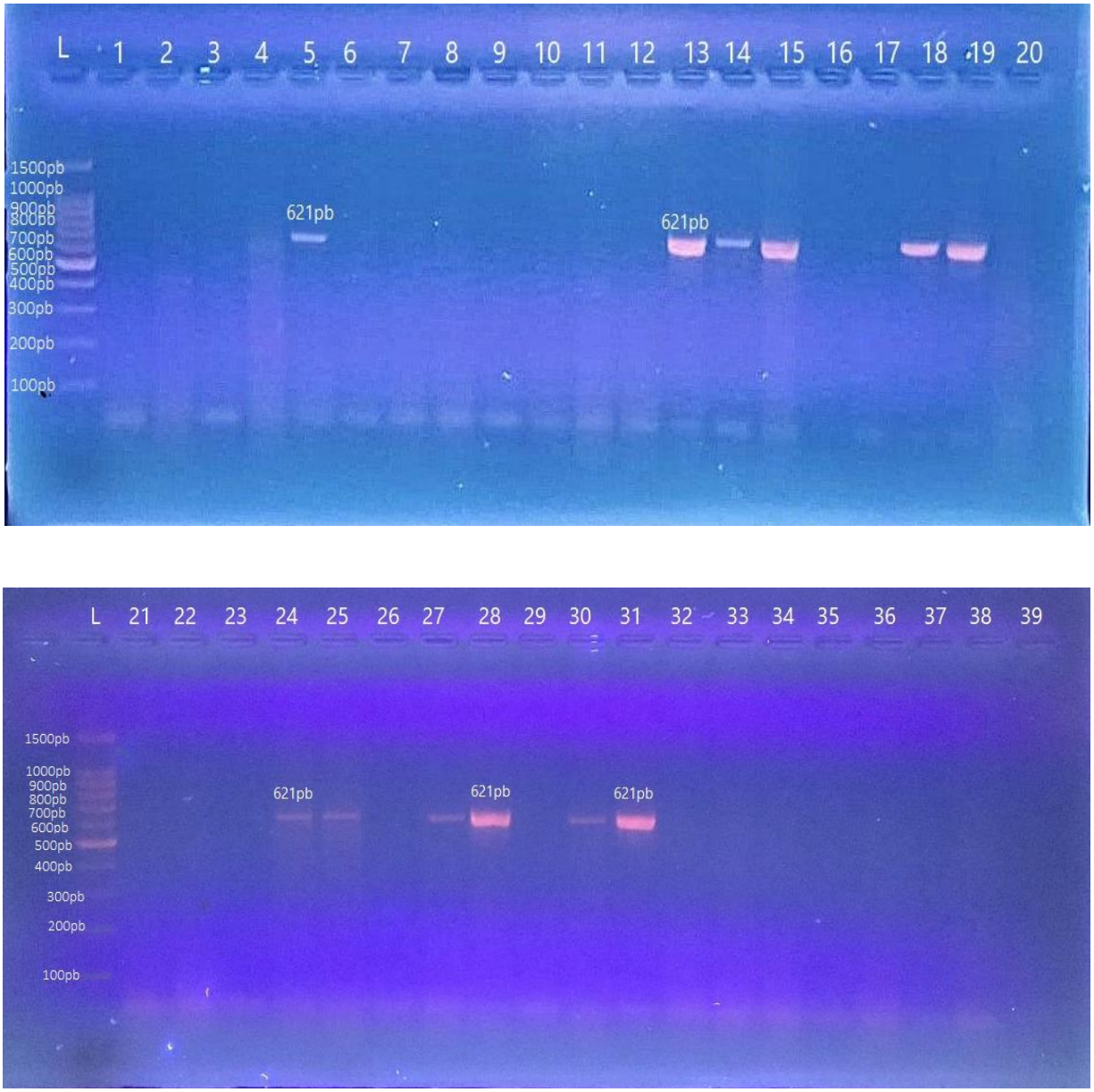


Figure 4-12: Electrophoresis of PCR product of carbapenemase gene(*blaNDM* ,621 bp) from *Klebsiella pneumoniae* isolates. Using 1.5% agarose , 75 volts for 90 min.

4.7.5 Gene *blaVIM* detection

The *blaVIM* is most frequently found in class I integrons that are related to transposons or plasmids (Liao *et al.*, 2021). This isolate exhibited resistance to the majority of beta-lactams but sensitivity to aztreonam. A gene cassette encoded the VIM. The *blaVIM* gene was placed on a no conjugative plasmid

that is electroporation-transferable (Liapis *et al*., 2019).This may enhance dissemination in Iraqi hospitals. However, the current study predicts that the finding of VIM-type carbapenemases in Kerbala hospitals will exacerbate the problem of carbapenem resistance. The *blaVIM* gene has a high prevalence 24/40(60%) isolates as figure 4-12, this result agrees with (Al-Hasnawi *et al*., 2020) in Najaf and (Jarallah & Abbas, 2014) in Hilla and disagree with (Pourgholi *et al*., 2022), the most frequent gen *bla OXA-48*, followed by gen *blaVIM*.

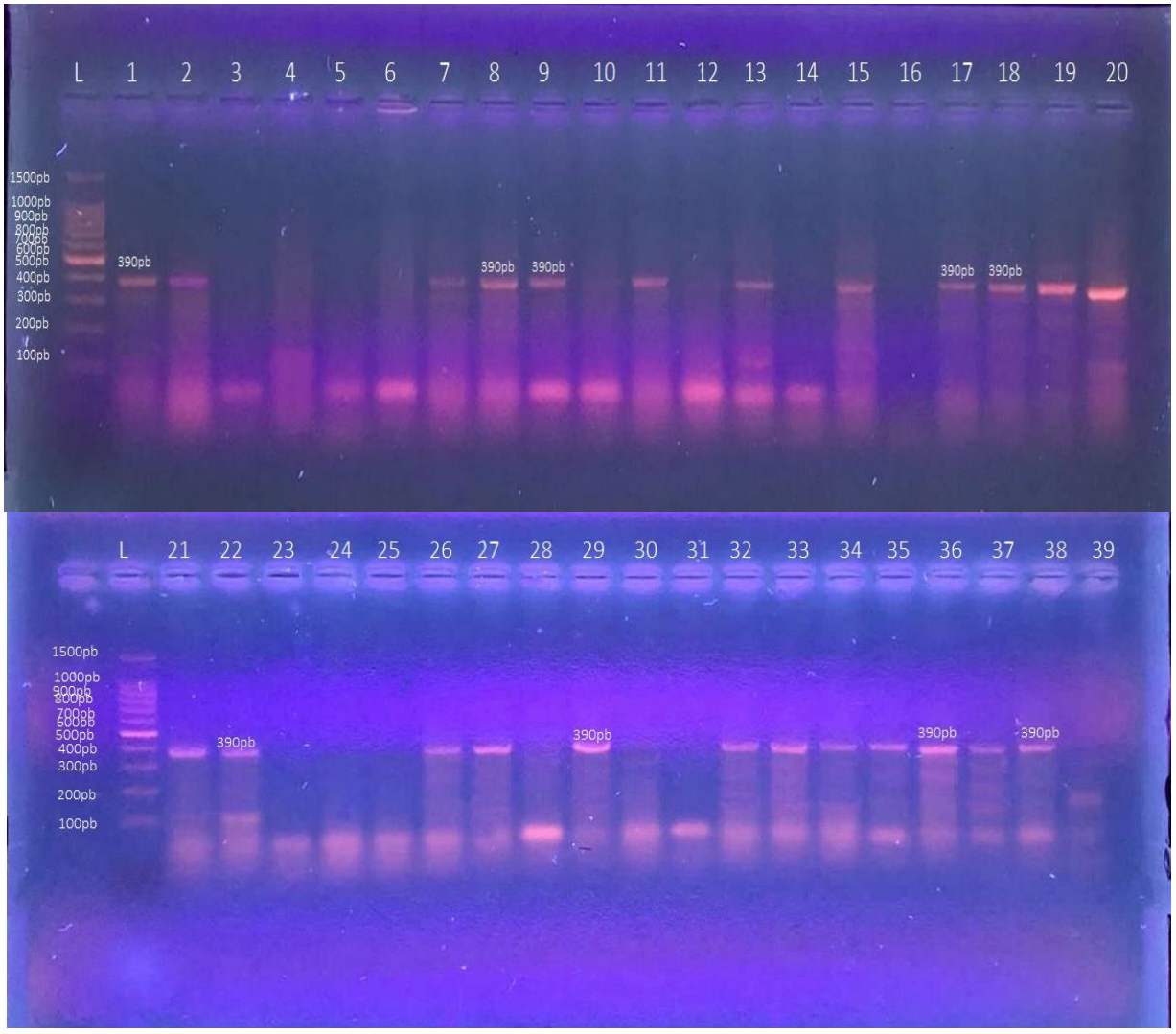


Figure 4-13: Electrophoresis of PCR product of carbapenemase gene (*blaVIM* ,390 bp) from *Klebsiella pneumoniae* isolates. Using 1.5% agarose , 75 volts for 90 min.

The results showed that 33/40 (82.5%) of the isolates had at least one carbapenemase gene, while 7/40 (17.5%) of the isolates did not have any carbapenemase gene as shown in figure 4-8 , this is consistent with the result of the phenotype test(Combined Disc Synergy Test). The percentage of carbapenemase genes among *K. pneumoniae* isolates were 2.5% for *blaKPC*, 12.5% for *blaIMP*, 30% for *blaNDM*, 45% for *blaOXA48* and 60% for *blaVIM* as shown in figure 4-9.

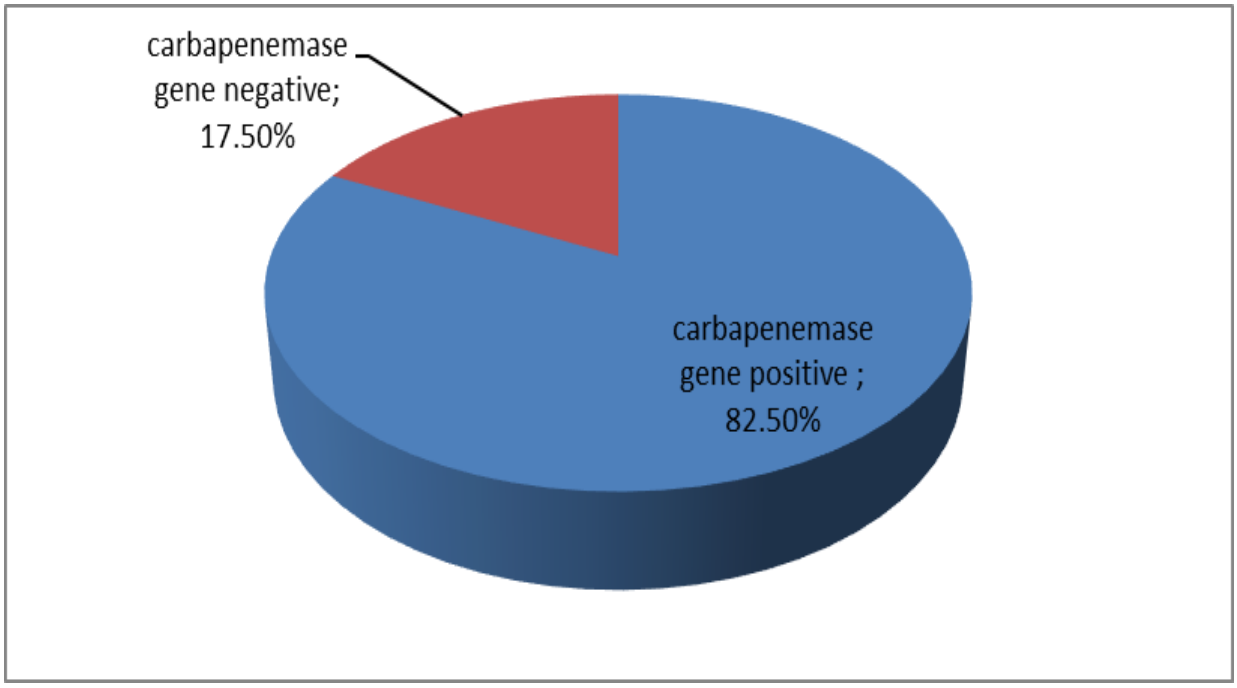


Figure 4-14: percentage of carbapenemase genes among *Klebsiella pneumoniae* isolates.

4.7 The Pattern Co-Existence of Carbapenemase Gene

The genotypic identification of Carbapenemase genes in *K. pneumoniae* isolates revealed that (82.5%) of isolates would have more than one gene, with the presence of four genes on the same isolate being the highest association of genes. Because of the coexistence of genes, the bacteria developed strong resistance to antibiotics, making infections challenging to treat, as show table 4-13.

Table 4-13: Genotypes of Carbapenemase genes among MDR isolates of *K. pneumoniae.*

No. of Genes	Genotype	MDR	n (%)
1	*blaKPC*	0	13(33%)
	blaNDM	2	
	blaOXA48	2	
	blaVIM	9	
2	*blaIMP/blaNDM*	0	14(36%)
	blaIMP /blaVIM	1	
	blaNDM /blaOXA48	5	
	blaVIM / blaOXA48	8	
3	*blaNDM /blaOXA48/ blaVIM*	1	4(10%)
	blaIMP / blaNDM/ blaVIM	2	
	blaIMP / blaNDM / blaOXA48	1	
4	*blaNDM /blaOXA48 /blaVIM / blaIMP*	1	2(5%)
	blaIMP / blaNDM/ blaVIM / blaKPC	1	
		33	33(85%)

Based antimicrobial susceptibility assay, PCR and carbapenemase gene typing, substantial prevalence of highly virulent MDR *K. pneumoniae* isolates were present in clinical specimens. Due to the multidrug resistance produced by the emergence of drug-inactivating enzymes, particularly beta-lactamases. The carbapenemase enzyme is increasingly being identified in bacteria that cause nosocomial infections (Pongchaikul *et al.*,2022).

Through the investigation of the current study, it was found that resistance to lactam drugs may be related to the inability of antibiotics to reach their sites of action by reducing the entry into their outer cell walls through the porin channels. Active efflux pumps serve as another mechanism of resistance, removing the antibiotic from its site of action before it can act. The mutation responsible for drug resistance is usually a modification at a specific site on bacterial chromosomes, and the capture, accumulation, and dissemination of resistance genes are largely due to the actions of mobile genetic elements (MGE). Bacterial efflux proteins are proteins identified primarily in Gram-negative bacteria but also exist in Gram-positive bacteria. Bacterial efflux pumps are associated with outer membrane channels. The transcription of genes involved in the efflux pumps is checked by local regulators, and the overexpression of efflux genes is regulated by the mutation mechanism. A mutation of the antibiotic target can develop antibiotic resistance.

The modification of antibiotics by hydrolysis is a major mechanism of antibiotic resistance that has been relevant since the first use of antibiotics. Class A includes extended-spectrum β-lactamases (ESBLs) and *Klebsiella pneumoniae* carbapenemase enzymes, class B includes metallo-β-lactamases (MBLs), and class D includes oxacillinases. The spread of carbapenemases, which are enzymes that inactivate beta-lactam antimicrobials, has occurred in different ways.

Conclusions and Recommendations

Conclusions and Recommendations

Conclusions

1. Assess the dissemination and types of carbapenemases producing K. pneumoniae in Kerbela hospitals for epidemiological purposes.
2. It as noted that multidrug resistance was common among local isolates of bacteria in the current study.
3. The two antibiotics, Imipenem and Cefotaxime, are those to which most of the isolates in the current study showed resistance.
4. Antibiotic resistance to five or six classes can signal the advent of XDR or even PDR isolates.
5. A high prevalence of Carbapenemases genes among MDR *K. pneumoniae* isolates.

Recommendations

1. Increasing future studies to show the importance of these bacteria in their possession of these genes and factors.
2. Rapid detection of these enzymes using methods with a short turnaround time is essential to ensure early recognition and timely implementation of control measures in hospitals.
3. In view of the existence of a rise in the resistance of bacteria to antibiotics, suggest finding medicinal alternatives from medicinal herbal extracts that have the ability to cause an effect on the growth of these.

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الخلاصة

تضمنت هذه الدراسة تشخيص بكتريا *Klebsiella pneumoniae* المسؤولة عن العديد من الاصابات بعد عزلها من مصادر سريرية مختلفة مثل الدم , القشع , الحروق , الجروح , البلعوم , الاذن , الادرار , المهبل كما انه التحري عن بعض عوامل الضراوة لهذه البكتريا عبر الكشف عن الجينات *blaKPC, blaIMP, blaVIM, blaNDM, blaOXA48* المشفرةعن انزيمات الكاربيبنيميز المحللة لمضادات البيتا لاكتام .

اسفرت النتائج عن الحصول على 68 عزلة من اصل 275 عينة تم جمعها من المرضى المراجعين و الراقدين في المستشفيات والعيادات الخارجية في محافظة كربلاء المقدسة ومن كلا الجنسين ولمختلف الاعمار للمدة المحصورة مابين الاول من شهر التاسع ولغاية نهاية شهر كانون الاول عام 2022م . وقد شخصت خلال الفحص المظهري على الاوساط الزرعية وسط اكار الدم وسط المكونكي وباستخدام تقنية الفايتك .

تم اجراء فحص الحساسية للعزلات البكتيرية التي تم الحصول عليها في هذه الدراسة تجاه 12 نوع من المضادات الحيوية العائدةالمستخدمة في علاج الاصابات , بكتريا *K.pneumoniae* والتي تضمنت Meropeneme , Imipenem, Cefipeme , Cefotaxime, Ceftazidime, Gentamicin, Aztreonam, Levofloxacin, Ciprofloxacin, Trimethoprim-sulfamethoxazole, Amikacin, Piperacillin / Tazabactam واتضح من النتائج ان اعلى نسبة مقاومة كانت لمضاد Imipenem 80% و تليه المضاد Cefotaxime بنسبة 74% ,و بينما كانت اقل نسبة مقاومة المضاد Levofloxacinو المضاد Piperacillin/Tazabactam بنسبة 37% كما تبين ايضا من نتائج هذا الفحص ان 40 عزلة منها كانت متعددة المقاومة للمضادات الحيوية (Multi drug resistant).

اشتملت هذه الدراسة ايضا على الفحص المظهري للمقاومة المتعددة وباستخدام تقنية الاقراص المتازرة للكشف عن وجود انزيمات البيتا لاكتام المعدنية التي تحتوي على عنصر الزنك وكانت النتيجة موجبة لثلاثة وثلاثين عزلة وسالبة لسبع منها ..

أكدت نتائج الكشف الجزيئي لدراستنا الحالية وجود الجين *blaVIM* بأعلى بنسبة(60%)في عزلات بكتريا *K.pneumoniae* تالية جين *blaOXA48* بنسبة 45% ثم جين *blaNDM*30 % ومن بعدها جين *blaIMP* بنسبة 12,5% بينما اتضح تواجد الجين *blaKPC* باقل نسبة (2,5%) ل33 عزلة من اصل 40 عزلة بينما تبين أن سبع عزلات خالية من اي جين .

جمهوريـة العـراق
وزارة التعليم العالـي والبحث العلمـي
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قســم علــوم الحيـاة

الكشف الجزيئي لبعض أنزيمات الكاربابنيميز في بكتريا *Klebsiella pneumoniae* المعزولة من مصادر سريرية مختلفة

رسالة مقدمة الى

مجلس كلية العلوم – جامعة كربلاء

وهي جزء من متطلبات نيل درجة الماجستيرعلوم في علوم الحياة

من قبل الطالبة

نبراس صالح غافل

بكلوريوس علوم الحياة/ جامعة المستنصرية -2004م

بإشراف

أ.م.د كوكب عبدالله حسين السعدي

2023م 1445 هـ