

Prevalence Trends and Insights of Carbapenem-Resistant *Klebsiella pneumoniae* Using Diverse Phenotypic Assays

Archana Mani, Anees Pathima, Sasikala Shanmugam*

Department of Microbiology and Biotechnology, Presidency College (Autonomous), Chennai, Tamil Nadu, INDIA.

ABSTRACT

Introduction and Objectives: The emergence and the spread of carbapenem-resistant *Klebsiella pneumoniae* significantly threaten global public health. The detection of carbapenemase is crucial for early targeted therapy and improved clinical outcomes. The current study sought to evaluate the prevalence of carbapenemase-producing *Klebsiella pneumoniae* and emphasized different phenotyping approaches to detect carbapenemase producers. **Materials and Methods:** A sum of 72 *Klebsiella pneumoniae* isolates were collected and screened for carbapenem susceptibility. Antibiotic sensitivity testing and carbapenem screening were performed. Carbapenemase production among *Klebsiella pneumoniae* strains was evaluated using four phenotypic methods including MHT, mCIM, Carba NP and CD method. **Results:** Out of 72 strains, 54 isolates were multidrug-resistant and suspected as carbapenemase producers. Among carbapenem-resistant isolates tested, 53.7% of isolates were positive for MHT, 68.5 % of strains were positive for mCIM, 48% were positive for the Carba NP method and 33.33% were positive for the Combined Disc Method. **Conclusion:** This study sheds light on the screening methods in the evaluation of carbapenemase-producing *Klebsiella pneumoniae*, and the necessity to screen the pathogens ensuring the rational usage of available antibiotics.

Keywords: Carbapenem resistance, *Klebsiella pneumoniae*, Phenotypic methods.

Correspondence:

Dr. Sasikala Shanmugam

Associate Professor and Head of the Department, Department of Microbiology and Biotechnology, Presidency College (Autonomous), Chennai-600005, Tamil Nadu, INDIA.
Email: drsasikala27@gmail.com

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INTRODUCTION

Klebsiella pneumoniae (*K. pneumoniae*) is an opportunistic Gram-negative bacterium from the Enterobacteriaceae family that causes infections in immunocompromised and hospitalized patients. *K. pneumoniae* is the most prevalently found species in the *Klebsiella* genus.^[1] *K. pneumoniae* is the primary cause of nosocomial infections including pneumonia, septicemia, and urinary tract infections with greater clinical significance.^[2] It is a facultative anaerobic bacteria that poses an alarming risk of sickness and fatality in immunocompromised patients.^[3] The World Health Organization's assessment of the global status of antimicrobial resistance stated that *K. pneumoniae* is the second most prevalent bacteria that exhibits Multi-Drug Resistance (MDR).^[4]

Carbapenems are frequently considered as last resort antibiotics and recommended as the preferred medication for infections brought on by Gram-negative bacteria when patients become critically ill.^[5] The emergence of antimicrobial-resistant

Enterobacterales is consistently recognized as a rising global menace. Antimicrobial Resistance (AMR) is a serious public health problem accounting for about 700,000 fatalities annually worldwide. It is estimated that by the end of the year 2050, this might rise to 10 million.^[6]

Treatment of infections becomes challenging due to the rise of carbapenem resistance which is often linked to antimicrobial resistance to various antibiotic classes.

As a result, extensive research is required to monitor the epidemiological traits of Carbapenem-Resistant *K. pneumoniae* (CR-Kp) isolates.^[7] Carbapenem resistance is mediated by three major mechanisms that include enzymatic hydrolysis, weakly carbapenemase-active ESBL and AmpC, and modification of penicillin-binding proteins.^[8]

Understanding the prevalence rate of pathogens resistant to carbapenem is crucial in terms of preventing antimicrobial therapy failure. For routine diagnostic laboratories, the phenotypic approach serves as the primary basis for detection.^[9] Phenotypic assays are less expensive and used to detect Carbapenemase activity existing and emerging drug resistance.^[10] Using various phenotypic assays, the current study intended to investigate the rate of *K. pneumoniae* that produces carbapenemase.



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MATERIALS AND METHODS

Klebsiella pneumoniae isolates

This present Observational study was carried out from July 2023 to March 2024 in the Department of Microbiology, Presidency College, Chennai. This study comprised 72 non-duplicate MDR isolates in total. *K. pneumoniae* strains were recovered from a range of clinical specimens, distributed in urine (39), pus (20), wound swabs (7), and tracheal secretions (6). Clinical samples were collected and organisms were identified based on conventional bacteriological methods. Repetitive and contaminated samples were excluded from the study.

Antimicrobial Susceptibility Testing (AST)

In accordance with CLSI standards, isolates of *K. pneumoniae* were tested for antibacterial susceptibility using the Kirby-Bauer disc diffusion method.^[11] The antibiotics used were amikacin (30 µg), gentamicin (10 µg), amoxicillin-clavulanic acid (20/10 µg), cefuroxime (30 µg), ceftazidime (30 µg), cefepime (30 µg), imipenem (10 µg), meropenem (10 µg), ciprofloxacin (5 µg), and norfloxacin (10 µg).

Screening of carbapenem resistance

Carbapenem antibiotics were included in the AST panel and screened for carbapenemase production. Those strains, that showed resistance to a minimum of one carbapenem antibiotic (meropenem/imipenem) were considered as carbapenem-resistant *K. pneumoniae* and subjected to further confirmatory investigations which include, MHT, mCIM, carba NP, and CD method for the detection of carbapenemase production.

Phenotypic methods

A total of 72 MDR strains of *K. pneumoniae* were tested. Carbapenemase producers were phenotypically confirmed using the following methods.

Modified Hodge Test (MHT)

On an MHA plate, a 0.5 McFarland-adjusted suspension of *E. coli* ATCC 25922 was swabbed and left to dry. A meropenem (10 µg) disc was placed in the plate. Test isolates were streaked as a straight line and incubated at 35±2°C for 16-24 hr. The positive result is indicated by the zone of inhibition in the clover-leaf pattern indicates a positive result.^[12]

Modified Carbapenem Inactivation Method (mCIM)

2 mL of tryptic soy broth (TSB) was taken and inoculated with test organisms. The inoculum was mixed for 10-15 sec and 10 µg of Imipenem disc was added. Then the tubes were incubated for 4 hr at 35±2°C. A lawn culture was made with *E. coli* ATCC 25922 on MHA plates prior to the carbapenem inactivation step

and allowed to dry. The Imipenem disc was removed from the broth using sterile forceps and positioned over the MHA plates. Two test discs can be placed and studied per plate. The plates are then incubated at 35+/-2°C for 18-24 hr. If the IZ (Zone of Inhibition) around the disc is 6-10 mm, it is a positive result for carbapenemase production and if the IZ around the disc is 11-19 mm, it is an indeterminate result and if the IZ is >=20 mm, it is considered as a negative result for carbapenemase production according to CLSI guidelines.^[13]

Carbapenemase Nordmann-Poirel(Carba NP)

0.05% Phenol Red and 0.1 mMol/L of ZnSO₄·7H₂O in Clinical Laboratory Reagent Water (i.e., 2 mL of 0.5% Phenol Red+180 µL ZnSO₄·7H₂O+16.6 mL Clinical Laboratory Reagent Water) were mixed together to make Carba NP Solution A. The pH is set to 7.8±0.1 and stored at 4°C in an amber-colored bottle. Solution B was prepared by mixing 12 mg/mL imipenem-cilastatin, with Solution A (i.e., 0.12 g Imipenem-Cilastatin+10 mL Solution A). 2 loopful (10 µL) of bacterial culture are suspended in 200 µL of Tris-HCl lysis buffer was added and vortexed. 100 µL of the bacterial lysate was aliquoted into 2 tubes and labeled as "a" and "b" respectively. A and B solutions were added to "a" and "b" tubes and incubated at 37°C for 2 hr. If tube "a" is red and tube "b" is yellow, it is positive or orange in color and considered as negative if both the tubes are red in color. Plain Solution A and B were used as a reagent control.^[14]

Combined Disc Method (CD Method)

CD method was performed using the Imipenem-Ethylenediamine-Tetra-Acetic Acid (EDTA) to detect MBL production. Test strains were inoculated onto the MHA plate. Two imipenem (10 µg) disc was kept on MHA plate, imipenem disc alone, and imipenem with 10 µL of 750-µg of EDTA. After overnight incubation, a difference of ≥7 mm IZ surrounding the imipenem+EDTA disc is interpreted as MBL positive.^[15]

Quality controls

Positive control-*K. pneumoniae* ATCC BAA 1705.

Negative control-*E. coli* ATCC 25922.

Statistical analysis

The data were interpreted and analyzed using Microsoft Excel. Quantitative data were presented in the form of numbers and percentages in tables and figures.

RESULTS

A total of 72 *K. pneumoniae* strains were isolated from various samples including urine, pus, wound swab, and tracheal secretion. Antibiogram results revealed that among 72 strains, 54 (75%) strains were multidrug-resistant. Among all the *K. pneumoniae* tested, CR-Kp was predominantly present in pus samples (12/20)

followed by wound swabs (4/7), urine (22/39) and tracheal secretion (2/6) (Table 1).

Out of ten antibiotics tested, the most sensitive antibiotic was gentamycin (38.88%), the second most sensitive antibiotic was ciprofloxacin (36.11%), whereas the highest percentages of antimicrobial resistance were detected in cefuroxime which showed 80.55% resistance followed by imipenem (73.61%) and ceftazidime. In addition, meropenem and imipenem resistance was shown in 73.61% and 65.27% of *K. pneumoniae* isolates, respectively. 73.61% of *K. pneumoniae* were classified as probable carbapenemase producers because they exhibited resistance to carbapenem antibiotics (Figure 1).

Additionally, the MHT assay findings showed that 25 bacteria were MHT negative and 29 (53.7%) were MHT positive for the synthesis of carbapenemase (Figure 2), 6% were intermediate, and 68% were negative. Of the 54 isolates, the mCIM test identified 37 (68.5%) of positive for carbapenemase production and 17 (31.48%) isolates were reported as negative. A total of 26 (48%) isolates were positive and 28 (51.85%) were negative by Carba NP test (Figure 3).

The CD Method showed 18 (33.33%) positive and 36 (66.66%) negative results. Out of 54 carbapenemase-producing isolates tested, 26 (48%) and 18 (33.33%) were found to be positive by the Carba NP and CD method, respectively. Our study demonstrated the highest positivity of carbapenemase production by mCIM (68.5%) followed by MHT (53.7%), Carba NP (48%) and CD method (33.3%).

Our study demonstrated the highest positivity of carbapenemase production by mCIM (68.5%) followed by MHT (53.7%), Carba NP (48%) and CD method (33.3%). Among the strains that showed resistance to one of the carbapenems, more detection was observed by mCIM test than MHT with regard to Carbapenemase

production. Some carbapenemase producers were missed by MHT which were detected in mCIM test.

DISCUSSION

The prevalence of CR-Kp has increased worldwide very rapidly in the last decade. This is more critical, especially in the Indian set up because of the predominance of carbapenemase-encoded genes in the CR-Kp isolates. This study evaluated the prevalence of carbapenem resistance and AMR patterns of MDR *K. pneumoniae* isolates. Our research revealed that a substantial percentage of *K. pneumoniae* produced carbapenemase. It was found that every isolate that produced carbapenemase was multidrug resistant.

In the current study, CLSI-recommended methods were used for Carbapenemase enzyme detection among 72 MDR *K. pneumoniae* isolates. First, the carbapenem susceptibility of all 72 MDR *K. pneumoniae* isolates was assessed by Kirby Bauer disk diffusion. In this study, MHT, mCIM, Carba NP, and the Combined Disc method were performed individually.

CLSI suggested MHT in 2009 as a carbapenemase screening technique. It is a simple and inexpensive test that can be done in clinical practices. However, this method has the disadvantage of producing false-negative results. Mucoid colonies may interrupt with results.

A total of 54 MDR *K. pneumoniae* strains were tested, out of which 29(53.7%) isolates were positive by the MHT. Pawar, S. K.,

Table 1: Distribution of CR-Kp isolates in different types of samples (mCIM).

Sample type	Carbapenemase producers
Urine	56.41%
Pus	60%
Wound swab	57.14%
Tracheal secretion	33.33%

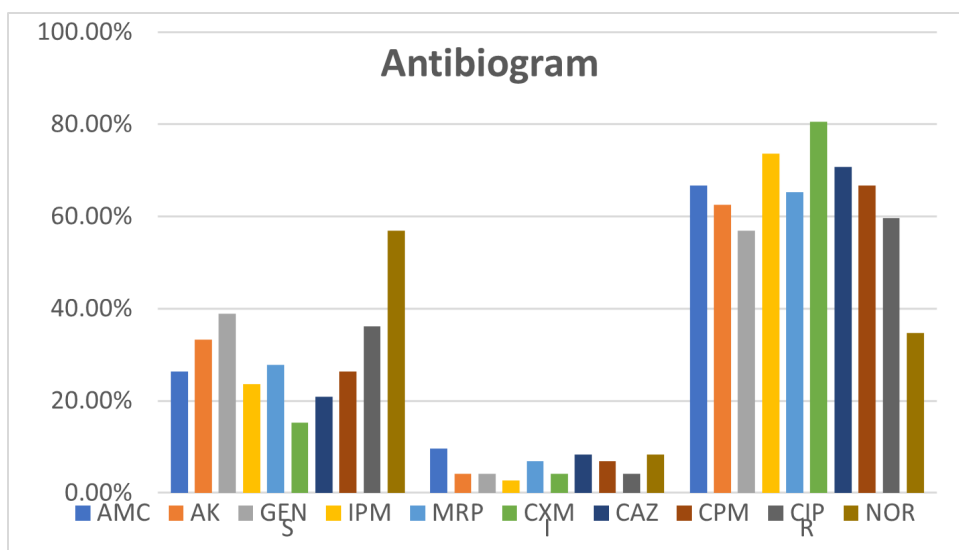


Figure 1: Antibiotic sensitivity patterns of *K. pneumoniae* isolates (n=72).

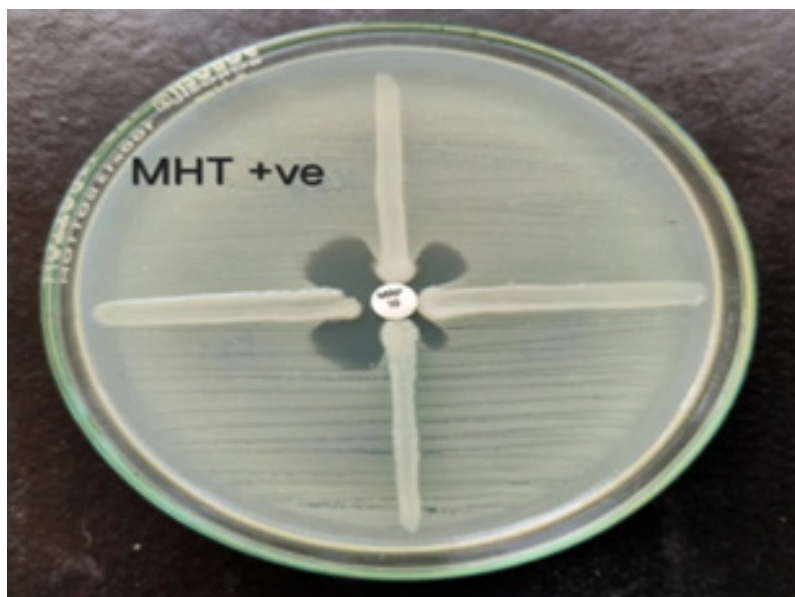


Figure 2: Showing a positive result for Modified Hodge Test.

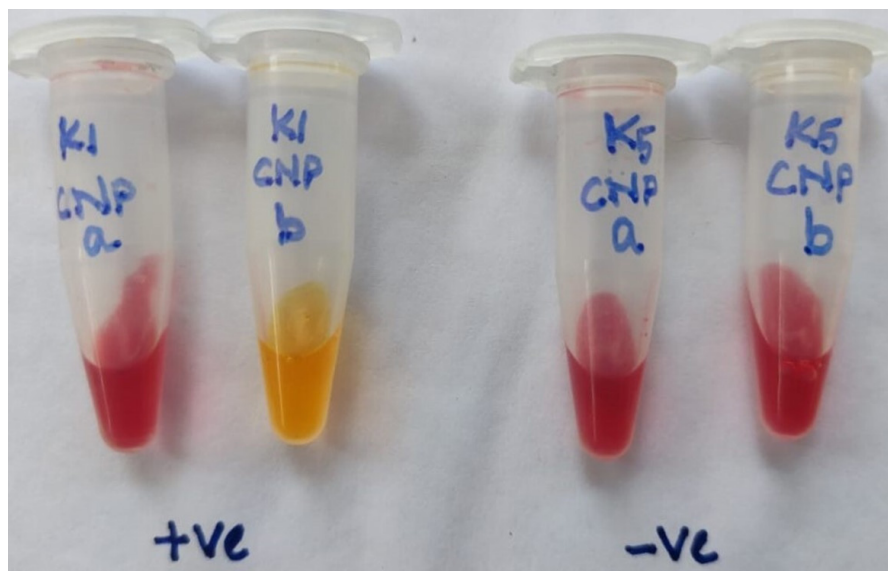


Figure 3: Figure showing Carba NP Test Positive and Negative results.

et al., 2018 reported 76% (50/66) of strains as Carbapenemase producers by MHT.^[16] Amjad A., *et al.*, 2011 have also stated that MHT detected 69% (138/200) of positive strains.^[17]

The mCIM, was proposed as a standardized method in Supplement M100 (2017) of the CLSI Standards. This method can be used by laboratories to identify the epidemiology of carbapenem-resistant Enterobacteriaceae. They proposed that mCIM might be carried out regularly in a lab. It is an excellent and easier-to-perform method for screening carbapenemase production. On the other hand, mCIM is the most sensitive, affordable, reproducible, and subjective approach.

The highest prevalence of carbapenem resistance among CR-Kp in the present study was observed by mCIM (68.5%). While another research conducted by Gallego, M. A. *et al.*, 2022 reported that 96% (46/48) of strains were carbapenemase producers.^[18] Current study findings are on par with the study conducted in Karnataka, India (Kachari *et al.*, 2023) which reported that out of 34 isolates, 20 isolates (58.8%) were positive by mCIM.^[19]

The Carba NP test was established by Patrice Nordmann, Laurent Poirel, and Laurent Dortet in 2012. This method was endorsed by CLSI in the year 2015. It is a chromogenic assay that rapidly detects carbapenemase production compared to other phenotypic methods. Additionally, it is inexpensive and simple to do regularly.

The Carba NP test's advantage is that it can be regarded as a quick biochemical test that can identify CR-Kp isolates in less than 2 hr.

In the present study, we found that out of 54 isolates, 26 (48%) isolates were Carbapenemase producers by the Carba NP method. Another study done by Datta. S. *et al.*, 2017 reported the uppermost prevalence that out of 132 *K. pneumoniae* isolates 114 (86.4%) were positive by Carba NP method.^[20] Khare. A.P. *et al.*, 2022 reported less prevalence than other studies that out of 150 isolates, 65 (43 %) strains of *K. pneumoniae* were positive for carbapenemase production.^[21] Similarly, Tejasvi. K. *et al.*, 2019 also revealed that 22.6% (34/150) of isolates were Carbapenemase producers.^[22]

In our study, we obtained 33.3% (18) of positivity for carbapenemase production by Combined Disc Method. While Uzoamaka *et al.*, reported that out of 18 isolates, 10 (55.55%) were positive.^[23] While Kulkarni *et al.*, 2022 reported that out of 71 Imipenem-resistant strains, 31 (43.66%) showed positive for MBL production.^[24]

The constraint of the MHT and mCIM tests is high turnaround time (18- 24 hr). However, they are time-consuming, as results can be interpreted after overnight incubation. Whereas the Carba NP test can be reported after 2 hr. Though genotypic methods are considered as the most reliable method, it has disadvantages. It is cost-effective and cannot be done routinely due to its budgetary issues. Diagnostic perfection comes only with the test which has less turnaround time.

CONCLUSION

The distribution of CR-Kp isolates in clinical settings was investigated in the present study. A high frequency of *K. pneumoniae* which produces carbapenemase, was observed in the current investigation. The dissemination of CR-Kp isolates represents a significant challenge to clinicians in treating infections, which prompts to practice of different phenotypic techniques for the detection of carbapenem resistance, together with strict infection control measures. In conclusion, regular monitoring of *K. pneumoniae* that produces carbapenemase is required to determine the best empirical antibiotic treatment.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

CR-Kp: Carbapenem Resistant *Klebsiella pneumoniae*; **MHT:** Modified Hodge Test; **mCIM:** Modified Carbapenem Inactivation method; **Carba NP:** Carbapenemase Nordmann- Poirel; **MDR:** Multidrug Resistance; **AMR:** Antimicrobial Resistance; **AST:** Antimicrobial Susceptibility Testing; **ATCC:** American Type Culture Collection; **MBL:** Metallo beta lactamases; **CDM:** Combined Disc Method; **EDTA:** Ethylene diamine tetra acetic acid; **IZ:** Zone of inhibition; **TSB:** Tryptic soy broth; **EDTA:** Ethylenediamine-tetra-acetic acid.

ETHICAL CONSIDERATIONS

This study does not contain any human participants or animals.

AUTHORS' CONTRIBUTIONS

Archana Mani-Designed the study, investigation, data curation, written draft manuscript. Anees Pathima-Investigation and data curation. Dr. S. Sasikala- Reviewed the results, supervised, revised and approved the final version of the manuscript.

SUMMARY

This study aimed to determine the prevalence of carbapenemase-producing *Klebsiella pneumoniae* and emphasize different phenotyping approaches for detecting carbapenemase producers. Out of 72 strains, 54 were multidrug-resistant and suspected carbapenemase producers. Out of these, 53.7% were positive for MHT, 68.5% for mCIM, 48% for Carba NP, and 33.33% for Combined Disc Method. This highlights the importance of screening pathogens for effective antibiotic use.

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