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Distribution of *blaKPC*, *blaNDM*, and *blaOXA-48*-like Genes among Carbapenem-Resistant Clinical *Klebsiella pneumoniae* Isolates Using Real-Time PCR

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Abstract

Carbapenem-resistant *Klebsiella pneumoniae* is an important cause of difficult-to-treat hospital infections. Resistance is frequently mediated by carbapenemase genes, particularly *blaKPC*, *blaNDM*, and *blaOXA-48*-like. Real-time PCR detection of these genes can support infection-control decisions and guide antimicrobial stewardship. This study determined the distribution of *blaKPC*, *blaNDM*, and *blaOXA-48*-like among carbapenem-resistant clinical *K. pneumoniae* isolates and evaluated their association with specimen source, hospital ward, and antimicrobial resistance profile. A cross-sectional clinical microbiology study was performed on 124 non-duplicate carbapenem-resistant *K. pneumoniae* isolates recovered from routine human clinical specimens. Isolates were identified by culture, biochemical testing, and automated identification. Antimicrobial susceptibility testing was performed using an automated system and disk diffusion, with interpretation according to current clinical breakpoints. Genomic DNA was extracted from pure colonies. Short-amplicon TaqMan real-time PCR assays were used to detect *blaKPC*, *blaNDM*, and *blaOXA-48*-like. At least one carbapenemase gene was detected in 112/124 isolates (90.3%). *blaNDM* was the most frequent gene, detected in 68 isolates (54.8%), followed by *blaOXA-48*-like in 54 isolates (43.5%) and *blaKPC* in 18 isolates (14.5%). Dual-gene carriage was detected in 31 isolates (25.0%), mainly *blaNDM* + *blaOXA-48*-like. Triple-gene carriage was detected in 5 isolates (4.0%). Gene-positive isolates showed significantly higher multidrug resistance than gene-negative carbapenem-resistant isolates. Short-amplicon real-time PCR provided a rapid and image-free method for detecting clinically important carbapenemase genes in *K. pneumoniae*. The predominance of *blaNDM* and *blaOXA-48*-like suggested a local resistance pattern requiring continuous surveillance.

Keywords: *Klebsiella pneumoniae*; carbapenem resistance; *blaNDM*; *blaKPC*; *blaOXA-48*-like; real-time PCR

التوزيع الجزيئي لجينات ***blaKPC*** و ***blaNDM*** و ***blaOXA-48-like*** التوزيع الجزيئي لجينات ***blaKPC***و ***blaNDM***و ***blaOXA-48*-like** بين عزلات ***Klebsiella pneumoniae*** السريرية المقاومة للكاربابينيم باستخدام تفاعل البلمرة المتسلسل اللحظي قصير المقطع

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الخلاصة

تُعدّ *Klebsiella pneumoniae* المقاومة للكاربابينيم من الأسباب المهمة للعدوى المستشفوية صعبة العلاج. بسبب مقاومة البكتيريا لجينات *blaKPC* و *blaNDM* و *blaOXA-48*-like. يمكن للكشف عنها بأستخدام (Real-time PCR) أن يدعم قرارات مكافحة العدوى ويسهم في توجيه الاستخدام للمضادات الميكروبية. هدفت الدراسة إلى تحديد توزع جينات *blaKPC* و *blaNDM* و *blaOXA-48*-like بين عزلات *K. pneumoniae* وأُجريت الدراسة على 124 عزلة من *K. pneumoniae* المقاومة للكاربابينيم، من عينات سريرية واستُخلص الحمض النووي الجينومي من المستعمرات، ثم استُخدام TaqMan Real-time PCR للكشف عن الجينات *blaKPC* و *blaNDM* و *blaOXA-48*-like. حيث ظهر جين كاربابينيماز واحد في 112 من أصل 124 عزلة (90.3%). وكان الجين *blaNDM* الشائع ، إذ كُشف عنه في 68 عزلة(54.8%) ، و *blaOXA-48*-like في 54 عزلة(43.5%) ، ثم الجين *blaKPC* في 18 عزلة (14.5%). وكُشف عن حمل مزدوج للجينات في 31 عزلة (25.0%)، وكان الأكثر شيوعًا هو *blaNDM* + *blaOXA-48*-like، في حين كُشف عن حمل ثلاثي للجينات في 5 عزلات (4.0%). كما أظهرت العزلات الحاملة لهذه الجينات مقاومة متعددة للمضادات الحيوية بصورة معنوية أعلى مقارنة بالعزلات المقاومة للكاربابينيم والخالية من هذه الجينات وتشير غلبة الجينين *blaNDM* و *blaOXA-48*-like إلى وجود نمط مقاومة محلي يستدعي المراقبة المستمرة والترصد الدوري.

1. Introduction

Carbapenem-resistant *K. pneumoniae* is a major concern for clinical microbiologists because this organism is linked to hospital-acquired infections, treatment failures, extended hospitalizations, and increased mortalities. This organism can infect immunocompromised and hospitalized patients and is the cause of infection of the urinary tract, blood, lung, and wounds, and cause infections of the abdomen and of associated devices [1]. *K. pneumoniae* is especially significant now because many of its strains of the organism are multidrug resistant, including the resistance of carbapenems which are the drugs of choice for the treatment of serious infections due to extended spectrum β-lactamase-producing Enterobacterales. Due to the most recent surveillance studies and clinical studies, it is evident that the spread of carbapenem-resistant Enterobacterales, particularly, carbapenem-resistant *K. pneumoniae*, continues to be a significant public-health concern, particularly, in hospital settings [2,3].

Carbapenemase production is primarily responsible for much of the clinical threat posed by carbapenem-resistant *K. pneumoniae*. A particular concern are carbapenemases, which are enzymes that can inactivate carbapenems and a broad spectrum of β-lactam antibiotics. In *K. pneumoniae*, the most common carbapenemase families are KPC, NDM, OXA-48-like, VIM, and IMP family enzymes. Yet, there is much variance in the dominance and distribution of these genes at the regional and global levels. In some geographical areas, *blaKPC* is predominant, whereas in other areas *blaNDM*, *blaOXA-48*-like, or a combination of different genes of carbapenemases are present. This significant variance necessitates the importance of local molecular surveillance for the rational choice of treatment and infection-control measures [4-6].

Among these genes, *blaNDM* is particularly critical since NDM metallo-β-lactamases are resistant to avibactam and multiple β-lactamase inhibitors. This makes NDM-producing *K. pneumoniae* infections challenging to treat as they may present with distinct NDM phenotypes. Such infections may necessitate combination therapy with aztreonam and a myriad of other agents depending on the results of microbiological testing. Clinical evidence shows the increasing frequency of *blaNDM* with or without *blaOXA-48*, especially in multi-drug resistant clinical samples from hospitalized and intensive care patients [7-10].

The *blaOXA-48*-like genes are also of clinical concern as the OXA-48-like carbapenemases appear to result in heterogeneous carbapenem resistance. Some OXA-48-like producing isolates may have an intermediate or near borderline carbapenem resistance. This represents a phenotypic detection challenge in standard clinical laboratory settings particularly in the presence of OXA-48-like genes, extended-spectrum β-lactamases (ESBL), changes in porins, or other carbapenemase genes. The growing numbers of K. pneumonia susceptibility evaluations have provided evidence of the increasing importance of the detection of OXA-48-like carbapenemases especially in clinical microbiology since they are rapidly expanding concerning the therapeutic arms of the hospital and the challenging nature of the problem [11-14].

The *blaKPC* gene is ingrained in many international biomedical studies as one of the most relevant carbapenemase determinants. There are instances of the KPC-producing *K. pneumoniae* variants causing outbreaks and ICU transmissions, as well as Classification Level 4 Clonal Lineages in High-Risk Bloodstream Infections. KPCs are uncommon in some areas; however, the prevalence of NDMs or OXA-48-like is significant. The KPCs are still relevant because the other factors may alter treatment, mainly due to the presence of new β-lactams combined with β-lactamase inhibitors. *K. pneumoniae* with KPC has been the subject of some of the most impactful studies with respect to severe clinical infections, including ICU related bloodstream infections and sepsis. This has allowed the studies of *blaKPC* to advocate for inclusion or expansion of targeted molecular screening panels [2, 5, 15].

The inclusion of routine antimicrobial susceptibility testing for the identification of variants of the carbapenemase resistant organism is of significance. It is, however, less than ideal in isolation, mainly because testing identifies organism resistance. However, the results of testing, including the carbapenemase used, significantly contributes to the selection of the organism for a control response, relaying of transmission observations to other laboratory personnel, and the use of other precautionary measures [1, 4, 16].

Recent studies show that rapid methods based on molecular or immunological techniques to detect carbapenemases (carbapenem-hydrolyzing enzymes of the bacterial genus that include Klebsiella, Enterobacter, Serratia, Salmonella, and Erwinia) are of great benefit since they limit the time required to detect high risk resistant isolates and support timely infection control practices. Unfortunately, many laboratories are lacking cost-effective and relatively simple molecular diagnostic panels designed to detect the local prevalence of specific carbapenemases. A three-gene real-time PCR panel that includes the *blaKPC*, *blaNDM*, and *blaOXA-48*-like genes is feasible since these resistance determinants are widely studied and are suitable to be addressed by real-time PCR using short amplicons [3, 4, 14].

Hence, the current study was designed to detect the presence of the *blaKPC*, *blaNDM*, and *blaOXA-48*-like genes among the carbapenem-resistant *K. pneumoniae* clinical isolates using short-amplicon real time PCR. The study was designed utilizing culture identification, antimicrobial susceptibility, Ct values, and amplification curve analyses. This method utilizes local molecular surveillance of carbapenem-resistant *K. pneumoniae* and would be of aid in improving infection control and antimicrobial restraint planning.

2. Methods

2.1 Study design and setting

This study used a cross-sectional design and was conducted in the microbiology laboratory unit at the College of Science, Alfarahidi University, Baghdad, and the Mazaya University College, Dhi Qar, Iraq. It included non-duplicate *K. pneumoniae* bacterial isolates obtained via routine diagnostic clinical specimens from laboratories. Patient's data was obtained anonymously and included only one clinically obtained isolate to prevent duplication. This study was laboratory based and used the isolates and data directly obtained from the routine laboratory.

2.2 Study population and specimens

The specimens used in the study included blood, urine, sputum, and sputum endotracheal aspirate, as well as a variety of specimens including swabs and sterile body fluids. Only those confirmed to be *K. pneumoniae* and exhibiting resistance or reduced sensitivity to a given carbapenem were included. Isolates were excluded if they were obtained from repeated specimens of a patient, isolates obtained from mixed cultures, isolates whose identity was unverified, and isolates whose DNA extraction or real time PCR internal control failed.

2.3 Sample size

A total of 120 carbapenem-resistant K. pneumoniae isolates which included non-duplicate samples were included in the study. This sample size was statistically appropriate to estimate the occurrence of the three carbapenemase genes and assess the variability in gene distribution based on specimen source, clinical ward, and resistance to antimicrobials.

2.4 Culture and preliminary identification

Clinical specimens were cultured using standard bacteriologic media based on the specimen type. For urine specimens, MacConkey and blood agar were used, while positive blood cultures created a need for subculturing using blood and MacConkey agar. Respiratory and wound samples were also cultured on blood, MacConkey, and chocolate agar as necessary and were incubated aerobically between 35-37 °C for 18-24 hours.

MacConkey agar colonies that were large, slimy, and lactose-fermenting were presumptive *K. pneumoniae* colonies. With the aid of Gram staining and traditional biochemical methods, presumptive identification was performed. Once the identification was done, isolates were confirmed using an automated bacterial identification system, such as VITEK 2 Compact, Phoenix, MicroScan, or MALDI-TOF MS, depending on what was available at the laboratory.

2.5 Testing for antimicrobial resistance

Testing for antimicrobial resistance was done using an automated system and/or disk diffusion that met the laboratory requirements. The test antimicrobial resistance panel included imipenem, meropenem, ertapenem, amikacin, gentamicin, ciprofloxacin, levofloxacin, cefotaxime, ceftazidime, cefepime, piperacillin–tazobactam, trimethoprim–sulfamethoxazole, tigecycline, and colistin that were at the laboratory. These were all tested to determine multidrug resistance patterns, possible treatment options, and resistance beyond carbapenems.

When evaluating for reduced carbapenem resistance, antibiotics measured were imipenem, meropenem, or ertapenem. When available, meropenem and imipenem. Quality control was completed using standard reference strains such as Escherichia coli ATCC 25922 and *K. pneumoniae* ATCC 700603.

2.6 Extraction of DNA

For the extraction of DNA, pure overnight colonies were used. DNA was extracted using 200 µL of phosphate-buffered saline or nuclease-free water. The tubes were incubated at 95–99°Cfor ten minutes to slowly break down the cell membranes. The tubes were then placed in an ice bath for 3-5 minutes. The tubes were then centrifuged at 13,000 rpm for 3 minutes. The supernatant was transferred to a new sterile tube for DNA extraction. The QIAamp DNA Mini Kit, Qiagen, Hilden, Germany", was used following the manufacturer's instructions for Gram-negative bacteria. The DNA was assessed for both concentration and purity using a NanoDrop spectrophotometer. The DNA samples were kept if the A260/A280 ratio was about 1.7–2.0. The collected DNA samples were stored at −20°C until the real-time PCR was carried out.

For this study, the real-time PCR targets were the *blaKPC*, *blaNDM*, and *blaOXA-48*-like genes. The CDC KPC/NDM protocol for real-time PCR identified automated pipettes for the CDC PCR test to use for both the primer set and the probe for the respective target genes (Table 1).

Table 1- Real-time PCR primers and probes used for detection of carbapenemase genes.

Target gene	Primer/probe name	Sequence 5′–3′	Amplicon size	Reference
blaKPC	KPC-F	GGC CGC CGT GCA ATA C	<150 bp	[17]
blaKPC	KPC-R	GCC GCC CAA CTC CTT CA	<150 bp	[17]
blaKPC	KPC-Probe	FAM-TG ATA ACG CCG CCG CCA ATT TGT-BHQ	<150 bp	[17]
blaNDM	NDM-F	GAC CGC CCA GAT CCT CAA	<150 bp	[17]
blaNDM	NDM-R	CGC GAC CGG CAG GTT	<150 bp	[17]
blaNDM	NDM-Probe	HEX-TG GAT CAA GCA GGA GAT-BHQ	<150 bp	[17]
blaOXA-48-*like*	OXA48-RT-F	TCT TAA ACG GGC GAA CCA AG	125 bp	[18, 19]
blaOXA-48-*like*	OXA48-RT-R	GCG TCT GTC CAT CCC ACT TA	125 bp	[18, 19]
blaOXA-48-*like*	OXA48-RT-Probe	6-FAM-AGC TTG ATC GCC CTC GAT TTG G-TAMRA	125 bp	[18, 19]
16S rRNA internal control	16S rRNA-F	TGG AGC ATG TGG TTT AAT TCG A	Internal control	[19]
16S rRNA internal control	16S rRNA-R	TGC GGG ACT TAA CCC AAC A	Internal control	[17]
16S rRNA internal control	16S rRNA-Probe	CY5-CA CGA GCT GAC GAC AR*C CAT GCA-BHQ	Internal control	[17]

2.7 Real-time PCR reaction mixture

Each reaction was prepared in a final volume of 20 µL. A standard TaqMan reaction contained (Table 2):

Table 2- Reaction components and volumes for RT-PCR

Component	Volume per reaction
2× qPCR Probe Master Mix	10 µL
Forward primer, 10 µM	0.8 µL
Reverse primer, 10 µM	0.8 µL
Probe, 10 µM	0.4 µL
DNA template	2 µL
Nuclease-free water	6 µL
Total	**20 µL**

2.8 Real-time PCR cycling conditions

The following cycling program was used (Table 3):

Table 3- Cycling program for RT-PCR

Step	Temperature	Time	Cycles
Initial enzyme activation	95°C	3 min	1
Denaturation	95°C	3 sec	35–40
Annealing/extension	60°C	30 sec	35–40

2.9 Statistical analysis

SPSS, GraphPad Prism and R were used for calculations. Categorical parameters are expressed as percentages. Continuous parameters are expressed as mean ± standard deviation and median (interquartile range) values. The chi-square test or Fisher's exact test compared the frequency of the carbapenemase genes with respect to the specimen type, ward, sex, and resistance phenotype. The difference in the median of MIC values among the groups is given by the Mann-Whitney U test and the Kruskal-Wallis test. The p-value < 0.05 was considered significant.

3. Results

3.1 Distribution of isolates according to specimen type

The specimen source where the most isolates were collected was the urine, and the second was the sputum/endotracheal aspirate, blood, wound swabs, and other body fluids. A majority of isolates were collected from patients in the hospital, specifically from the ICU and surgical wards (Table 2).

Table 4- Distribution of isolates according to specimen type

Specimen type	Number	Percentage
Urine	46	38.3%[a]
Sputum/endotracheal aspirate	28	23.3%[b]
Blood	22	18.3%[c]
Wound/pus	18	15.0%[c]
Body fluids	6	5.0%[d]
Total	120	100%

Different superscript letters within the same column indicate significant differences between groups at $P < 0.05$.

3.2 Antimicrobial resistance profile

Every isolate demonstrated a form of resistance toward one carbapenem. Resistance profiles show a heavy predominance of ertapenem resistance, with some resistance to meropenem and imipenem. A few of the resistance profiles towards piperacillin-tazobactam, fourth, and third-generation cephalosporins, and fluoroquinolones, showed a similar trend of high-level resistance. Resistance was lowest against amikacin and tigecycline (Table 3).

Table 5- Antimicrobial resistance profile

Antibiotic	Resistant isolates	Resistance percentage
Ertapenem	120/120	100%[a]
Meropenem	104/120	86.7%[b]
Imipenem	98/120	81.7%[c]
Ceftazidime	116/120	96.7%[d]
Cefepime	112/120	93.3%[d]
Ciprofloxacin	101/120	84.2%[b]
Gentamicin	82/120	68.3%[e]
Amikacin	54/120	45.0%[f]
Tigecycline	26/120	21.7%[g]
Colistin	18/120	15.0%[h]

Different superscript letters within the same column indicate significant differences between groups at $P < 0.05$.

3.3 Carbapenemase Gene Frequency

Carbapenemase, and a higher positivity rate of 85.0% for at least one of the three designated carbapenemase genes, was detected for 102/120 isolates through real-time PCR assays. Resistance profiles showed a dominant resistance to *blaNDM*, and a lesser predominance to *blaOXA-48*-like and *blaKPC* genes. The highest resistance was to multiple

genes, followed by single gene carriage of 57 isolates. The highest dominant resistance profile was to the *blaNDM* and *blaOXA-48*-like genes. The highest rate of multiple carbapenemase gene resistance was observed for blood isolates, and for respiratory isolates. The most frequent sign of a positive *blaNDM* urine isolate was due to the high prevalence of urine specimen type (Table 4).

Table 6- Carbapenemase Gene Frequency

Gene	Positive isolates	Percentage
blaNDM	76/120	63.3%[a]
blaOXA-48-*like*	58/120	48.3%[b]
blaKPC	22/120	18.3%[c]
Any target gene	102/120	85.0%[d]
Negative for all three genes	18/120	15.0%[e]

Different superscript letters within the same column indicate significant differences between groups at $P < 0.05$.

3.4 Relationship Between Gene Carriage and Carbapenem Resistance

The dual gene-included isolates exhibited a higher resistance to imipenem and meropenem than the single gene systems exhibited. The dual and twin-positive systems showed the highest meropenem and imipenem resistance. The highest prevalence of resistance occurred with the dual and triple combined systems (Table 5-7).

Table 7- Co-occurrence patterns

Genotype pattern	Number	Percentage among all isolates
blaNDM *only*	32	26.7%[a]
blaOXA-48-*like only*	18	15.0%[b]
blaKPC *only*	7	5.8%[c]
blaNDM + blaOXA-48-*like*	29	24.2%[a]
blaNDM + blaKPC	8	6.7%[c]
blaKPC + blaOXA-48-*like*	4	3.3%[c]
blaNDM + blaKPC + blaOXA-48-*like*	4	3.3%[c]
Negative for all three genes	18	15.0%[b]

Different superscript letters within the same column indicate significant differences between groups at $P < 0.05$.

Table 8- Gene distribution according to specimen type

Specimen type	blaNDM	blaOXA-48-like	blaKPC	Multiple genes
Urine (n=46)	29[a]	19[a]	7[a]	14
Respiratory (n=28)	18[b]	15[b]	6[a]	12
Blood (n=22)	17[b]	13[c]	6[a]	11
Wound/pus (n=18)	9[c]	8[d]	2[b]	6
Body fluids (n=6)	3[d]	3[e]	1[b]	2

Different superscript letters within the same column indicate significant differences between groups at $P < 0.05$.

Table 9- Association between gene carriage and carbapenem resistance

Genotype group	Meropenem resistance	Imipenem resistance	p value
Single-gene positive	46/57 (80.7%)	43/57 (75.4%)	0.021
Multiple-gene positive	43/45 (95.6%)	42/45 (93.3%)	0.032
Negative for three genes	15/18 (83.3%)	13/18 (72.2%)	0.0012

Different superscript letters within the same column indicate significant differences between groups at $P < 0.05$.

4. Discussion

In the present study, the majority of carbapenem-resistant *K. pneumoniae* isolates recovered from human clinical samples contained carbapenemase genes. The majority of the positive isolates lend support to the usefulness of implementing RT-PCR in routine susceptibility testing, which is likely to overcome the finding of the phenotypic resistance. Resistance, thus, highlights the importance of this study, given that the type of carbapenemases produced may determine the outcome of infection control strategies, whether to change the choice of antimicrobial agents, as well as the means and methods of local surveillance (1, 13, 14).

The finding of dominant *blaNDM* in the recent isolates was the most diagnostically significant. Reports of *K. pneumoniae* with NDM in the clinical arena have become more common, notably in cases of hospitalized, ICU, and broad-spectrum antimicrobial recipient patients. NDM determinants are metallo-β-lactamases and are, in part, challenging due to the inability of avibactam and various other β-lactamase inhibitors to impede their activity. Therefore, infection control of NDM-producing pathogens may be a challenge requiring diagnostic and therapeutic measures that differ from KPC-producing pathogens. In recent years, treatment recommendations and research have focused on the use of aztreonam, cefiderocol, and either susceptibility-focused therapy or treatment strategies for other NDM-producing Enterobacterales (7, 8, 10]. It is in this context that the high frequency of *blaNDM* identified within the present study suggests that regional treatment strategies and policies are of necessity to focus on more than just the current reported carbapenem resistance.

The second most prominent gene was *blaOXA-48*-like. This was primarily scheming concerning the clinical importance of the OXA-48-like carbapenemases. This was because

OXA-48-like derivatives tend to be difficult to predict using the routine phenotypic patterns. Some OXA-48-like-producing isolates have been shown to exhibit a varying range of carbapenem MIC values, and the ability to identify these isolates becomes a great deal more difficult when the range of resistance to the carbapenem is borderline or when additional ESBL are present. In addition to having the presence of a porin defect. Some recent evaluations regarding the detection methodologies have reported that one of the common characteristic features of carbapenem-resistant *K. pneumoniae* was an OXA-48-like positivity, which demands the inclusion of rapid diagnostic panels [11-13]. This research illustrated the high prevalence of *blaOXA-48*-like suggesting the need for rapid routine molecular diagnostic screening, particularly in the case of facilities where the presence of OXA-48-like producers exists.

The frequency of *blaKPC* was lower than that of *blaNDM* and *blaOXA-48*-like. This result illustrated that KPC was the most dominant subtype of the carbapenemases in the studied isolate collection. Consequently, its presence was of greater importance as KPC-producing *K. pneumoniae* was reported several times to be associated with serious hospital outbreaks, bloodstream infections, as well as the most concerning high-risk clonal lineages. This study illustrated how KPC-producing *K. pneumoniae* was commonly reported from ICU and bloodstream infections, including cases where these isolates produced KPC along with other carbapenemase genes [2, 5, 15]. Hence, the lesser the frequency of *blaKPC*, did not warrant its removal from the diagnostic screening panels because of its positive clinical and epidemiological significance.

A significant finding in this study was the co-occurrence of carbapenemase genes, specifically the *blaNDM* + *blaOXA-48*-like combination. This combination has been associated with severe resistance and has been increasingly recorded across several hospital environments. The co-carriage of NDM and OXA-48-like enzymes, in addition to broad-spectrum and particular agents of the β-lactam class, may pose serious threats to the efficacy of therapeutic choices. Genç et al. [8] stated that the availability of diverse options of antimicrobial agents is necessary to combat *K. pneumoniae* specifically resistant to carbapenems, especially if such strains present complex combinations of carbapenemases. The co-production of NDM and OXA-48 by strains also highlighted a serious issue. Thus, the authors think that co-carriage of these genes suggests that the resistance mechanisms functioning in conjunction may have significant therapeutic relevance and should not be stated separately.

ICU and blood isolates displayed a high number of multiple-gene carriage. ICU patients typically possess a multitude of risk factors. These factors include greater risk of exposure to mechanical ventilation, invasive devices, and severe multiple antibiotic therapy. There is a high risk of developing multidrug resistant infections. This risk can be compounded by extended stays and repeated exposure to many healthcare employees. ICU environments can serve as a reservoir for some carbapenamase-producing Gram-negative bacteria. Some of these include bacteria with the resistant genes *blaNDM*, *blaOXA-48*-like, and *blaKPC* [9, 12]. At the isolation and study of multiple carbapenemase genes, blood and respiratory isolates appeared to be greater in number than urinary isolates. This implies that invasive infections at some point along a gradient of multiple resistances and greater complexity of multidrug resistances exist. However, to establish this firmly, more data concerning clinical outcomes and larger studies are necessary.

The phenotypic and molecular resistance profiles linked to substantive and universal resistance due to the absence of resistance genes in some last and reserved drugs (e.g., aminoglycosides, fluoroquinolones, and carbapenems, and some cephalosporins) are resistant. This extended resistance pattern has proven to be highly concerning in many studies

documenting the multidrug resistance of carbapenem resistant K pneumoniae [2, 3, 11, 20]. The incredibly greater resistance of multiple-gene positive isolates indicates possible co-carbapenamase with multiple-gene and multiple drug resistance. This speaks to the emergency of multidrug and resistance conflicting genes spread. Clonal spread of resistant colonies of great concern remains. Although sequenced, the clear genomic and resistance patterns of phenotypes indicate that great concern remains due to the spread of conflicting resistance genes and the absence of direct genomic surveillance.

The identification of carbapenem-resistant isolates not expressing *blaKPC*, *blaNDM*, or *blaOXA-48*-like showed significant results. It was possible that these isolates contained other carbapenemases, such as blaVIM or blaIMP, or these isolates may have resisted carbapenems with non-carbapenemase mechanisms. These mechanisms can include porin loss in conjunction with ESBL or AmpC production or membrane permeability changes due to efflux-pump activity. When possible, the latest real-time PCR techniques have suggested utilizing blaVIM or blaIMP in conjunction with other mechanisms, especially in the context of results that show phenotypic carbapenem resistance [4, 16]. Thus, from a practical and targeted standpoint, the three-gene panel was a rational approach in the show example, but it should be deemed a resistance-mechanism screening approach rather than a comprehensive resistance-mechanism investigation.

A clear major asset of the study was the inclusion of real-time PCR. Convention PCR protocols mandate the use of a series of steps such as an electrophoresis of an agarose gel, capturing an image of the gel, and visually interpreting the sized bands. These methods can pose a high risk of generating contamination and may elicit an expectation from reviewers to validate results via the use of a gel. In contrast, real-time PCR enables the use of a closed-tube detection system, amplification curves, and the incorporation of the necessary positive and negative controls as well as internal controls and objective Ct values with the aids of implemented controls. The CDC recommends the use of primer and probe systems as well as extraction controls, negative controls, and 16s internal amplification controls [1]. Previous studies have shown real-time PCR to be a great, practical, and rapid approach to detecting major carbapenemasaes in clinical and surveillance isolates [4, 16].

Another strength of the study was the design of the short amplicons. Because of the general efficiency of short amplicons in real-time PCR and the usage of routine diagnostic DNA preparations like boiled or partially degraded DNA, the design of the PCR in this study was able to utilize simple and ready-to-use methods available in clinical microbiology applications. The lab's short primer set PCR designs for the genes *blaKPC*, *blaNDM*, and *blaOXA-48*-like were less than 150 bp. This length allowed for rapid amplifications in the lab and interpretations in terms of their Cycle of Threshold (Ct) values. This PCR design was in congruence with other carbapenemase screening molecular diagnostics, which used rapid amplification and short amplicons [1, 4]. Contemporary European *K. pneumoniae* standard and blood-settler *K. pneumoniae* studies state that the community combines and coordinates controls for carbapenem resistant *K. pneumoniae* and *K. pneumoniae* focused controls and blood-settler Integrative Continuous managing K. clinical cases [1; 2; 13].

The current analysis recognizes some deficiencies. Whole-genome sequencing falls short in some respects. First, while this analysis targeted three prevalent carbapenemase genes (i.e. *blaKPC*, *blaNDM*, *blaOXA-48*-like), more rare genes (i.e. VIM, blaIMP, others) were not considered. Second, this analysis used primarily a lab-based approach, thus leading to a number of overlooked differences in clinical outcomes, including (but not limited to) treatment outcomes, mortality, and antibiotic use. Regardless of these shortcomings, a number of practical lab-based solutions (i.e. imaging) were developed that create a secondary framework for evaluating the molecular presence of prevalent carbapenemase genes.

In summary, *blaNDM* and *blaOXA-48*-like were the most frequently detected carbapenemase genes, whereas *blaKPC* was the least prevalent among the carbapenem-resistant clinical *K. pneumoniae* isolates. Co-carriage, particularly *blaNDM* + *blaOXA-48*-like, represented a significant resistance mechanism and was concomitant with more severe carbapenem resistance and the presence of associated hospital sources. Implementation of short-amplicon real-time PCR was proven advantageous as it provided a fast, effective, and image-free mechanism of continual documentation of the laboratory. These results substantiate the need for targeted real-time PCR to be incorporated into everyday clinical microbiology to surveil C. difficile C. pneumoniae, especially in settings with a high multi-drug resistant bacterial load.

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Ethical approval

The study was approved (2654-12-5-2025) by the Committee for Research Ethics depending on the fact that bacterial isolates recovered during routine diagnostic work with direct patient intervention was performed.

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Conflict of interest

The authors declare no conflict of interest.

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