

RESEARCH ARTICLE

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A Comprehensive Study of Phenotypic and Genotypic Techniques to Detect Metallo- β -lactamases in Carbapenem-resistant *Escherichia coli* (*E. coli*) and *Klebsiella pneumoniae* (KP) Strains Derived through Numerous Clinical Specimens in Advanced Health care Facilities

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Abstract

In recent years, an increase in *Escherichia coli* and *Klebsiella pneumoniae* resistant to carbapenem was reported globally. Due to their high prevalence and extensive range of medical conditions, *Escherichia coli* and *Klebsiella pneumoniae* are both confirmed to be major public health concerns. Furthermore, carbapenem resistance restricts treatment options for individuals infected with these bacteria. Consequently, early detection of carbapenem resistance is essential for starting effective therapy, achieving successful management, and avoiding the infection from spreading further in the future. This study's objective was to identify the phenotypic and genotypic identification of Metallo- β -lactamases (MBL) in carbapenem-resistant *E. coli* and *K. pneumoniae* in advanced healthcare facilities. Meropenem resistance was tested in *E. coli* and *K. pneumoniae* using the Kirby-Bauer disc diffusion technique. MBL was discovered using a combination of Disc diffusion testing and the Modified Hodge Test. The Polymerase Chain Reaction was used to determine the genotypes of the *bla* NDM-1 genes that express these enzymes. Out of 427 strains, including 223 *E. coli* and 204 *K. pneumoniae*, 35 (8.2%) consisted of carbapenem-resistant, and 29 (82.85%) showed phenotypically verified as metallo beta-lactamase producers by using the Combined disc test and 20 (57.14%) using the Modified Hodge test. Polymerase Chain Reaction tests for genes detect those three different strains all showed the *bla* NDM-1 gene. Carbapenemase production and MBL can be recognized with the help of phenotypic combination disc and MHT tests in labs. Since both tests showed 100% concordance, laboratories may use the less expensive CDT instead of the MHT. The current study supports institutional antibiotic stewardship programmes to manage antibiotic use and prevent CRE worldwide.

Keywords: Carbapenem-resistant, Metallo- β -lactamase, Modified Hodge Test, *bla* NDM-1, Gene Detection

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INTRODUCTION

Escherichia coli and *Klebsiella pneumoniae*, which are intolerant to the antibiotic carbapenem, are becoming more common and spreading. This is considered a worldwide threat to health, especially in developing countries. Carbapenems are β -lactam Antibiotics with a wide range of effects that most β -lactamases can't break down.¹ Infectious diseases caused by multidrug-resistant organisms were ultimately treatable after their debut in 1980. Unfortunately, *Enterobacteriaceae* and other Gram-negative organisms' developed resistance to carbapenems is now a global health crisis.² Class A β -lactamases, such as *K. pneumoniae* carbapenemase (KPC) and Guiana extended-spectrum (GES), class D β -lactamase OXA-48, and Ambler class β -lactamases MBLs such as IMP, VIM, and NDM all contribute to this resistance via plasmid-mediated clavulanic acid inhibition.³ In the year 2000, the presence of *K. pneumoniae* carbapenemase (KPC) was first reported in the United States. Subsequently, it has been found in many other countries and has spread all over the world.^{4,5}

New Delhi metallo- β -lactamase (NDM) was initially found in *E. coli* and *K. pneumoniae* strains derived through a Swedish sufferer who visited India.⁶ There are six typical methods for demonstrating MBL emergence: the Modified Hodge test (MHT), imipenem/ceftazidime-EDTA Double Disc Synergy test, the impregnated imipenem discs, and a decrease of at least four log units in MIC values when using the imipenem-EDTA combination.⁷

Using straightforward methods for detecting MBL development among Gram-negative bacilli clinical strains is vital. This research aimed to identify metallo- β -lactamases to carbapenem-resistant *E. coli* and *K. pneumoniae* by analyzing their phenotypes and genomes. These strains were obtained through numerous clinical specimens at highly developed healthcare facilities.

MATERIALS AND METHODS

The current investigation was conducted between February 2021 to November 2022 by the Department of Microbiology at the Adesh Medical

College and Hospital in Shahabad, Kurukshetra.

427 isolates of the family Enterobacteriaceae were identified in pus, blood, urine, CSF, and other body fluids collected from IPD and ICU patients to the Microbiology lab. Detection of the strains was accomplished using standard microbiological procedures. On Muller-Hinton agar plates with widely accessible discs (Hi-media), antimicrobial sensitivity was tested using the Kirby-Bauer disc diffusion procedures, and the outcomes were evaluated followed by CLSI guidelines.^{8,9}

Imipenem-EDTA disc test in combination for phenotypic MBL production detection

All carbapenem-resistant strains were tested with imipenem-EDTA and appropriate positive and negative controls. Mueller Hinton agar plates were inoculated according to CLSI guidelines. On the MHA plate, a 10-mcg imipenem disc and a combination of 10-mcg imipenem-EDTA (10/750 mcg) discs were incubated at 37°C for 16-18 hours. The imipenem-EDTA disc enhanced the inhibition zone by 7 mm compared to the imipenem disc alone, indicating an MBL-positive strain.¹⁰

Modified Hodge test phenotypic detection MBL production

CLSI-recommended Modified Hodge testing was used on each strain.¹¹ While an *E. coli* ATCC 25922 lawn culture was being performed at a 1:10 dilution on the Mueller Hinton's agar plate, a disc containing ten mcg of Imipenem was placed in the middle of the test area. The test organisms were then placed in a straight line starting from the disc's edge to the plate's edge. Before being analyzed, plates were kept at 37°C for 24 hours. In a positive Modified Hodge test, the *E. coli* 25922 strain formed an indentation within the disc diffusion zone approximating a clover leaf-like structure, indicating the production of MBL. In a negative test, the *Escherichia coli* ATCC 25922 strain did not generate an indentation along the growth streak of the test organism within the disc diffusion zone^{12,13} as shown in Figure 1.

Controls used in the test procedure were as follows.

K. pneumoniae ATCC BAA-1705 MHT (Positive).

K. pneumoniae ATCC BAA-1706 MHT (Negative).

Genotypic characterization of MBL production

Genotypic recognition was done using a marker-based method to identify the *bla* NDM gene at the Centre for Interdisciplinary and Biomedical Research, Adesh University, Bathinda.

DNA extraction

The DNA was extracted from bacterial strains using the Xploregen kit-based procedure. 1-2 colonies from an overnight growing culture were suspended in 100 µl ultrapure water and cooked at 90°C for twenty minutes. Five minutes were spent spinning the culture at 15,000 rpm to collect the liquid that rose to the surface.

The pellets were stored at 20°C until they were needed. The manufacturer's method was used to get the DNA out of the pellets. The DNA integrity was tested using nano-quant (260/280 ratio). The agarose gel was also used to test the DNA's integrity.

Polymerase chain reaction and DNA sequencing

Primers with sequences 5'-GGTTTG GCGATCTGGTTTTTC-3' (NDM-Forward primer) and 5' -CGGAATGGCTCATCACGATC-3' (NDM-Reverse primer) were used in a polymerase chain reaction to amplify the *bla* NDM-1, which resistance genes. We amplified under the circumstances

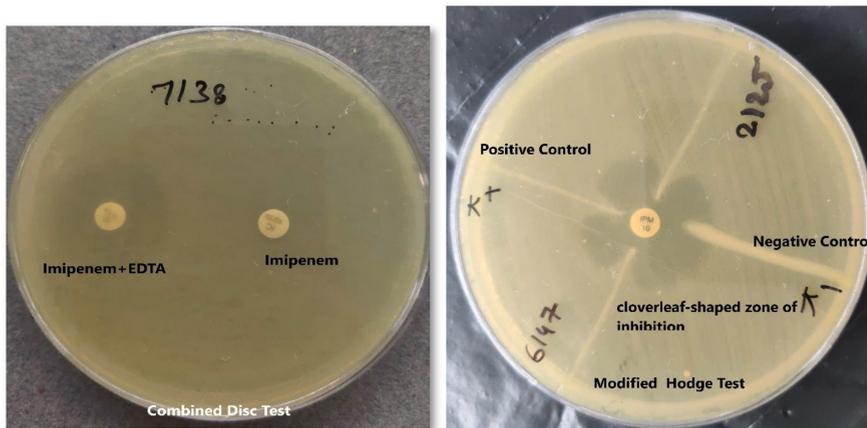


Figure 1. Modified Hodge Test and Combined Disc Test

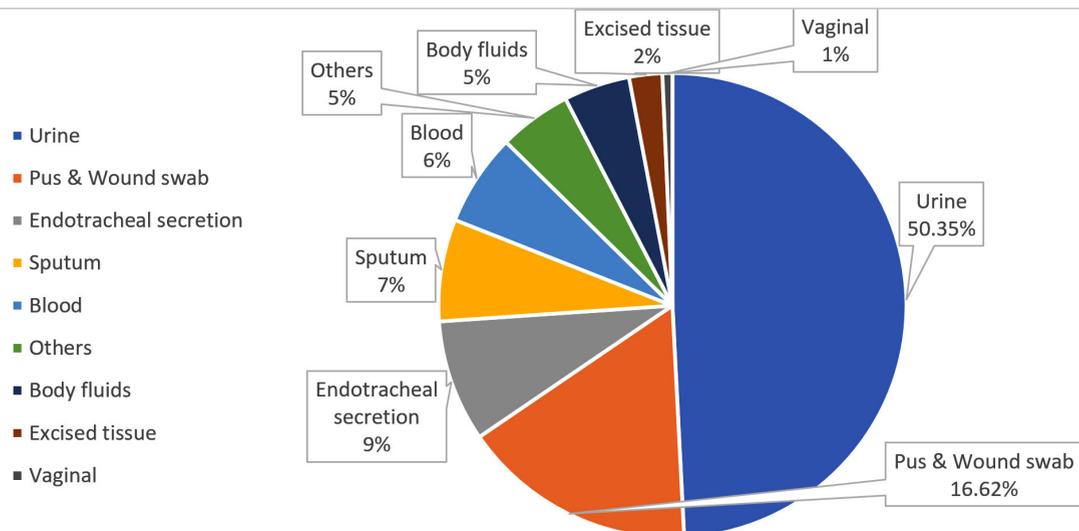


Figure 2. Division of *E. coli* and *K. pneumoniae* from numerous specimens

prescribed by the kit's (Xploreagen kit) protocol, which called for a Polymerase Chain Reaction. The Ethidium Bromide loading reagent was added to the mixture of amplified PCR products and then electrophoresed at 100 volts for at least thirty-five minutes.

RESULTS

427 strains were discovered in diverse clinical specimens. As shown in Table, the most strain came from urine (215, or 50.35%), followed by pus and wound swabs (71, or 16.62%), ET secretion (37, or 8.66%), sputum (31, or 7.25%), and blood (28, or 6.55%) shown in Figure 2.

Carbapenem resistance was found in 35 of these 427 strains (223 *E. coli* and 204 *K. pneumoniae*). Out of 35 carbapenem-resistant strains, CDT identified a greater proportion of MBL producers than MHT producers. In contrast to the modified Hodge test, which found five *E. coli* and fifteen *K. pneumoniae* specimens obtaining MBL, the Combined disc test revealed

nine *E. coli* and twenty *K. pneumoniae* strains as resulting MBL.

Analysis of genotypes (*bla* NDM-1)

As confirmed by the detection of the *bla* NDM-1 gene in two *K. pneumoniae* and one *E. coli*, shown in Figure 3.

DISCUSSION

This investigation aimed to determine if MBL-producing *E. coli* and *K. pneumoniae* were obtained through various patient specimens. Carbapenem-resistant *Escherichia coli* (*E. coli*) and *Klebsiella pneumoniae* (*KP*) strains derived through numerous clinical specimens in advanced Health care facilities were resistant to carbapenem. This study discovered Carbapenem-resistant bacteria in 23 (11.27%) *K. pneumoniae* strains and 12 (5.38%) *E. coli* strains. In one *E. coli* and two *K. pneumoniae* specimens, the *bla* NDM-1 gene was discovered using a polymerase chain reaction.

There were 35 (8.19%) carbapenem-resistant strains out of 427.

Hedge et al. also reported 16% resistance towards Imipenem, concordant with this study.¹⁴ According to Agarwal et al., 14.27% of *Enterobacteriaceae* are resistant to the antibiotic meropenem.⁹ This value is comparable to Balan et al.¹⁵ (15%) and Behera et al.¹⁶ (19.50%) reported in this study.

35 out of 427 (8.19%) of the strains in our study made metallo-β-lactamases. Other studies also show a lower prevalence of MBL production. As an example, a study that was carried out by Kaur et al.² revealed a prevalence of MBL of 6.4%, and a study by Deshmukh DG et al.¹⁷ reported a prevalence of MBL of 2.9%.

In a similar investigation, Meel et al. discovered that the prevalence of MBL producers 10 (8.84%) of *Klebsiella pneumoniae*.¹⁸

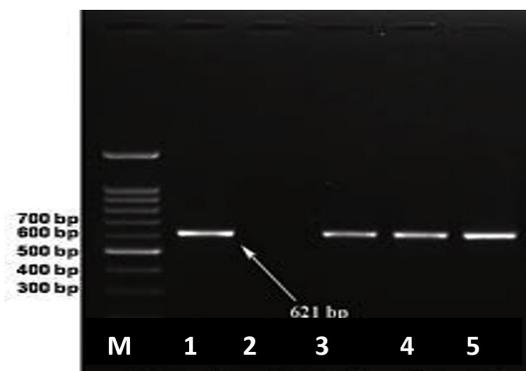


Figure 3. Agarose gel electrophoresis (1.2%) of *bla* NDM-1 PCR-amplified product in *K. pneumoniae* and *E. coli*. Lane M: showing DNA Ladder. 1 well is 16S DNA Control, Lane 3, 4, 5: PCR amplicon proportions (621 bp) for the *bla*NDM-1

Table. MBL-positive strains determined by phenotypic testing

Organism	No. of isolates	Carbapenem-resistant isolates	MBL positive by Combined disc test	MBL positive by Modified Hodge test
<i>E. coli</i>	223	12 (5.38%)	9/12(75%)	5/12 (42%)
<i>K. pneumoniae</i>	204	23(11.27%)	20/23 (87.1%)	15/23 (65.2%)
Total	427	35 (8.2%)	29/35 (82.8%)	20/35 (57.2%)

The polymerase chain reaction was used in the current work to amplify the *bla* NDM-1 gene. Only 8.33% (1/12) and 8.69% (2/23), respectively, of the 35 carbapenem-resistant specimens of *K. pneumoniae* and *E. coli* were determined. According to the investigation, *K. pneumoniae* and *E. coli* strains that produce carbapenem are responsible for 8% of all illnesses.

Similar results were reported by Bora et al. in 2016; 8.67% (19/219) of the *K. pneumoniae* strain tested positive for *bla* NDM-1.¹⁹

In a study by Mohasen et al., *bla* NDM 1 was determined in three strains of *K. pneumoniae*, but the carbapenemase gene was not discovered in any of the *E. coli* specimens.¹¹ The three NDM-1-positive strains were susceptible to polymyxin-B, but almost every other drug tested, except carbapenems, did not work against them. In a study by Sahin et al., Metallo- β -lactamases were found with the MBL antibiotic gradient test. Two of the 43 strains had MBL enzyme, but only one of these two strains was *bla* NDM-1 positive.²⁰

CONCLUSION

Thus, the phenotypic technique, the combined disc test and the MHT test can be used to identify carbapenemase production and MBL in laboratories. Since both tests showed complete concordance, laboratories may use the CDT instead of the MHT, which is expensive. When genotypic assays are unavailable, employ phenotypic ones. Drug-resistant microorganisms can be reduced by following antimicrobial stewardship programs and antibiotic policies, including carbapenem antimicrobials.

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None.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

None.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

Not applicable.

REFERENCES

1. Yong D, Toleman MA, Giske CG, et al. Characterization of a new metallo-beta-lactamase gene, *bla* NDM-1, and a novel erythromycin esterase gene carried on a unique genetic structure in *Klebsiella pneumoniae* sequence type 14 from India. *Antimicrob Agents Chemother.* 2009;53(12):5046-5054. doi: 10.1128/AAC.00774-09
2. Kaur J, Khanna P, Chand K. Phenotypic Detection of Carbapenem Resistance among *Escherichia coli* and *Klebsiella* Isolates Obtained from Various Clinical Samples. *Int J Curr Microbiol Appl Sci.* 2017;6(10):1566-1573. doi: 10.20546/ijcmas.2017.610.188
3. Queenan AM, Bush K. Carbapenemases: the versatile beta-lactamases. *Clin Microbiol Rev.* 2007;20(3):440-458. doi: 10.1128/CMR.00001-07
4. Amira ME, Areej ME, Hemat KA, Ramadan HI, Heba IA. Phenotypic and genotypic detection of lactams resistance in *Klebsiella* species from Egyptian hospitals revealed carbapenem resistance by OXA and NDM genes. *Afr J Microbiol Res.* 2016;10(10):339-347. doi: 10.5897/AJMR2015.7871
5. Gupta V, Singh M, Datta P, Goel A, Singh S, Prasad K, Chander J. Detection of various beta-Lactamases in *Escherichia coli* and *Klebsiella sp.*: A study from Tertiary Care Centre of North India. *Indian J Med Microbiol.* 2020;38(3-4):3906. doi: 10.4103/ijmm.IJMM_20_253
6. Collee JC, Fraser AG, Marmion BP, Simmons A. Mackie and McCartney practical medical microbiology 14th edition, 131-140. Church hill. Livingstone, London. 2006.
7. Fazlul MKK, Deepthi S, Farzana Y, Najnin A, Rashid Ma, Munira B, Srikumar C, Nazmul. Detection of Metallo- β -Lactamases-Encoding Genes Among Clinical Isolates of *Escherichia Coli* in a Tertiary Care Hospital, Malaysia. *arXiv preprint arXiv:1904.05198;2019.* doi: 10.48550/arXiv.1904.05198
8. Kamel NA, El-Tayeb WN, El-Ansary MR, Mansour MT, Aboshanab KM. Phenotypic screening and molecular characterization of carbapenemase-producing Gram-negative bacilli recovered from febrile neutropenic pediatric cancer patients in Egypt. *PLoS one.* 2018;13(8):e0202119. doi: 10.1371/journal.pone.0202119
9. Agarwal N, Chauhan S, Singh P, Sharma VK. Phenotypic detection of carbapenem resistance and metallo-beta-lactamase among Enterobacteriaceae from clinical

- samples in tertiary care hospital. *Int J Res Med Sci.* 2019;7(6):2373-2376. doi: 10.18203/2320-6012.ijrms20192530
10. Dortet L, Poirel L, Nordmann P. Rapid identification of carbapenemase types in Enterobacteriaceae and Pseudomonas spp. by using a biochemical test. *Antimicrob Agents Chemother.* 2012;56(12):6437-6440. doi: 10.1128/AAC.01395-12
 11. Mohsen SM, Hamzah HA, Al-Deen Mustafa MI. Phenotypic and molecular study of carbapenem-resistant Enterobacteriaceae in a referral hospital in the East coast Malaysia. *Int J Health Allied Sci.* 2018;7(1):17-22. doi: 10.4103/ijhas.IJHAS_96_17
 12. Srivastava P, Bisht D, Kumar A, Tripathi A. Prevalence of Carbapenem-Resistant *Escherichia coli* and *Klebsiella pneumoniae* in Rural Uttar Pradesh. *Journal of Datta Meghe Institute of Medical Sciences University.* 2022;17(3):584-588. doi: 10.4103/jdmimsu.jdmimsu_76_22
 13. Nagaraj S, Chandran SP, Shamanna P, Macaden R. Carbapenem resistance among *Escherichia coli* and *Klebsiella pneumoniae* in a tertiary care hospital in south India. *Indian J Med Microbiol.* 2012;30(1):93-95. doi: 10.4103/0255-0857.93054
 14. Archana Hegde M, Tejashree A. Genotyping of Carbapenem-Resistant *Klebsiella pneumoniae* from Clinical Isolates. *Int J Curr Microbiol Appl Sci.* 2019;8(05):825-835. doi: 10.20546/ijcmas.2019.805.098
 15. Balan K, Sireesha P, Setty CR. Study to detect incidence of carbapenemase among Gram-negative clinical isolates from tertiary care hospital. *J Dent Med Sci.* 2012;1(6):08-12. doi: 10.9790/0853-0160812
 16. Behera B, Mathur P, Das A, Kapil A. Ertapenem susceptibility of extended spectrum beta-lactamase-producing Enterobacteriaceae at a tertiary care centre in India. *Singapore Med J.* 2009;50(6):628-632.
 17. Deshmukh DG, Damle AS, Bajaj JK, Bhakre JB, Patwardhan NS. Metallo-β-lactamase-producing clinical isolates from patients of a tertiary care hospital. *J Lab Physicians.* 2011;3(2):93-97. doi: 10.4103/0974-2727.86841
 18. Meel P, Maheshwari M, Arora B, Malhotra V. Phenotypic detection of Metallo-β-lactamase mediated carbapenem resistance in gram-negative bacilli of family Enterobacteriaceae in a tertiary care hospital in north India. *J Crit Rev.* 2019;7(3):2020. doi: 10.31838/jcr.07.03.64
 19. Bora A, Ahmed G. Detection of NDM-1 in clinical isolates of *Klebsiella pneumoniae* from Northeast India. *J Clin Diagn Res.* 2012;6(5):794-800.
 20. Sahin K, Tekin A, Ozdas S, et al. Evaluation of carbapenem resistance using phenotypic and genotypic techniques in Enterobacteriaceae isolates. *Ann Clin Microbiol Antimicrob.* 2015;14(1):1-6. doi: 10.1186/s12941-015-0105-1