A Molecular Resistance Mechanisms Detection in *Klebsiella pneumoniae* through Carbapenem Enzymatic Genes

Halah Raheem Mohammed Almidhatee [1*], Zainab Hayder Jaber Alkufaishi [2], Anwar Kadhim Hussein AL-saffar [3], Jwan Ahmed Ali Alhamawandi [4]

Abstract

Background: *Klebsiella pneumoniae*, a Gram-negative bacterium, shows significant challenges in healthcare due to its high virulence and antibiotic resistance. The spread of carbapenem resistance, particularly mediated by enzymes like *Klebsiella pneumoniae* carbapenemase (KPC) and New Delhi metallo-beta-lactamase (NDM), further complicates treatment options. Methods: Clinical specimens (n=300) were collected from Hilla Hospital in Iraq between March 2023 and September 2023. *Klebsiella pneumoniae* isolates were identified using culture methods and molecular techniques, followed by antimicrobial susceptibility testing (AST) using the Disk Diffusion Test (DDT) and genetic detection of resistance mechanisms through PCR. Results: In the study, among 25 *Klebsiella pneumoniae* isolates from clinical specimens, significant antibiotic resistance was observed, particularly against β-lactams, aminoglycosides, and fluoroquinolones. Piperacillin and carbenicillin exhibited the highest resistance rates at 80% and 81.2%, respectively. Resistance to imipenem and meropenem was 8.4% and 19.8%, respectively, while gentamicin (82%), amikacin (72.6%), cefepime (60.8%), cefotaxime (70.2%), ceftazidime (70.2%), and ceftriaxone (71.6%) also showed notable resistance. Molecular analysis via PCR revealed the presence of 16S rRNA gene in all isolates, indicating bacterial diagnosis. bla-NDM was the predominant MBL gene, present in 48% of isolates, followed by blaKPC in 72%, contributing to antibiotic resistance through beta-lactamase synthesis. Conclusion: *Klebsiella pneumoniae* strains, especially those carrying carbapenemase genes, exhibit widespread antibiotic resistance, limiting treatment options. Enhanced surveillance, molecular characterization, and infection control measures are essential for addressing the growing threat of antimicrobial resistance posed by *Klebsiella pneumoniae*, particularly strains producing blaKPC.

Keywords: *Klebsiella pneumoniae*, Carbapenem resistance, PCR detection, Antimicrobial susceptibility, Molecular characterization

Significance | Identifying carbapenem resistance mechanisms in *Klebsiella pneumoniae* might be a treatment strategies against the rising antibiotic resistance globally.

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Introduction

Carl Friedlander was the first to describe and name *Klebsiella pneumoniae* in 1882, initially dubbing it "encapsulated bacillus." Subsequently, in 1886, it was officially designated as Klebsiella. This Gram-negative, encapsulated, and nonmotile bacterium is commonly encountered in various environmental niches. Its clinical significance lies in its propensity to cause pneumonia, particularly in diabetic patients (El-Badawy et al., 2017).

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Klebsiella pneumoniae exhibits a remarkable ability to colonize both the gastrointestinal (GI) tract and oropharynx of humans. Its high virulence and resistance to antibiotics enable it to breach the body's defenses and cause infection. Between 4 and 9 percent of all nosocomial bacterial infections are attributed to *Klebsiella pneumoniae*, with hospital-acquired pneumonia being a frequent consequence (Ahmadi et al., 2022).

Carbapenemases, enzymes primarily encoded on various plasmids, play a pivotal role in the spread of carbapenem resistance. *Klebsiella pneumoniae* carbapenemase (KPC) is a notable example of such enzymes, facilitating the dissemination of carbapenem resistance. Notably, *Klebsiella pneumoniae* is a major producer of carbapenemases, with KPCs being predominantly responsible for carbapenem resistance (Bunyan Al-Salem, 2022).

Several antimicrobial resistance genes are harbored within KPC, with particular emphasis on the blaKPC gene, which significantly contributes to the escalation of antibiotic resistance during the widespread dissemination of KPC. Consequently, strains carrying the blaKPC gene pose substantial challenges to healthcare systems worldwide.

Another concerning enzyme is the New Delhi metallo-beta-lactamase (NDM), encoded by the mobile resistance gene blaNDM. NDM possesses the ability to hydrolyze carbapenems, a critical class of antibiotics used in the treatment of various bacterial infections (Hussein et al., 2022). The global dissemination dynamics of blaNDM remain poorly understood due to its wide distribution across different Gram-negative bacteria on multiple plasmids, typically found within highly recombining and transposon-rich genomic regions (Zhao et al., 2022).

Klebsiella pneumoniae, characterized by its encapsulated, Gram-negative nature, poses significant challenges in clinical settings due to its virulence, antibiotic resistance, and ability to cause a range of infections, notably pneumonia. The emergence and spread of carbapenemases, particularly KPC and NDM, underscore the urgent need for robust infection control measures and the development of novel therapeutic strategies to combat this formidable pathogen.

Our study aimed a comprehensive exploration of antibiotic susceptibility patterns in *Klebsiella pneumoniae*, unraveling the intricate interplay between microbial resistance mechanisms and therapeutic efficacy. The findings underscore the urgent need for judicious antibiotic stewardship practices and the development of novel therapeutic strategies to mitigate the burgeoning threat of antibiotic resistance in clinical settings.

Materials and Methods

Clinical Specimens Collection and Processing

Clinical specimens were procured from Hilla Hospital in Iraq, constituting 25 out of 300 (8.3%) samples collected between March 2023 and September 2023. These specimens comprised blood (55), urine (45), wound swabs (100), and burn swabs (100). Upon collection, the samples underwent culturing on MacConkey agar, Nutrient agar, blood agar, and brain heart infusion broth. Diagnosis of *Klebsiella pneumoniae* was facilitated through imaging tests in conjunction with blood or sputum samples. Identification procedures involved PCR tests and utilization of the Vitek-2 system, with adherence to parameters outlined in the CLSI-2023 document. These protocols ensured accurate identification and characterization of the pathogen across a diverse panel of clinical specimens.

Antimicrobial Susceptibility Testing (AST)

Antimicrobial Susceptibility Testing (AST) was performed on 25 (8.3%) *Klebsiella pneumoniae* isolates using the Disk Diffusion Test (DDT) to ascertain resistance patterns. This involved in vitro antibacterial susceptibility testing against selected antibacterials, including Piperacillin, Ceftazidime, Cefotaxime, Carbenicillin, Ceftriaxone, Imipenem, Gentamicin, Amikacin, Ciprofloxacin, and Meropenem. DDT was conducted on Muller-Hinton agar medium, wherein antibiotic discs were placed and incubated at 37 degrees Celsius for interpretation. Results were evaluated according to the guidelines set forth by the Clinical and Laboratory Standards Institute (CLSI) in 2023, aiming to assess the susceptibility of *Klebsiella pneumoniae* isolates to various antimicrobial agents. Additionally, genetic detection of mechanisms was carried out.

DNA Extraction and PCR Testing

DNA extraction from bacteria was accomplished using DNA extraction kits, followed by PCR tests for gene detection in *Klebsiella pneumoniae*, employing primers as specified in Table (1). PCR conditions for thermocycling were tailored to the requirements outlined in Table (2), facilitating accurate gene amplification and detection.

Results

A total of 25 *Klebsiella Pneumoniae* isolates were identified from among 300 clinical specimens, including samples of blood (55), urine (45), sputum (100), and burn swabs (100). This accounts for 8.3% of the specimens, as illustrated in Figure (1). These isolates were sourced exclusively from Hilla Teaching Hospital in Iraq and collected between March 2023 and September 2023. The isolation process involved culturing on various media, namely MacConkey agar, Nutrient agar, blood agar, and brain heart infusion broth. Identification criteria were based on specific characteristics, such as the appearance of red colonies on Nutrient agar, along with results from biochemical tests indicating positive outcomes for Capsule, Catalase, Citrate, gas production, MUG Test, and growth in KCN, while yielding negative results for other tests. Confirmation of *Klebsiella Pneumoniae* diagnosis for all isolates was achieved through utilization of the VITEK2-system, ensuring a

comprehensive and dependable assessment of these clinical specimens.

The distribution of *Klebsiella Pneumoniae* isolates is depicted in Figure (1).

The results of the Antibiotic Susceptibility Test (AST) of *Klebsiella Pneumoniae* isolates are shown in Figure (2).

The molecular detection results of *Klebsiella Pneumoniae* isolates are presented in Table 3.

Discussion

The present study on Antibiotic Susceptibility Testing (AST) for *Klebsiella Pneumoniae* revealed diverse patterns of antibiotic resistance and sensitivity among 25 isolates, as depicted in Figure 2. Results showed that 81.2% and 80% of the isolates were resistant to carbenicillin and piperacillin, respectively. Moreover, 60% of the isolates exhibited resistance to the β-lactam/β-lactamase inhibitor combination agent piperacillin-tazobactam. Resistance rates to third-generation cephalosporins were as follows: cefotaxime (70.2%), ceftriaxone (71.6%), and ceftazidime (70.2%). Additionally, 65.8% of the isolates demonstrated resistance to the fourth-generation cephalosporin, cefepime. Notably, resistance to imipenem and meropenem among the isolates was noted at rates of 8.4% and 19.8%, respectively. Aminoglycoside antibiotics showed an overall resistance percentage, with the highest levels observed in gentamicin (82%) and amikacin (72.2%). Furthermore, 80.4% of the isolates exhibited resistance to ciprofloxacin among fluoroquinolones. Antibiotic resistance was identified across various classes, including multi-drug resistance (MDR), extensive drug resistance (XDR), and pan-drug resistance (PDR), encompassing resistance to aminoglycosides, carbapenems, cephalosporins, β-lactam/β-lactamase inhibitor combinations, quinolones, monobactam, and antibacterial penicillin. These findings underscore the diverse antibiotic resistance profiles manifested by the *Klebsiella Pneumoniae* isolates. The susceptibility of *Klebsiella pneumoniae* isolates to antibiotics was assessed using the Kirby-Bauer disk diffusion method. Out of the tested isolates, 25 (8.3%) exhibited positive results for multiple drug resistance.

During the investigation, 80% of the isolates showed resistance to piperacillin, indicating an escalation in antibiotic resistance compared to previous reports from Al-Hilla Teaching Hospital [Abdel-Halim et al., 2022]. Piperacillin susceptibility among *Klebsiella pneumoniae* isolates varies widely, ranging from 31.7% to 64.5%. Similarly, carbenicillins, also known as carboxypenicillins, exhibit significant variability in efficacy, particularly against ampicillin-resistant *Klebsiella pneumoniae* strains. A study by El-Tawab et al. (2022) indicated a moderate rate of resistance to piperacillin among the isolated *Klebsiella pneumoniae* strains. In a recent investigation, tests conducted in Al-Hilla revealed that carbenicillin performed relatively better [Ben Sallem et al., 2022]. Despite this, 13.3% of the isolates in the current study exhibited drug resistance, although piperacillin-tazobactam was found to be moderately active against *Klebsiella pneumoniae* isolates.

Several studies have identified piperacillin-tazobactam as the most effective antimicrobial agent against *Klebsiella pneumoniae* [Güzel et al., 2022]. The study revealed a cefepime resistance rate of 60.8%, surpassing the 50% rate reported in another local study. Cefepime, a fourth-generation antibiotic, demonstrates increased resistance against β-lactamases, particularly AmpC β-lactamases, and possesses an extended spectrum, along with enhanced permeability through the porins present in the outer membrane of Gram-negative bacteria. Carbapenems, though highly effective against *Klebsiella pneumoniae*, are expensive and not readily available in the Hilla province. However, recent studies have highlighted the potent activity of carbapenems against *Klebsiella pneumoniae*. Imipenem, in particular, has shown significant efficacy against this bacterium in previous research [Ahmadi et al., 2022]. The focus of this study is on the activity of carbapenems, specifically meropenem and imipenem, against *Klebsiella pneumoniae*. Consistent with earlier findings, meropenem exhibited greater activity compared to imipenem, with resistance rates of 22.4% and 20.4%, respectively, aligning with [Nigiz et al., 2022] but contradicting [Kudaer et al., 2022], which also found meropenem to be superior to imipenem. *Klebsiella pneumoniae* isolates from hospitals in Hilla seem to have reduced susceptibility to fluoroquinolones, likely due to increased or cumulative usage of these medications. Susceptibility to fluoroquinolones appears to be declining more rapidly (17.6%) compared to other antimicrobial classes, as evidenced by comparisons with earlier studies conducted in Hilla. Every *Klebsiella pneumoniae* isolate in this investigation exhibited multiple antibiotic resistance.

Discrepancies in the definitions of multidrug resistance have hindered meaningful comparisons of MDR rates among studies, apart from the current one. The susceptibility to carbapenems was initially assessed in all 120 *Klebsiella pneumoniae* isolates following the guidelines outlined by the Clinical and Laboratory Standards Institute (CLSI). The screening was conducted using the disk diffusion method with antibiotic disks containing 10μg of meropenem and imipenem each. According to the CLSI 2023 recommendations, isolates were classified as susceptible to imipenem and meropenem if the inhibition zone diameter was ≥19 mm, intermediate if it ranged from 16 to 18 mm, and resistant if it was ≤15 mm. The susceptibilities of the isolates to imipenem and meropenem are detailed in Figure 2, based on the results of the susceptibility tests. Throughout the study period, meropenem demonstrated a higher activity (19.8%) compared to imipenem (8.4%). However, among the 40 isolates showing resistance to both

Table 1. Primers used in this research.

Primers	Sequence (5'_3')	ref
16SrRNA	*TAATGCTTTGATCGGCCTTG*	**El-Morsi, *etal*.. (2022).**
	TGGATTGCACTTCATCTTGG	
bla$_{NDM}$	*GGAATAGAGTGGCTTAAYTCTC*	**El-Morsi, *etal*., (2022).**
	CCAAACYACTASGTTATCT	
bla-kpc	CGTTCTTGTCTCTCATGGCC	**El-Morsi, *etal*., (2022).**
	CCTCGCTGTGCTTGTCATCC	

Table 2. The Conditions for Genes Detection Used in this Study.

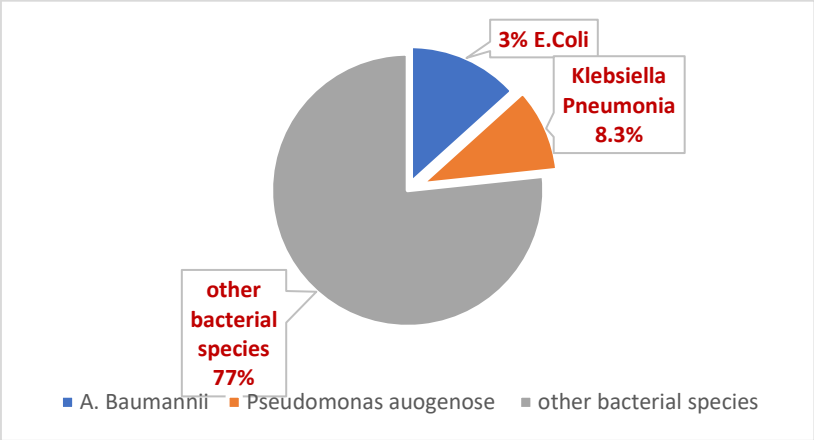
Primers	Temperature (°C) / Time					Number of cycles	Amplicon size (bp)
	Initial Denaturation	Cyclinc condition			Final Extention		
		Denaturation	Anealing	Extention			
16SrRNA	94/3 min	94/30 sec	57/30 sec	72/40 sec	72/5min	25	505
Bla$_{NDM}$	95/5 min	95/30 sec	53/30 sec	72/1 min	72/5min	35	621
bla$_{KPC}$	95/5 min	95/30 sec	52/30 sec	72/1 min	72/5min	35	230

Table 3. Percentage of specimens used in this study

Type of specimens	*Klebsiella Pneumoniae* isolates	Total NO.	Percentage %
Blood	5	55	9.09%
Uine	5	45	11.11%
Sputum	10	100	10%
Burn swab	10	100	10%
Total	40	300	13.3%

Table 4. Distribution of Enzymatic genes in 25 *Klebsiella Pneumoniae* isolates

Genes	No. of Isolates (%)
bla-$_{NDM}$	12(48%)
bla-$_{KPC}$	18 (72%)
16SrRNA	25 (8.3%)



Figures 1. Distribution Isolation and identification of *Klebsiella Pneumoniae* from Different Clinical specimens.

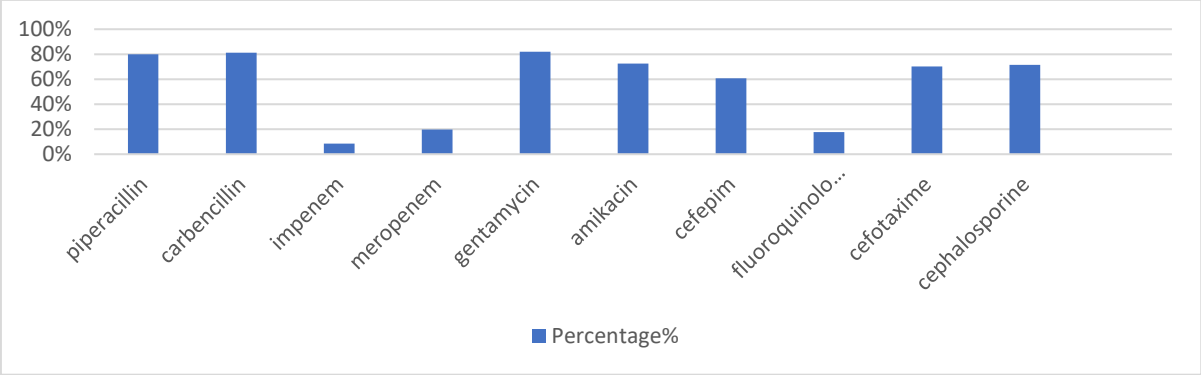


Figure 2. Antibiotic Susceptibility Test of *Klebsiella Pneumoniae* isolates toward Antibiotics (No. 25).

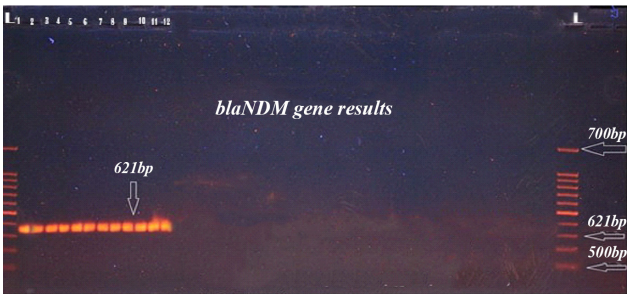


Figure 3. The electrophoreses of PCR amplified products of bla_{NDM} gene was performed at 60 volts for 2 hr. (500-l500bp ladder). Lanes (1-12) of *Klebsiella Pneumoniae* isolates show positive results with bla_{NDM}(621 bp).

Figure 4. The electrophoreses of PCR amplified products for $bla_{KPC}$was performed at 60 volts for 2 hr. (700-l500bp ladder), and Lanes (1-18) of *Klebsiella Pneumoniae* isolates show positive results with bla_{KPC} (230bp).

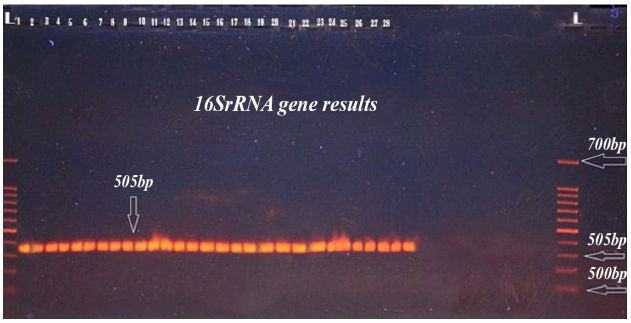


Figure 5. The electrophorese of PCR amplified products of 16SrRNA was performed at 60 volts for 2 hr. (100-l500bp ladder), Lanes (1-25) of *Klebsiella Pneumoniae* isolates show positive results with 16SrRNA (505bp).

imipenem and meropenem, 23 exhibited cross-resistance to both antibiotics.

Additionally, we tested for the presence of carbapenemase producers among the isolates resistant to carbapenem. Using phenotypic and molecular characteristics, the polymerase chain reaction (PCR) method was employed to evaluate the capacity of these carbapenem-resistant *Klebsiella pneumoniae* isolates to produce carbapenemase in classes A (serine-β-lactamases) and B (metallo-β-lactamases, MBL). Carbapenemase production in *Klebsiella pneumoniae* appears to contribute to carbapenem resistance, as illustrated in Figures (3) and (4) below, due to the loss of outer membrane proteins and the synthesis of Metallo-β-lactamases (MBLs). Figure (4) illustrates the various mechanisms by which resistance to carbapenems is contributed to in *Klebsiella pneumoniae*. Strains with a specific type of MBL (MBLs) have also been increasingly reported worldwide in recent years. Strains producing MBLs seem to be associated with more severe infections, prolonging the duration of nosocomial outbreaks. To ensure that reduced susceptibility to carbapenems is not due to carbapenemase production, phenotypic tests using the PCR method can be conducted in the hospitals of Hilla.

To study the molecular mechanisms underlying carbapenemase synthesis, the detection of isolates carrying carbapenemase genes was conducted through PCR amplification of genes such as BlaNDM and BlaKPC, associated with MBL as depicted in Figure (5). Carbapenem-resistant *Klebsiella pneumoniae* (74%) was identified using the Kirby-Bauer disk diffusion method, with Figure (6) below illustrating the capsule types of isolates and their antibiotic resistance patterns. The observed peaks in antibiotic resistance could be attributed to increased MBLs (68%) in the isolates. This finding suggests that the expression of MBLs enhances the ability of microorganisms to resist various classes of beta-lactam antibiotics. Conclusion: Episodes of *Klebsiella pneumoniae* infection are becoming increasingly challenging to treat due to the widespread resistance of most strains to multiple antibiotics, particularly β-lactams. This resistance is largely attributed to porin loss, carbapenemase synthesis (MBLs), and mediated efflux pumps. The expression of MBLs, utilized by certain microorganisms to develop resistance to a range of beta-lactam antibiotics, poses significant epidemiological risks. Strains carrying MBLs appear to be more pathogenic in hospitals, challenging to treat, and may spread more rapidly among patients, contributing significantly to nosocomial outbreaks. These epidemiological risks associated with MBLs stem from their ability to resist multiple beta-lactam antibiotics.

The arms may be treated with antibiotics; however, the expression of MBLs enables the hydrolysis of the majority of molecules of any β-lactam antibiotics. The prevalence of nosocomial infections and outbreaks caused by MBL-producing strains can be attributed to various factors, with the resistance of *Klebsiella pneumoniae* to diverse β-lactam antibiotics being most significant. Consequently, it is believed that the continued use of β-lactam antibiotics will further promote the proliferation and spread of MBL-producing strains worldwide. Regarding the detection of carbapenem resistance in *Klebsiella pneumoniae*, which is closely linked to factors like porin loss, carbapenemase synthesis (MBLs), and mediated efflux pumps, a phenotypic test utilizing PCR to detect the presence of carbapenemase can be conducted in hospitals in Hilla. Below, we outline our use of the PCR method to identify isolates in *Klebsiella pneumoniae* tissue carrying carbapenemase genes.

Additionally, the major MBLs have been identified. All *Klebsiella pneumoniae* isolates indicate the presence of genes associated with MBLs. As depicted in Figure (5), the polymerase chain reaction (PCR) method was utilized to assess *Klebsiella pneumoniae* isolates for the presence of carbapenemase genes. This method targets genes specific to MBLs, such as blaNDM and blaKPC. *Klebsiella pneumoniae* isolate 19 was identified as carrying DNA or rRNA genes. Given that genes like 16SrRNA are present in all isolates, *Klebsiella pneumoniae* isolate 19 likely contains a blaNDM gene, while isolate 40 is likely to harbor a blaKPC gene. This inference arises from the prevalence of blaNDM genes (48%) in the majority of *Klebsiella pneumoniae* cells and the smaller proportion carrying blaKPC genes (72%).

Conclusion

In conclusion, the presence of 16SrRNA has complicated our understanding of antibiotic resistance, and the dissemination of carbapenemase genes exacerbates this challenge. *Klebsiella pneumoniae* isolates commonly harbor the 16SrDNA sequence, rendering them highly resistant to multiple antibiotics. The spread of carbapenemase genes intensifies this resistance by hydrolyzing beta-lactam antibiotics, including carbapenems, rendering them ineffective in treating infections. This poses a serious concern as it limits treatment options for patients infected with this bacterial strain.

Understanding the mechanisms of carbapenemases is crucial for developing effective drugs and vaccines. Carbapenemases constitute a diverse array of antibiotic resistance mechanisms, including metallo-beta-lactamases (MBLs) that utilize metal ions like zinc for their catalytic function. The spread of MBL genes and other carbapenemase genes requires thorough surveillance, molecular characterization, and comprehension of their resistance mechanisms to inform targeted interventions such as drug development and infection control measures.

Furthermore, antimicrobial stewardship, emphasizing the responsible use of antibiotics, and stringent infection control practices are vital components in addressing the escalating burden of antimicrobial resistance. Particularly, BlaKPC-producing strains

of *Klebsiella pneumoniae* have posed significant challenges for healthcare systems worldwide, necessitating comprehensive strategies to mitigate their impact on patient care and public health.

Author contributions

HA.R.M.A., Z.H.J.A., A.K.H.A., J.A.A.A. conducted the experiments, performed the statistical analysis, wrote, edited, and reviewed the article.

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Competing financial interests

The authors have no conflict of interest.

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