Development of a PCR-based method for the detection of *Klebsiella pneumoniae* Carbapenem resistance genes in clinical specimens

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Abstract

Background: Extended-spectrum beta-lactamase-producing *Klebsiella pneumoniae* (ESBL-KP) has shown increasing resistance to carbapenems, which are among the last-resort antibiotics. Carbapenem-resistant *K. pneumoniae* (CRKP) poses significant challenges in healthcare settings due to its resistance to even potent antibiotics, making infections difficult to treat and control. CRKP is often associated with severe complications such as pneumonia, bloodstream infections, and urinary tract infections, particularly in immunocompromised patients.

Aim: This study emphasizes the need for specific identification protocols, individualized therapy, and effective infection prevention measures to control the spread of CRKP.

Materials and Methods: A total of 300 clinical specimens were collected from patients at Al-Hilla Teaching Hospital between April and September 2024, including blood (3), urine (2), wounds (10), and burns (10). Bacteria were isolated using standard media, identified using the VITEK-2 system, and subjected to antibiotic susceptibility testing according to CLSI guidelines (2024). PCR was employed to detect carbapenem resistance genes (*blaKPC*, *blaNDM*, *blaOXA*).

Results: Of the 300 specimens, 25 (8.33%) were identified as *K. pneumoniae*. PCR-based methods enabled rapid detection of resistance genes: *blaKPC* (96%), *blaNDM* (80%), and *blaOXA* (72%). Biochemical tests confirmed positive results for urease, methyl red, citrate, and indole tests. The VITEK-2 system facilitated extensive phenotypic and antibiotic susceptibility testing. Resistance patterns varied, with susceptibility highest to carbapenems and lowest to ampicillin (100% resistance). Molecular detection of the 16S rRNA gene achieved 100% accuracy.

Conclusion: The findings highlight the critical threat posed by CRKP and underscore the importance of molecular diagnostics in detecting resistance genes. This research contributes to understanding carbapenem resistance mechanisms, guiding the development of effective treatment strategies.

Keywords: *Carbapenem-resistant, Klebsiella pneumoniae*, *bla$_{-OXA}$*, *bla$_{-KPC}$*, *bla$_{-NDM}$*

Introduction

It is a Gram-negative facultative anaerobe that could have been isolated from most of hospital-acquired infections including; pneumonia, UTI septicemia, and wound infections [1]. Highly capable of; more severe or even life-threatening infection is the result of increasing emergence of resistance to multiple antibiotics but principally carbapenem antibiotic class which may be used in MDR bacterial infections. CRKP is a new strain that has proven to be a big threat to public health in many countries, it caused more death, longer hospital stays, and fewer treatment plans available [2]. Carbapenems were not active against *K. pneumoniae* clinical isolates with resistant mechanisms because they received the carbapenemase genes that encode proteins capable of hydrolysing carbapenem agents. Carbapenemase genes include gems blaOXA blaKPC, and blaNDM among the clinically important gems in *K. pneumoniae*. These genes can be transmitted horizontally via plasmids, further enhancing the spread of resistance among bacterial populations.

blaOXA (Oxacillinase): The blaOXA gene family is related to the action of beta-lactam antibiotics and carbapenems commonly used in clinics. Variants such as OXA-48 are particularly notorious for their ability to confer resistance to carbapenems while maintaining susceptibility to certain cephalosporins, complicating treatment regimens.

blaKPC (Klebsiella pneumoniae Carbapenemase): blaKPC produces a class A beta-lactamase enzyme which can also cleave carbapenems and other beta-lactam drugs such as penicillins and cephalosporins [3]. KPC-producing K. pneumoniae has been widely reported in healthcare settings and is associated with high mortality rates due to limited treatment options.

blaNDM (New Delhi Metallo-beta-lactamase): blaNDM spans a metallo-beta-lactamase that featuyres zinc ions to deem carbapenems and other beta-lactams. blaNDM gene was initially reported in *K. pneumoniae* in 2008 from New Delhi, India and it has emerged as an epidemic thereafter. This means that its presence may be associated with widespread antibiotic resistance as most of the NDM-producing strains are resistant to multiple antibiotics including aminoglycosides and fluoroquinolone [4]. The increased spread of these

carbapenemase genes among *K. Pneumoniae* isolates is a huge concern to public health, results in hospital-acquired outbreaks, and reduces the management options for patients with infections due to these organisms. This underlines the need for molecular monitoring and timely identification of resistance genes such as *blaOXA*, *blaKPC* and *blaNDM* to control the distribution of CRKP and improve the therapies used in those patients [5].

Materials and Methods

Clinical specimens and culture characteristics

25 *K. pneumoniae* Collected from different specimens (10 wound, 10 burn, 3 blood and 2 urine) from patients admitted to Al-Hilla Teaching Hospital between April, and 2024-September, 2024. All isolates culturing in blood agar and MacConkey agar. The Results of the identification of *Klebsiella pneumoniae* on MacConkey agar showed pink lactose fermenter mucoid colonies on MacConkey agar. Positive test for urease, Voges-Proskauer, and Simmon citrate, but negative for oxidase, methyl red, motility test, indole and TSI test showed A/A with gas, without H2S.

Antimicrobial susceptibility testing

Twenty-five Isolate of K. pneumoniae identified on Mulleur Hinton agar was tested for completely carbapenem groups such as meropenem, imipenem, ertapenem and doripenem by the disc diffusion method. It uses different antibiotics to skin-invasion but commonly used ones include Ampicillin, Ceftriaxone, Aztreonam, Cefotaxime, Ceftazidime, Cefepime, Cefixime, Piperacillin, Ciprofloxacin, Trimethoprim, Amoxicillin-clavulanic acid, Cefoxitin, Nalidixic acid, Piperacillin-tazob Amoxicillin-clavulanic acid, Cefoxitin, Nalidixic acid, Piperacillin-tazobactam, Gentamicin, Levofloxacin. Antibiotic-impregnated disks are placed on an agar plate inoculated with the bacterial isolate overnight 37 at 24hr. The size of the inhibition zone shows the susceptibility zone diameter as used for comparison with CLSI (2024).

Viteck-2 system

The study results showed the role of the VITEK2 System in the identification *K. pneumoniae* faster and more accurate than traditional methods given the speed of detection and efficiency of diagnosis of samples within conditions far from pollution.

Polymerase chain reaction

The PCR method allows for the precise identification of the bacterium and its associated carbapenemase genes (*blaOXA*, *blaKPC*, *blaNDM*) that confer resistance to carbapenem antibiotics. The PCR protocol involves several key steps, from DNA extraction to amplification and detection of target genes.

DNA extraction

Before performing PCR, genomic DNA must be extracted from clinical isolates of *K. pneumoniae*. The quality and purity of the extracted DNA are critical for successful amplification. Clinical specimens (blood, urine, wound, and burn swabs).

DNA extraction method: A variety of methods can be used, including commercial kits or traditional methods like silica-based membranes.

DNA quantification: DNA concentration is measured using a spectrophotometer to ensure adequate input for PCR.

PCR setup

PCR is performed using gene-specific primers to amplify regions of the target genes (*blaOXA-48*, *blaKPC*, *blaNDM*, and 16S rRNA as a control for *Klebsiella pneumoniae*). The primers used in the present study with sequences and amplicons are as in Table 1.

Table 1: Primer sequences and PCR product sizes for target genes in *Klebsiella pneumoniae*

Target genes	Sequences (5'-3')	Product size (bp)	Annealing Tm
16SrRNA	5'-AGAGTTTGATCCTGGCTCAG-3'	897	55°C
	5'-TACGGTTACCTTGTTACGACTT-3'		
bla-NDM	5'-GGTTTGGCGATCTGGTTTTC-3'	621	52°C
	5'-CGGAATGGCTCATCACGATC-3'		
bla-KPC	5'-CATTCAAGGGCTTTCTTGCTGC-3'	798	53°C
	5'-AAGCCTTGGTTGTTGATTAG-3'		
blaOXA-48	5'-GCGTGGTTAAGGATGAACAC-3'	438	55°C
	5'-CATCAAGTTCAACCCAACCG-3'		

PCR reaction mixture

- Template DNA (~50–100 ng)
- Forward and reverse primers (0.5 µM each)
- Taq DNA polymerase (1.25 U)
- dNTPs (200 µM)
- MgCl2 (1.5 mM)
- PCR buffer (1x)
- Nuclease-free water to adjust the total reaction volume (25µL).

PCR Cycling Conditions

The PCR cycling conditions must be optimized for the specific primers and template DNA. A typical PCR cycle for carbapenemase gene detection includes:

- Initial Denaturation: 95°C for 5 minutes
- Denaturation: 95°C for 30 seconds
- Annealing: 55,.52,53 and 55°C (16SrRNA, blaKPC, blaNDM and blaOXA-48) for 30 seconds
- Extension: 72°C for 1 minute
- Final Extension: 72°C for 10 minutes
- Cycle Number: 30–35 cycles.

Assessment of concentration and quality of DNA isolated from K. pneumoniae strains

The levels of DNA and its concentration are very important parameters which directly affect our downstream molecular analyses (PCR). DNA concentrations of the purified samples at 50ng/µl 260 / 280 absorbance of the purified samples.

Results

Distribution of *Klebsiella pneumonia* in the different specimens

25 out of 300 clinical specimens were diagnosed as *Klebsiella pneumonia* collected from 3 blood, 2 urine, 10 wound and 10 burns as in Figure 1.

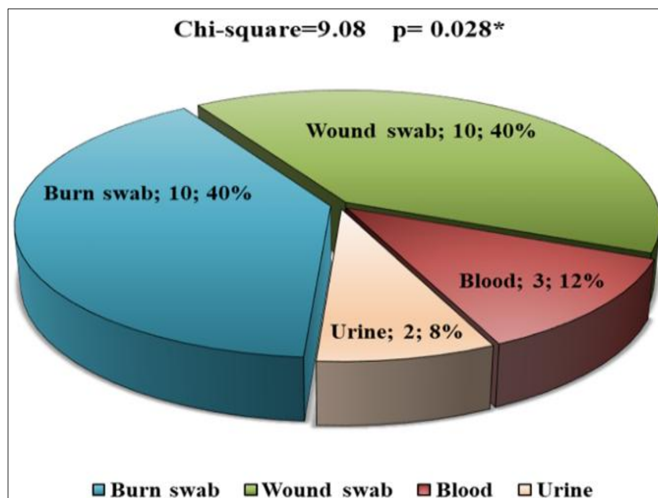

Fig 1: Percentage of K. pneumoniae isolates according to different sources of isolation

Antimicrobial Susceptibility Testing (AST)

The results are presented in a table format:

Table 2: Antimicrobial Susceptibility Testing (AST) Results for *Klebsiella pneumoniae*

Antibiotic	Resistance (%)
Ampicillin	100%
Ceftriaxone	95%
Aztreonam	95%
Cefotaxime	95%
Ceftazidime	95%
Cefepime	95%
Cefixime	90%
Piperacillin	90%
Ciprofloxacin	79%
Trimethoprim	74%
Amoxicillin-clavulanic acid	74%
Cefoxitin	69%
Nalidixic acid	68%
Levofloxacin	63%
Piperacillin-tazobactam	68%
Gentamicin	63%
Imipenem	95%
Doripenem	97%
Ertapenem	98%
Meropenem	99%

Viteck-2system

The results provide insights into the biochemical characteristics of the *Klebsiella pneumoniae* strain tested by Viteck-2system in Table 3.

Table 3: Biochemical test results for *Klebsiella pneumoniae by Viteck -2 system*

Test Symbol	Results	Test Symbol	Results	Test Symbol	Results	Test Symbol	Results
APA	-	ADO	+	Dtre	+	Del	+
H2S	-	LIP	-	SUCT	+	SKG	+
BGLU	+	AGLU	-	TYrA	+	BGUR	-
.SAC	+	ODC	-	CIT	+	PHOS	+
GLYA	+	GGAA	-	Dtre	+	CMT	+

[Positive+, Negative_]

Molecular analysis results for *Klebsiella pneumoniae*

The molecular analysis revealed the following results for the detection of the carbapenem resistance genes in *Klebsiella pneumoniae* isolates.

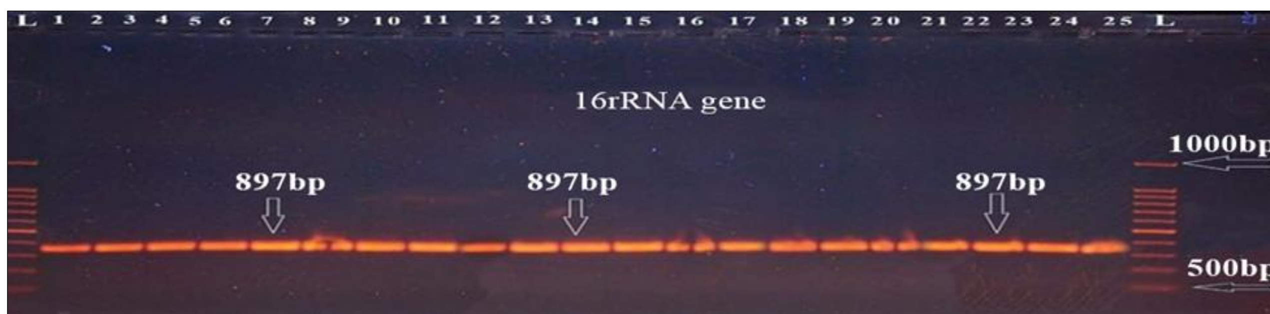

Fig 2: The *16SrRNA* gene product (897 bp) was detected using agarose gel electrophoresis. The DNA isolated from *Klebsiella pneumoniae* samples tested positive in isolates 1 -25 isolated.

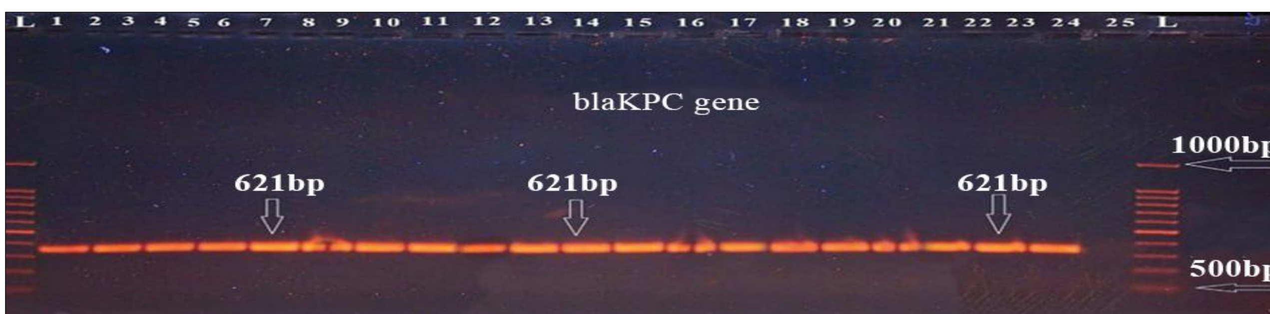

Fig 3: The KPCgene product (621 bp) was detected using agarose gel electrophoresis. The DNA isolated from *Klebsiella pneumoniae* samples tested positive in isolates 1 -24 isolated.

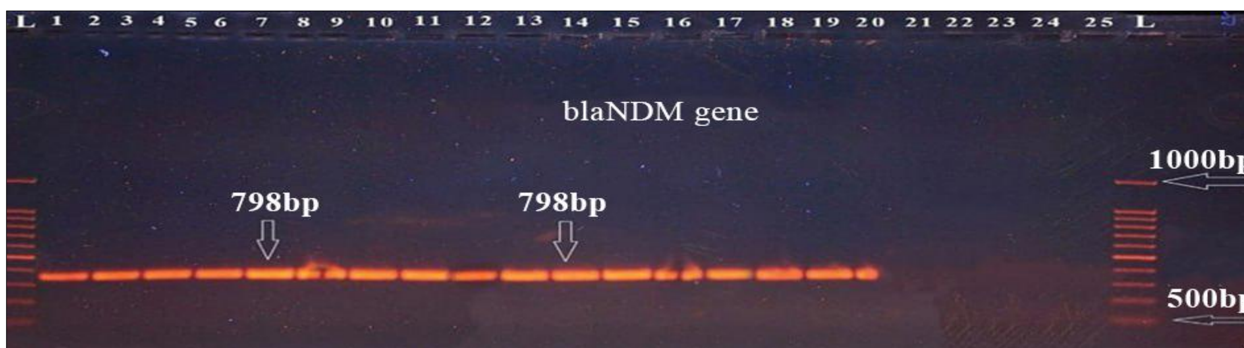

Fig 4: The bla-*NDM* gene product (798 bp) was detected using agarose gel electrophoresis. The DNA isolated from *Klebsiella pneumonia* samples tested positive in isolates 1 -20 isolated.

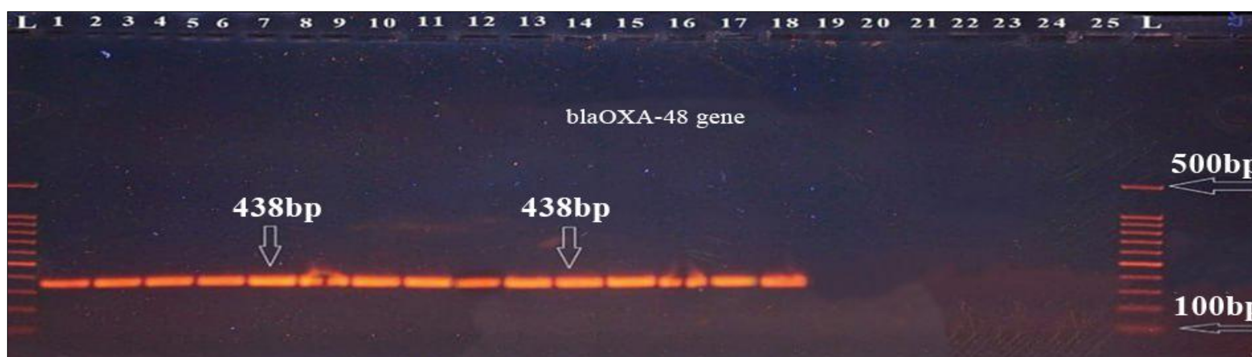

Fig 5: The *blaOXA-51* gene product (438 bp) was detected using agarose gel electrophoresis. The DNA isolated from *Klebsiella pneumoniae* samples tested positive in isolates 1 -18isolated.

Measurement of concentration and purity of DNA

At the end of DNA extraction, the concentration and purity of DNA by Nanodrop, the results showed a concentration between (50 ng/µl) and a purity (1.69 - 2.02nm).

Discussion

Klebsiella pneumonia is a big factory pathogen that has multifaceted roles in clinical infections. It is known to be a healthcare-associated opportunistic pathogen found commonly

in blood, urine, burns and wounds within immunocompromised, critical ICU care patients or those with invasive devices. Those including urinary tract infection – UTI in catheterized patients, bloodstream infections – BSI associated with high mortality, and respiratory infections such as ventilator-associated pneumonia – VAP. K. pneumoniae also is isolated from open wounds and burn specimens with high frequencies and HAI for the same reasons. A study by [6], identified that as much as 30% of catheter-associated UTIs in hospitalized patients are caused by *K. pneumoniae,* the high frequency of urinary isolates demonstrated in Figure 1. The blood isolates component was substantial in this study as supported by [7] work which revealed *Klebsiella pneumoniae* as having a prevalence rate of 25% of BSIs among ICU patients. This shows the rising tendency of *K. pneumoniae* in serious infection and especially in intensive and sub-intensive care units where invasive procedures contribute to bacteremia. Furthermore [8], observed that it is *K. pneumoniae* the most common pathogen in patients with mechanical ventilators as the cause of VAP; accounting for 20%. In common with [9], this bacterium was also found to be among the most common causes of wound infections in burn units. The AST profile of *K. pneumonia* showed a high level of resistance to a number of antibiotics among the isolates used in this work. This is consistent with the reports by clinics all over the world of the challenges of handling multidrug-resistant *K. pneumoniae* strains. In the present study, the rate of resistance was 100% to ampicillin and 95% to several third-generation cephalosporines such as ceftriaxone, cefotaxime, ceftazidime and cefepime. These findings resemble the study by [10], who recognized that ESBLs were the main causes of beta-lactam resistance in K. pneumoniae isolates as most of the time it was resistant to these antibiotics. The new strain of ESBL has greatly reduced the use of cephalosporin to a very minimal level exposing carbapenem as a resort antibiotic. In the present work, resistance was detected towards piperacillin 90% and piperacillin-tazobactam 68% which suggests the emergence of CRKP. Furthermore, there was a 79% resistance rate to ciprofloxacin a 5inolone commonly used in managing UTIs. This is in concordance with Kumar *et al*., 2023 proposing that quinolone resistance in *K. pneumoniae* depends on mutations in the QRDRs and efflux pumps exercise. High resistance rates of both ciprofloxacin and levofloxacin at 63% in this study evidence the extensive spread of quinolone-resistant K. pneumoniae strains mainly in hospitals. Based on the current study, 63% of the *K. pneumonia* isolates were resistant to gentamicin similar to the research conducted by [11], who showed that the resistance to aminoglycoside was on the rise in *K. pneumonia* isolates. The common combination partner is beta-lactams or carbapenems for serious infection management; however, steadily increasing resistance is reducing their synergy. Moreover, the resistance percentage against trimethoprim and amoxicillin-clavulanic acid was 74%, probably because of the increase in resistance of the involved pathogen to these two most commonly used antibiotics [12], reported comparable resistance rates of *K. pneumoniae* isolated from patients with urinary and respiratory tract infections. Sixty-five point six per cent of the *K. pneumoniai* isolates were identified as positive through the Vitek-2 system for adenosine deaminase (ADO), D-trehalose fermentation (Dtre), succinic acid utilization (SUCT), skatole production (SKG), Available negative results were for APA; H2S; and LIP. The first three tested enzyme activities included Alpha-glucosidase (AGLU), beta-galactosidase (BGUR), and ornithine decarboxylase (ODC) [13], have also noted comparable biochemical patterns of *Klebsiella pneumoniae* strains, with adenosine deaminase and beta-glucosidase activity being identified in 77% and 71% of the strains respectively. These enzymatic functions were determined to be related to higher pathogenicity and antibiotic resistance in *K. pneumoniae.* Furthermore, the study by [14], also established a significant correlation between citrate usage and pathogen capability to cause UTI, which supports the hypothesis that these metabolic features played a relevant role in the ecological success of K. pneumoniae in the host organism. This study also presents high-level resistance to carbapenem antibiotics among the K. pneumoniae isolates, encompassing imipenem (95%), [15], also found comparable results where resistance rate was 90% for imipenem and 92% for meropenem [16], reported still higher doripenem resistance patterns, which ranged up to 85% of the isolates. Specifically, with a doripenem resistance rate of 97% we could conclude that although the use of this antibiotic is not ineffective it is steadily becoming so. In molecular diagnosis, 100% positivity for the 16S rRNA gene in the isolates confirmed that efficient identification of *Klebsiella pneumoniae.* The 16S rRNA gene is another molecular marker routinely used to identify bacterial species and to demonstrate the presence of certain bacteria in clinical samples [17]. Thus, considering carbapenemase production–the marker of carbapenem resistance, the results in terms of the prevalence of the blaKPC gene in 96% of the isolates are disturbing. This finding is in agreement with the sick literature since the evolution of KPC-producing *K. pneumoniae* has received a lot of negative attention worldwide, especially in hospital-acquired infections [18]. It is, therefore, important to continue surveying and preventively addressing extended-spectrum β-lactamase–producing KPC isolates. Whereas, blaNDM was detected in 80 percent isolates meaning that preventative measures for New Delhi metallo-beta-lactamase which break down a number of beta-lactams/ carbapenems make the infections more challenging to treat. The high levels of NDM-producing strains are in concordance with other parts of the world where its trend is also on the increase. Besides, the blaOXA gene, associated with OXA-type beta-lactamases involved in carbapenem resistance was found in 72% of isolates and was prevalent in K. pneumoniae isolates [21, 22]. This fact of co-carriage of multiple resistance genes such as *blaKPC, blaNDM, blaOXA* demonstrated that the clinical isolates of *K. pneumoniae* harbor multiple resistance patterns making it difficult to treat and increases the risk of failure of therapy. It for these reasons that thought should be given to the implementation effective and more importantly enforceable antimicrobial stewardship programmes and strict infection control measure to prevent the spread of MDR It is becoming mandatory.

The relationship between blaKPC, blaNDM, and blaOXA genes in *Klebsiella pneumoniae*

All these genes code for different types of carbapenemase enzymes that can degrade carbapenems and other beta-lactam antibiotics and therefore infections from *K. pneumoniae* become a nightmare to manage [23, 41]. The blaKPC gene encodes a protein which is KPC enzyme, this being one of the

most extended carbapenemases in *K. pneumoniae*. KPC has been documented to hydrolyse all Classes of beta lactam antibiotics from carbapenem to cephalosporin and penicillin. Even greater worry is the fact that KPC covetable *K. pneumoniae* strains come with other resistance genes that make the bacterium resistant to several classes of antibiotics. The dissemination of the blaKPC gene throughout the globe has immensely contributed to the globalization of the CRKP mainly in hospitals [24, 42]. The blaNDM gene produces the NDM enzyme, metallo-beta-lactamase which catalyzes the hydrolysis of a wide range of other beta-lactam antibiotics together with carbapenems. Hence, treatment of *Klebsiella pneumoniae*-producing NDM is highly worrisome because NDM is not as easily defeated by-products such as clavulanic acid as KPC. KPC-producing *K. pneumoniae* has been emerging in various parts of the world over the last few years which is the source of the problem of multidrug resistance. In the presence of other co-resistance genes, including blaKPC, NDM influences the choice of the infection management team on the next course of action or treatment to give. The realization of OXA-type carbapenemases was inculcated through bla OXA gene, which has been subclassified into a large family of enzymes accountable for carbapenem resistance [25, 43]. This is true because belonging to the OXA carbapenemases, OXA-48 is primarily associated with *K. pneumoniae* and is the one with less activity against carbapenem hydrolysis compared to, for example, KPC or NDM. Although they Level huge resistance when they are presented along other beta-lactamase genes with specific reference to ESBLs in the strains where they are synthesized. or have porin mutations that restrict antibiotic entry [26, 44].

Co-occurrence and compounded resistance: It is not rare to find clinical isolates of K. pneumoniae harbouring both *blaKPC, blaNDM and blaOXA* simultaneously. This coexistence makes the phenotype's phenotype very resistant because any gene offers a specific method to break down carbapenems and other antibiotics. For example, while blaKPC and blaNDM provide resistance against a broad spectrum of beta-lactams, blaOXA enzymes can enhance resistance to specific carbapenems, especially when other mutations or enzymes are present [27, 45].

Treatment challenges: Such co-expression of these genes greatly restricts therapeutic management. Carbapenems which were usually used at the last resort against multidrug-resistant Gram-negative bacteria become ineffective, and the clinician is left with suboptimal or more toxic or nephrotoxic agents like polymyxins, tigecycline, or combinations. Additionally, the ability of *K. pneumoniae* to harbor multiple resistance genes increases the likelihood of treatment failure, extended hospital stays, and higher mortality rates [28].

Horizontal gene transfer and global spread: The genes include *blaKPC, blaNDM and blaOXA*, are frequently carried on the mobile genetic elements such as plasmids that can be transferred between bacteria through horizontal gene transfer, respectively [29, 46]. It adds further to the accelerated emergence of carbapenem-resistant *K. pneumoniae* across the globe and also the subsequent appearance of strains having mutual carbapenemase genes which becomes a formidable public health problem. The relationship between *blaKPC, blaNDM, and blaOXA* in *K. pneumoniae* highlights the complexity of carbapenem resistance mechanisms and their synergistic impact in driving multidrug resistance, making treatment of infections increasingly difficult and elevating the urgency for new therapeutic strategies and stringent infection control measures [30, 40].

Conclusion

The study highlights the significance of Carbapenem-resistant *Klebsiella pneumoniae*, as it carries genes that confer resistance to carbapenems, the last line of defense against infections caused by this bacterium. As a result, these bacteria have become increasingly dangerous due to the challenges in eradicating them. The findings also underscore the critical role of carbapenem-resistance genes and emphasize the need for further research into the mechanisms of resistance to develop effective treatments for infections caused by these pathogens.

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