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Research Article

Molecular Characterization of Carbapenem-Resistant Klebsiella Pneumoniae: Detection of Blandm and Other Resistance Genes by PCR

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ABSTRACT
Introduction: The global rise of carbapenem-resistant *Klebsiella pneumoniae* (CRKP) poses a significant public health challenge. Early molecular identification of carbapenemase genes is critical for guiding treatment and infection control.
Aim: This study aimed to characterize carbapenem resistance in phenotypically confirmed *K. pneumoniae* isolates by detecting major carbapenemase genes (*blaKPC*, *blaNDM*, *blaOXA-48*, *blaIMP*, and *blaVIM*) using multiplex PCR.
Methods: Twenty-six meropenem-resistant *K. pneumoniae* isolates, selected from 196 clinical samples at a tertiary care hospital, underwent genomic DNA extraction and multiplex PCR amplification targeting five carbapenemase genes. PCR products were analyzed via agarose gel electrophoresis.
Results: All 26 isolates (100%) harbored the *blaNDM* gene, while none tested positive for *blaKPC*, *blaOXA-48*, *blaIMP*, or *blaVIM*. The presence of a 621 bp amplicon confirmed *blaNDM* gene carriage.
Conclusion: The exclusive presence of *blaNDM* indicates its predominant role in carbapenem resistance in the region studied. Molecular diagnostics are indispensable for accurate detection and epidemiological surveillance of multidrug-resistant organisms.

Keywords: Klebsiella Pneumoniae, Carbapenem Resistance, Blandm, PCR, Carbapenemase Genes, Molecular Diagnostics.

INTRODUCTION

The emergence and rapid spread of carbapenem-resistant Enterobacteriaceae (CRE) represent a critical threat to global public health, especially within healthcare settings where vulnerable patient populations are at risk [1]. Among CRE, *Klebsiella pneumoniae* has gained prominence as a formidable nosocomial pathogen due to its propensity to acquire and disseminate multiple antibiotic resistance mechanisms [2, 3]. Carbapenems, including imipenem, meropenem, and ertapenem, have long been regarded as antibiotics of last resort for treating serious infections caused by multidrug-resistant Gram-negative bacteria [4]. However, the increasing prevalence of carbapenem-resistant *K. pneumoniae* (CRKP) significantly undermines the efficacy of these vital drugs, leading to limited therapeutic options, prolonged hospital stays, increased treatment costs, and elevated morbidity and mortality rates worldwide [5]. The mechanisms underlying carbapenem resistance in *K. pneumoniae* are diverse but primarily involve the production of carbapenemases—enzymes capable of hydrolyzing carbapenem antibiotics and conferring high-level resistance [6]. Carbapenemases belong to different molecular classes, classified based on their amino acid sequences and catalytic mechanisms, which also influence their inhibitor susceptibility and substrate spectrum [7]:

- **Class A Carbapenemases**, such as the *Klebsiella pneumonia* carbapenemase (KPC), utilize a serine residue at their active site to hydrolyze β-lactams [8]. KPC enzymes have been widely reported in the United States and Europe and represent a significant cause of carbapenem resistance [9, 10].
- **Class B Carbapenemases**, or metallo-β-lactamases (MBLs), including *New Delhi Metallo-β-lactamase* (*blaNDM*), *Verona Integron-encoded Metallo-β-lactamase* (*blaVIM*), and *Imipenemase Metallo-β-lactamase* (*blaIMP*), require divalent metal

ions (typically zinc) for their catalytic activity [11]. MBLs have a broad hydrolytic spectrum, effectively inactivating almost all β-lactams except monobactams such as aztreonam [12]. The *blaNDM* gene, first identified in 2008 from a patient in New Delhi, India, has since become one of the most widespread and clinically significant MBLs, owing to its rapid dissemination facilitated by plasmids and other mobile genetic elements [13].

- **Class D Carbapenemases** consist mainly of oxacillinases like *blaOXA-48*, which hydrolyze carbapenems but often with lower efficiency [14, 15]. Nevertheless, *blaOXA-48* and its variants have caused significant outbreaks, particularly in Europe and the Middle East [16].

The proliferation of carbapenemase genes is exacerbated by their frequent association with mobile genetic elements such as plasmids, transposons, and integrons [17]. These elements facilitate horizontal gene transfer not only within *K. pneumoniae* populations but also across different bacterial species and genera, accelerating the spread of resistance within healthcare and community environments [18]. This genetic mobility poses a formidable challenge to infection control practices and antimicrobial stewardship efforts [19]. Moreover, carbapenem resistance often coexists with resistance to other antibiotic classes, such as aminoglycosides, fluoroquinolones, and colistin, leading to multidrug-resistant (MDR) or extensively drug-resistant (XDR) phenotypes [20]. These MDR/XDR strains drastically limit effective treatment regimens, forcing clinicians to rely on less effective or more toxic alternatives, further complicating patient management [21]. Molecular epidemiology studies focusing on the identification and characterization of carbapenemase genes are essential for several reasons [22]. Firstly, they facilitate the early and accurate detection of resistance mechanisms, which is critical for initiating appropriate antimicrobial therapy [23]. Secondly, they enable tracking of the spread and evolution of resistance determinants within and between healthcare facilities [24]. Thirdly, they inform the design of targeted infection control interventions to prevent outbreaks [25]. Finally, understanding the local and regional prevalence of specific carbapenemase genes guides public health policies and antimicrobial stewardship programs [26]. Traditional phenotypic methods for detecting carbapenem resistance, such as susceptibility testing by disk diffusion or MIC determination, are indispensable but lack the specificity to identify the underlying resistance mechanisms [27, 28]. Phenotypic confirmatory tests for carbapenemase production, including the modified Hodge test and carbapenemase inhibitor synergy tests, have limitations related to sensitivity and specificity [29, 30]. Molecular diagnostics based on polymerase chain reaction (PCR) provide rapid, sensitive, and specific detection of carbapenemase genes, enabling precise characterization of resistance profiles [27, 31]. In many regions, particularly in South Asia, the epidemiology of carbapenem resistance in *K. pneumoniae* is dominated by *blaNDM*, with sporadic reports of other carbapenemase genes [9, 22, 32]. Despite the increasing recognition of this issue, there remains a paucity of data regarding the molecular characteristics of carbapenem-resistant *K. pneumoniae* in many tertiary care settings. This knowledge gap hampers effective clinical management and infection control measures [33, 34]. Therefore, this study aimed to investigate the molecular basis of carbapenem resistance among clinical *K. pneumoniae* isolates phenotypically resistant to meropenem by detecting the presence of five major carbapenemase genes (*blaKPC, blaNDM, blaOXA-48, blaIMP,* and *blaVIM*) using multiplex PCR. The findings will contribute to the understanding of carbapenem resistance mechanisms in this setting and support the development of targeted strategies to combat the spread of multidrug-resistant pathogens.

MATERIALS AND METHODS

Study Design and Sample Selection

This cross-sectional molecular study was conducted to investigate the presence of carbapenemase genes among clinical isolates of *Klebsiella pneumoniae* exhibiting resistance to carbapenems. The isolates were collected from various clinical specimens including blood, urine, sputum, wound swabs, and endotracheal aspirates submitted to the Microbiology Laboratory of a tertiary care hospital over a one-year period from September 2016 to August 2017.

A total of 196 non-duplicate *K. pneumoniae* isolates were identified using standard biochemical tests and confirmed by the automated identification system (such as VITEK 2 or MALDI-TOF, if applicable). Antimicrobial susceptibility testing was

performed using the E-test (bioMérieux, France) method to determine the minimum inhibitory concentrations (MICs) of meropenem. According to Clinical and Laboratory Standards Institute (CLSI) guidelines, isolates with an MIC ≥ 4 µg/mL were classified as meropenem-resistant. Among the 196 isolates, 26 (13.3%) met this criterion and were selected for further molecular characterization.

DNA Extraction

Genomic DNA from the meropenem-resistant *K. pneumoniae* isolates was extracted using the boiling lysis method, which is a rapid and cost-effective technique for preparing DNA templates for PCR.

- A loopful of an overnight bacterial culture grown on nutrient agar was suspended in 200 µL of sterile distilled water in a microcentrifuge tube.
- The suspension was heated in a boiling water bath at 100°C for 10 minutes to lyse the bacterial cells and release genomic DNA.
- Following boiling, the tubes were immediately chilled on ice for 5 minutes to prevent DNA degradation.
- The lysate was then centrifuged at 12,000 rpm for 10 minutes to pellet cellular debris.
- The clear supernatant containing genomic DNA was carefully collected and stored at –20°C until used as the DNA template for PCR amplification.

This method, while less purified than commercial DNA extraction kits, has been shown to provide DNA of sufficient quality for PCR-based detection of resistance genes.

Primer Sequences

Specific primers targeting five major carbapenemase genes—*blaKPC, blaNDM, blaVIM, blaIMP,* and *blaOXA-48*—were employed for multiplex PCR amplification. These primers were designed based on published sequences and validated for specificity and sensitivity in previous studies.

Gene	Primer Sequence (5'–3')	Product Size (bp)
blaKPC	F: CATTCAAGGGCTTTCTTGCTGC	798
	R: ACGACGGCATAGTCATTTGC	
blaNDM	F: GGTTTGGCGATCTGGTTTTC	621
	R: CGGAATGGCTCATCACGATC	
blaVIM	F: GATGGTGTTTGGTCGCATA	390
	R: CGAATGCGCAGCACCAG	
blaIMP	F: GGAATAGAGTGGCTTAAYTCTC	232
	R: GGTTTAAYAAAACAACCACC	
blaOXA-48	F: GCGTGGTTAAGGATGAACAC	438
	R: CATCAAGTTCAACCCAACCG	

All primers were synthesized commercially and diluted to working concentrations as per manufacturer recommendations.

Polymerase Chain Reaction (PCR) Conditions

The PCR assay was conducted in a total volume of 25 µL per reaction, using a commercially available PCR Master Mix containing Taq DNA polymerase, dNTPs, $MgCl_2$, and buffer components to optimize reaction conditions.

Each reaction mixture contained:

- 12.5 µL of PCR Master Mix (2X)
- 1 µL of forward primer (10 µM)
- 1 µL of reverse primer (10 µM)
- 2 µL of DNA template (extracted genomic DNA)
- 8.5 µL of nuclease-free water to adjust the final volume

Thermal cycling was performed on a programmable thermal cycler (e.g., Bio-Rad, Applied Biosystems) using the following conditions:

- Initial denaturation at 95°C for 5 minutes to ensure complete denaturation of double-stranded DNA
- 35 cycles of:
  - Denaturation at 95°C for 30 seconds
  - Annealing at 58°C for 30 seconds, optimized for primer binding specificity

- Extension at 72°C for 1 minute to synthesize the target amplicon
- Final extension at 72°C for 7 minutes to complete elongation of any remaining incomplete DNA strands

Negative controls (reaction mixtures without DNA template) and positive controls (known carbapenemase gene-carrying strains) were included in each PCR run to monitor contamination and validate assay performance.

Agarose Gel Electrophoresis

PCR products were separated and visualized using agarose gel electrophoresis to confirm the presence and size of amplified carbapenemase gene fragments.

- A 1.5% agarose gel was prepared by dissolving agarose powder in 1X TAE buffer and supplemented with ethidium bromide (0.5 µg/mL) for nucleic acid staining.
- Approximately 10 µL of PCR product mixed with loading dye was loaded into each well alongside a 100 bp DNA ladder (molecular weight marker) to estimate fragment sizes.
- Electrophoresis was conducted at 100 volts for approximately 45–60 minutes in 1X TAE buffer.
- Post-run, the gel was visualized under UV transillumination using a gel documentation system.
- Bands corresponding to the expected product sizes (as listed in Table 2.3) indicated the presence of the respective carbapenemase genes.
- The gel images were documented for record-keeping and further analysis.

RESULTS

A total of 196 *Klebsiella pneumoniae* isolates were recovered from various clinical specimens collected from patients of all ages and genders admitted to Government Rajaji Hospital, Madurai. The highest number of isolates was obtained from urine samples, accounting for 74 isolates (37.76%), followed by pus samples with 59 isolates (30.10%), and blood samples with 33 isolates (16.84%). Lower rates of isolation were observed in sputum (15 isolates, 7.65%), wound swabs (10 isolates, 5.10%), and tracheal aspirates (5 isolates, 2.55%) (Table 1). These findings indicate that *K. pneumoniae* is predominantly associated with urinary tract infections and wound infections in this hospital setting.

Table 1: Specimen-wise Distribution of Klebsiella pneumoniae Isolates (n=196)

Specimen	Number of Isolates	Percentage (%)
Urine	74	37.76
Pus	59	30.1
Blood	33	16.84
Sputum	15	7.65
Wound Swab	10	5.1
Tracheal Aspirate	5	2.55
Total	**196**	**100**

Confirmation of Carbapenem Resistance by MIC

A total of 32 *Klebsiella pneumoniae* isolates, which had shown resistant or intermediate zones to meropenem by the disc diffusion method, were further evaluated for Minimum Inhibitory Concentration (MIC) using the E-test. Among these, 26 isolates (81.25%) showed MIC values ≥ 4 µg/ml, confirming carbapenem resistance. The remaining 6 isolates (18.75%) had MIC values ≤ 1 µg/ml, indicating susceptibility (Table 2). No isolates exhibited intermediate MIC values (2 µg/ml). Based on these findings, the prevalence of carbapenem-resistant *K. pneumoniae* isolates in the study was determined to be 13.27% (26 out of 196).

Table 2: Meropenem MIC Distribution among 32 Suspected Isolates

MIC Range (µg/mL)	Number of Isolates	Percentage (%)
Susceptible (≤ 1)	6	18.75
Intermediate (2)	0	0
Resistant (≥ 4)	26	81.25
Total	**32**	**100**

Molecular Characterization of Carbapenem-Resistant Klebsiella pneumoniae

A total of 26 carbapenem-resistant Klebsiella pneumoniae (CRKP) isolates were subjected to PCR analysis to detect the presence of carbapenemase genes. All 26 isolates (100%) tested positive for the bla_NDM gene, confirming the widespread presence of the New Delhi Metallo-β-lactamase enzyme. None of the isolates harbored other carbapenemase genes such as bla_KPC, bla_VIM, bla_IMP, or bla_OXA-48 (Table 3).This finding indicates that bla_NDM is the predominant carbapenemase gene responsible for carbapenem resistance in the clinical isolates from this hospital setting. The absence of other carbapenemase genes suggests a limited diversity of resistance mechanisms in this population.

Table 3: Molecular Detection of Carbapenemase Genes in CRKP Isolates (n=26)

Carbapenemase Gene	Number of Positive Isolates	Percentage (%)	Number of Negative Isolates	Percentage (%)
bla_NDM	26	100	0	0
bla_KPC	0	0	26	100
bla_VIM	0	0	26	100
bla_IMP	0	0	26	100
bla_OXA-48	0	0	26	100

Comparative Analysis of Phenotypic and Molecular Detection

When compared with PCR, which served as the gold standard for the detection of carbapenemase genes, the performance of the phenotypic methods showed noticeable variation. The Combined Disc Test (CDT) demonstrated the highest concordance with molecular findings, successfully identifying carbapenemase production in 22 out of the 26 NDM-1 positive isolates, resulting in a sensitivity of 84.6%. The Rapidec Carba NP Test closely followed, detecting 21 positive cases, corresponding to a sensitivity of 80.77%. In contrast, the Modified Hodge Test (MHT) showed the least agreement, with only 16 of the 26 PCR-confirmed isolates testing positive, indicating a lower sensitivity of 61.54% (Table 4). These results highlight the superior diagnostic accuracy of molecular methods such as PCR for confirming carbapenemase production, particularly in identifying NDM-1-mediated resistance. Among the phenotypic assays, CDT proved to be the most reliable screening tool in resource-limited settings.

Table 4: Comparison of Phenotypic Tests with PCR Results (n = 26)

Phenotypic Test	No. of PCR-Positive Isolates Detected	Sensitivity (%)
Modified Hodge Test (MHT)	16	61.54%
Combined Disc Test (CDT)	22	84.60%
RapidecCarba NP Test	21	80.77%

DISCUSSION

In this study, 26 *Klebsiella pneumoniae* isolates phenotypically resistant to meropenem (MIC ≥ 4 µg/mL) were analyzed for carbapenemase genes using PCR. All 26 isolates (100%) were positive for blaNDM, while none harboredblaKPC, blaOXA-48, blaIMP, or blaVIM, highlighting the dominance of NDM-type carbapenemases in our setting. This is consistent with reports from other regions of India and South Asia, where NDM is often the predominant mechanism of carbapenem resistance in Enterobacterales [35, 36]. NDM enzymes, first reported in 2008 from a Swedish patient previously hospitalized in New Delhi, are now globally distributed and are commonly plasmid-mediated, which facilitates their rapid dissemination [37, 38]. The exclusive presence of blaNDM and absence of other carbapenemase genes in our isolates suggest a clonal or plasmid-driven spread of this resistance mechanism in the local healthcare environment. Other studies from North East and South India have

reported co-existence of NDM with OXA-48 or VIM in some cases, though at lower frequencies [39, 40]. The relatively limited gene diversity in our isolates may reflect geographic variation or recent transmission events within the hospital. Among the phenotypic detection methods, the Combined Disc Test (CDT) showed the highest agreement with PCR (84.6%), followed by RapidecCarba NP Test (80.77%), and the Modified Hodge Test (MHT) (61.54%). Similar findings have been reported elsewhere, where CDT demonstrates good sensitivity for detecting metallo-β-lactamases (MBLs), particularly NDM, due to EDTA's inhibitory effect on zinc-dependent enzymes [41]. Although the RapidecCarba NP test offers rapid detection, its performance may vary based on enzyme expression levels and isolate type [42]. MHT, once a widely used screening tool, is now considered suboptimal for detecting MBLs and has been removed from CLSI guidelines due to poor specificity and sensitivity, especially for NDM producers [43, 44]. Our study reinforces the superiority of molecular methods over phenotypic assays for carbapenemase detection. While PCR is more resource-intensive, it provides accurate, rapid identification of the resistance mechanism, which is crucial for guiding antimicrobial therapy and infection control. In contrast, phenotypic tests, although accessible and affordable in low-resource settings, may yield false negatives or miss less active enzyme variants. The clinical implications of NDM-mediated resistance are profound. NDM enzymes hydrolyze almost all β-lactams, including carbapenems, and are not inhibited by traditional β-lactamase inhibitors. Moreover, NDM-positive isolates often carry additional resistance determinants to aminoglycosides, fluoroquinolones, and colistin, leading to multidrug- or extensively drug-resistant (MDR/XDR) phenotypes [45, 46]. In such scenarios, treatment options are severely limited, often requiring toxic or less effective drugs like fosfomycin, polymyxins, or newer agents like cefiderocol [47]. From an epidemiological standpoint, the uniform detection of blaNDM in this cohort may suggest clonal spread of resistant strains or plasmid-mediated gene dissemination, which is increasingly being documented in hospital outbreaks [48]. Molecular epidemiology techniques such as MLST or whole-genome sequencing could clarify the transmission dynamics, but were beyond the scope of this study.

LIMITATIONS

This study has several limitations that should be considered. The molecular analysis was conducted on a relatively small number of isolates (n = 26), which may not fully represent the broader resistance landscape. As a single-center study, the findings may lack generalizability to other healthcare settings or geographic regions. Additionally, only five major carbapenemase genes (*blaNDM*, *blaKPC*, *blaOXA-48*, *blaIMP*, and *blaVIM*) were screened, potentially overlooking other emerging or rare resistance genes. The absence of molecular typing methods, such as MLST or PFGE, limited the ability to determine clonal relationships among isolates. Furthermore, the study did not evaluate the presence of co-resistance genes (e.g., ESBLs, AmpC, *mcr*) or mobile genetic elements, nor did it include clinical outcome data, which restricts insights into the clinical relevance and transmission dynamics of the resistant strains.

CONCLUSION

In conclusion, this study highlights the significant prevalence of carbapenem-resistant *Klebsiella pneumoniae* in a tertiary care hospital setting, with the blaNDM gene identified as the predominant mechanism driving resistance. The molecular detection via PCR proved to be a reliable and specific method for confirming carbapenemase production, outperforming phenotypic tests, although the Combined Disc Test showed good sensitivity and may serve as a practical screening tool in resource-limited environments. These findings emphasize the urgent need for routine molecular surveillance to guide effective antimicrobial therapy and implement targeted infection control measures. Continued monitoring and comprehensive molecular characterization are essential to curb the spread of multidrug-resistant pathogens and improve patient outcomes.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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