

# Molecular Detection of Carbapenem Resistance in Clinical Isolates of *Klebsiella pneumoniae* in Tertiary Care Hospital

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## Abstract

Antibiotic resistance has become a serious global threat, mainly due to misuse, overuse of antibiotics and non-compliance with infection control protocol. Superbugs are multidrug-resistant (MDR) and extended drug-resistant (XDR) bacteria, mainly *Klebsiella pneumoniae* and *Escherichia coli* from the Enterobacteriaceae family, which cause opportunistic infections and raise death rates and hospital expenditures. The present study was conducted at a tertiary care teaching hospital to study the epidemiology and molecular detection of carbapenem-resistant *K. pneumoniae* isolated from various clinical specimens. 240 *K. pneumoniae* isolates were collected from January 2020 to December 2021 at the Bacteriology laboratory, Index Medical College and Hospital, Indore. All isolates were analyzed for carbapenem resistance by the conventional disc diffusion method. All carbapenem-resistant isolates were tested for carbapenemase production using the phenotypic double-disk synergy test (DDST) and modified Hodge test (MHT) as per 2020 CLSI guidelines. All isolates were negative by phenotypic methods, further confirmed by conventional PCR to detect the gene responsible for carbapenemase production. 240 isolates of *K. pneumoniae* were included during the study periods. Out of 240 isolates, 102 isolates were found resistant to carbapenem drugs. All 102 isolates were confirmed carbapenemase and MBL producers by MHT and DDST tests. Among 102, 60 isolates were found to be MBL producers negative by MHT and DDST tests. Sixty phenotypic negative carbapenem-resistant isolates were tested by conventional PCR. One or more carbapenemase genes were detected in 61.0% of isolates. The *bla*<sub>KPC</sub> was detected in 13/60 (21%) isolates, followed by *bla*<sub>NDM</sub> 10/60 (16%) isolates, followed by *bla*<sub>VIM</sub> in 6/60 (10%), *bla*<sub>OXA-48</sub> in 5/60 (8%) and *bla*<sub>IMP</sub> in 3/60 (5%) isolates. *K. pneumoniae* produces carbapenemase, which enhances resistance to the carbapenem class of antibiotics. The simultaneous detection of these resistance genes expressed by *Klebsiella pneumoniae* might be managed by early detection and adhering to antibiotic policies that limit the use of antibiotics.

**Keywords:** Double Disk Synergy Test (DDST), Modified Hodge Test (MHT), Metallo-beta-Lactames (MBL), Polymerase Chain Reaction

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## INTRODUCTION

Antibiotic resistance has become a serious global threat, this is mainly due to misuse, overuse of antibiotics and non-compliance with infection control protocols.<sup>1-3</sup> The multidrug resistant (MDR) and extensive drug resistant (XDR), bacteria are called as 'Superbug' especially seen in Enterobacteriaceae family with *Klebsiella pneumoniae* followed by *Escherichia coli* which causes opportunistic infections and leads to increase in death rate which causes increase in expenses of hospital related costs.<sup>4-6</sup> In 2017, World Health Organization has classify carbapenem-resistant Enterobacteriaceae (CRE), on the global priority among top 10 global public health threats facing humanity.<sup>1,4,7,8</sup>

CRE is due to Beta-lactamases, Carbapenemases, mutation in bacteria and efflux pumps, it alters the expression and functions of porins and proteins that binds with penicillin and also combinations of these mechanisms lead to high levels of carbapenem resistance is seen in *K. pneumoniae*.<sup>9,10</sup> CRE classified into three different molecular classes like A, B and D, its examples are Serines carbapenemes, such as *K. pneumoniae* Carbapenemes, (KPC) are examples of carbapenemases in class A. Metallobactam lactamases (MBLs), like Verona integron encoded MBL, (VLM), New Delhi MBL (NDM), and imipenemase (IMP) are examples of class B, and OXA carbapenemases like *bla*<sub>OXA-48</sub> are examples of molecular class D.<sup>9-11</sup> High prevalence of CRE is reported from southern Europe and Asia than in other parts of the world.<sup>12</sup> CRE is mostly encounter in nosocomial isolates than community isolates.

Center for Disease Dynamics, Economics and policy 2021, stated in Indian scenario there is the markedly increase in *K pneumoniae*, it was 24% in 2008 and it is 59% in 2017. It is observed due to inadequate medical intervention, various co-morbidities and over use of antibiotics.<sup>13</sup>

For the detection of KPCs, several phenotypic tests have been established.<sup>14,15</sup> Presently Modified Hodge test (MHT) is preferred and accepted as accurate and sensitive method for detection of carbapenemase which is approved by CLSI guidelines.<sup>16,17</sup> MHT cannot be used as a confirmatory test for detection of the KPCs

because of difficult clarification and false positive results.<sup>18,19</sup> KPCs isolates producing AmpC and CTX-M  $\beta$ -lactamase are showing commonly as false positive results.<sup>20</sup>

For the identification of resistance conferring genes, gold standard method preferred is Polymerase Chain Reaction (PCR), which amplifies a specific nucleic acid target, to obtain a million or more copies which can then be easily detected by using nucleic acid staining techniques.<sup>21</sup>

In spite of high disease burden, limited Indian studies describe mechanisms of resistance caused due to *K. Pneumoniae* isolates in MDR, which highlights the requirement of comprehensive epidemiological surveillance results.<sup>22</sup> Therefore, the present study was conducted at a tertiary care teaching hospital with an aim to study epidemiology, antimicrobial susceptibility profile of carbapenem-resistant *K. pneumoniae* isolated from various clinical specimens.

## MATERIALS AND METHODS

A total of 240 *Klebsiella pneumoniae* isolates was collected from Index medical college and hospital Indore, during period January 2020 to December 2021. From the clinical specimens such as urine, CSF, wounds, blood, sputum; bacteria was identified using by standard Conventional methods.

### Culture Media and Chemicals

All required culture medium, antibiotics and chemicals (analytical grade) were procured from HIMEDIA Pvt. Ltd. Mumbai, India, and molecular testing reagents using QIAamp DNA mini kit (Qiagen India Pvt. Ltd.) for bacterial genomic DNA extraction and master mix (HotStarTaq Master Mix Kit(Qiagen Cat. No. 203443) were used in this study.

### Antibiotic susceptibility test

By using Kirby-Bauer method, antibiotic susceptibility test was done for all isolates according to CLSI standards guidelines 2020.<sup>23</sup> Selection of antibiotics was done as per CLSI 2020 guidelines. Following antibiotic discs used in the study were Amikacin (30 $\mu$ g), Gentamicin (10 $\mu$ g), Ceftazidime (30 $\mu$ g), Ciprofloxacin (5 $\mu$ g), Imipenem

(10µg), Meropenem (10µg), Aztreonam (30µg), Piperacillin / Tazobactam (100/10µg). Amoxiclave (30µg), Cefixime(5µg), Ceftriaxone (30µg), Cefuroxime (30µg), Cefepime (30µg), Tetracycline (30µg), Trimethoprim (5µg) Cefotaxime (30µg), Ampicillin (10µg), Nitrofurantoin (300µg).

### Carbapenemase screening

#### Phenotypic Methods

By performing Modified Hodge test (MHT), all isolates were screened for determination of carbapenemase production. To detect MBL (Metallo-beta-Lactames) production, a double disk synergy test (DDST) was done as per the CLSI guidelines 2020.<sup>23</sup>

**Table 1.** Primers are used for the detection of target genes

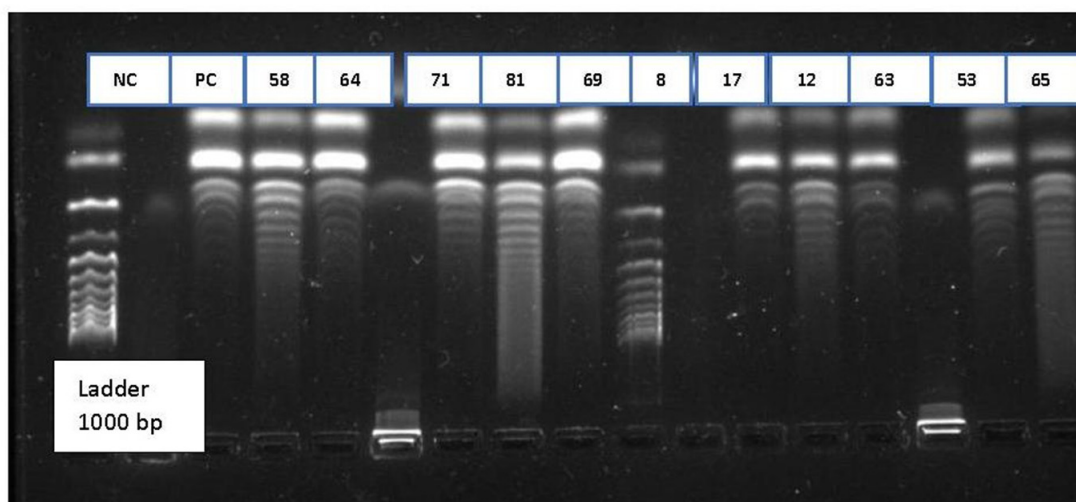
Gene target	Primers sequence (5'-3')	Product size (bp)
NDM – F	GGGCAGTCGCTTCCAACGGT	188
NDM –R	GTAGTGCTCAGTGTCGGCAT	
OXA -48F	TTGGTGGCATCGATTATCGG	390
OXA -48 R	GAGCACTTCTTTGTGATGGC	
VIM-F	GATGGTGTTGGTCGCATA	743
VIM-R	CGAATGCGCAGCACCAG	
IMP-F	GGAATAGAGTGGCTTAATTCTC	475
IMP-R	CCAAACCACTACGTTATCT	
KPC F	TGTCAGTGTATCGCCGTC	1000
KPC R	CTCAGTGCTCTACAGAAAACC	

#### Genotypic Methods

The genes responsible for carbapenemase production may be initiating the resistance to carbapenem groups antibiotics were detected by genotypic methods by using QIAamp DNA mini kit (Qiagen India Pvt. Ltd.) for bacterial genomic DNA extraction and master mix (HotStarTaq Master Mix Kit(Qiagen Cat. No. 203443) With the help of above this kit performed conventional PCR to

**Table 2.** Antibiotic-resistant pattern for *K. pneumoniae* isolates

Antibiotic	Total N= 240 (%)
Imipenem	102 (41.7)
Meropenem	102(41.7)
Ampicillin	240 (100)
Amoxyclav	217 (90.4)
Ceftazidime	240 (100)
Ceftriaxone	226 (94.1)
Cefotaxime	233 (97.1)
Cefuroxime	213(88.7)
Aztreonam	213 (88.7)
Cefixime	198(82.5)
Piperacillin+	220 (91.7)
Tazobactam	
Tetracycline	198 (82.5)
Cefepime	224 (93.3)
Trimethoprim	211(87.9)
Ciprofloxacin	194 (80.8)
Gentamicin	153 (63.8)
Amikacin	152 (63.3)
Nitrofurantoin	38(15.8)



**Figure 1.** Distribution of KPC genes in resistant isolates

detect the presence of genes such as NDM-1, OXA-48, KPC, VIM and IMP in *K. pneumoniae* isolates. The primers used in this study are enlisted below (Table 1).

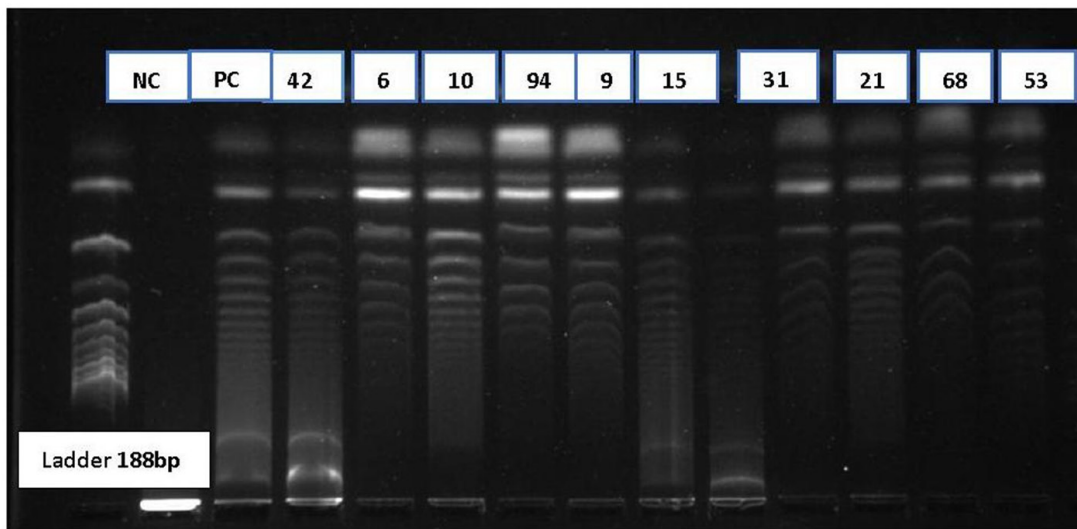
Master mix and PCR Cycling conditions were prepared as per kit standard protocol. Electrophoresis were used for the detection of amplicon band, using 2% agarose gel performed at 60-90 V for 30-45 mins. The gel documentation was done by using gel doc system (Bio Era, India). 100 bp DNA ladder (QiagenGelPilot) was used parallel to test for marking of molecular weight.

**RESULTS**

A Total of 240 isolates of *K. pneumoniae* isolates (126 in patients and 114 outpatients) were used in the study, among 108 isolates were collected from male and 132 from female

participants. Antibiotic resistant were showed in Table 2.

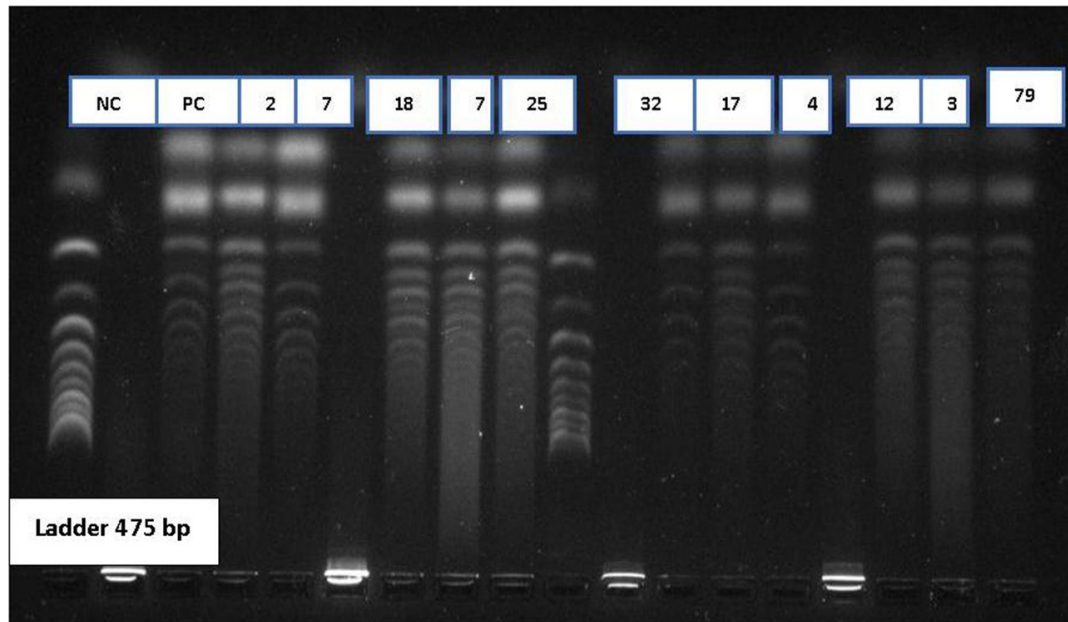
A total of 102 isolates observe to be resistant to carbapenem drugs. These were confirmed by phenotypic methods MHT and DDST for carbapenemase producers. Among 102 isolates, 60 isolates were negative to phenotypic methods. These 60 isolates were further confirmed by the molecular method using conventional PCR to detection of resistant genes. KPC Genes in resistant isolate shows in Figure 1 as the negative control (NC) Positive control (PC) and showing samples numbers with DNA Ladder 1000bp. NDM Genes in resistant isolate shows in Figure 2 as the negative control (NC) Positive control (PC) and showing samples numbers with DNA Ladder 188bp. IMP Genes in resistant isolate shows in Figure 3 as the negative control (NC) Positive control (PC) and showing samples numbers with



**Figure 2.** Distribution of NDMgenes in resistant isolates

**Table 3.** Frequency distribution of Carbapenemase genes family in various clinical specimens

Specimen type	Frequency of carbapenemase genes					No.
	<i>bla</i> <sub>NDM</sub>	<i>bla</i> <sub>KPC</sub>	<i>bla</i> <sub>OXA-48</sub>	<i>bla</i> <sub>VIM</sub>	<i>bla</i> <sub>IMP</sub>	
Blood	3	4	-	2	1	10
Urine	1	3	2	1	1	8
Wound	3	3	-	-	1	7
Sputum	1	2	1	2	-	6
CSF	2	1	2	1	-	6
Total	10 (16%)	13 (21%)	5 (8%)	6 (10%)	3 (5%)	37 (61%)



**Figure 3.** Distribution of IMP genes in resistant isolates

DNA Ladder 475bp. Of 60 Carbapenem-resistant isolates, 37(61%) were positive for one and multiple targeted genes. *bla*<sub>KPC</sub> was detected in 21% of isolates, followed by *bla*<sub>NDM</sub> 16.0%, *bla*<sub>VIM</sub> in 10.0%, *bla*<sub>OXA-48</sub> 8.0% and *bla*<sub>IMP</sub> in 5% (Table 3).

## DISCUSSION

In recent years, molecular diagnostic techniques have become a game changer for clinical laboratories of all sizes. Molecular diagnostics offer more powerful tools for earlier and more accurate detection of various diseases, including infectious diseases.<sup>12</sup>

Today, molecular diagnostic methods testing has become more routine, mainly due to the development of automated instrument systems that provide accurate and precise results utilizing polymerase chain reaction (PCR) and other molecular-based technologies for identification and DNA/RNA measurement of infectious pathogens, tumours, and human genes.<sup>24</sup>

Various genetic mechanisms, including efflux pumps, altered function and expression of porins and penicillin-binding proteins (PBPs), are considered to be involved in carbapenem

resistance in Enterobacteriaceae. However, several mechanisms are yet to be identified or discovered.<sup>25</sup>

A golden rule for building any clinical laboratory capacity in resource-limited settings is selecting the most appropriate workable, affordable, and sustained techniques. In the present study, an in-depth molecular characterization of *K. pneumoniae* was conducted by in-house conventional PCR. Nucleic acid-based assays for the detection of antimicrobial resistance may offer advantages over phenotypic methods.<sup>26,27</sup>

The inclusion criterion for molecular detection of carbapenem resistant genes was *K. pneumoniae* demonstrating carbapenem resistance by the E-test method.<sup>27</sup> For conventional PCR, the *bla*<sub>KPC</sub>, *bla*<sub>NDM</sub>, *bla*<sub>VIM</sub>, *bla*<sub>IMP</sub> and *bla*<sub>OXA</sub> were done using primers. Out of 60 *K. pneumoniae* isolates, from sequencing analysis, only three genes, namely *bla*<sub>KPC</sub>, *bla*<sub>IMP</sub> and *bla*<sub>NDM</sub> were detected.

In this study, the high prevalence of *bla*<sub>KPC</sub> gene13 (21%) was isolated, followed by *bla*<sub>NDM</sub> was detected in 10(16%), *bla*<sub>VIM</sub> in 6 (10%), *bla*<sub>OXA-48</sub> in 5 (8%) and *bla*<sub>IMP</sub> in 3 (5%). Various researchers



increasingly reported similar genes responsible for carbapenem resistance at a rapid velocity in *K. pneumoniae*. Baran et al.,<sup>28</sup> reported detection of carbapenemase-encoding genes in 81.7% (94/115) isolates. The genes detected in their study were *bla*<sub>KPC</sub> (78.3%), *bla*<sub>NDM-1</sub> (0.9%), and *bla*<sub>SME</sub> (2.6%). This study reported the increased isolation of carbapenem resistant *K. pneumoniae* and KPC as the commonest gene responsible for resistance.

In the study by Hamzan et al.,<sup>29</sup> only two genes, *bla*<sub>IMP4</sub> and *bla*<sub>NDM1</sub> were detected in *K. pneumoniae* using conventional PCR. Mohanty et al.<sup>30</sup> reported the highest frequency of NDM 1 in 65.6% and OXA 48 in 24.7%, OXA 181 23.6%, VIM in 6.4% and KPC in 2.1% of *K. pneumoniae* isolates. In their study *bla*<sub>IMP</sub> gene was not found in any of the isolates by using conventional PCR. A major advantage of conventional PCR is its cost effectiveness and ready access to conventional thermo cyclers that almost all research facilities. Veeraraghavan et al.<sup>31</sup> performed multiplex PCR for detection of resistance genes encoding  $\alpha$ -lactam resistance. In their study, among the carbapenemases co-expression of *bla*<sub>NDM</sub> and *bla*<sub>OXA48</sub>-like was observed in 28%, *bla*<sub>NDM</sub> in 19%, *bla*<sub>OXA48</sub>-like in 13% and *bla*<sub>KPC</sub> was not found. In the study of Vivan et al.,<sup>32</sup> the isolates that were phenotypically screened for carbapenemase production were subjected for genotypic confirmation by PCR (PCR) for KPC, metallo- $\beta$ -lactamases, OXA-48, and extended-spectrum beta-lactamase genes. PCR analysis demonstrated that all isolates carried *bla*<sub>KPC</sub> genes and sequencing showed that all strains belonged to KPC-2 subtype.

In the study by Pourgholi et al.,<sup>33</sup> *bla*<sub>KPC2</sub> has been detected in 8 out of 17 carbapenem resistant *K. pneumoniae* isolates from a tertiary teaching hospital. Ahmad et al.<sup>34</sup> from Aligarh, performed molecular characterization of novel sequence type of carbapenem-resistant New Delhi metallo- $\beta$ -lactamase-1-producing *K. pneumoniae* isolated from NICU. In their study, all 17 isolates were found to carry *bla*<sub>NDM</sub> (13 *bla*<sub>NDM-1</sub>, 1 *bla*<sub>NDM-4</sub> and 3 *bla*<sub>NDM-5</sub>), seven isolates carried *bla*<sub>OXA-48</sub>, 13 isolates had *bla*<sub>CTX-M-15</sub>, seven isolates carried *bla*<sub>CMY-1</sub> and five isolates were found to carry *bla*<sub>SHV-1</sub>. Galani et al.<sup>35</sup> detected 300 carbapenem resistant *K. pneumoniae* strains in hospitals across Greece and found KPC-2 (66.7%), NDM (16.7%),

VIM (7%) and OXA-48 (4%) whereas 14 strains carried both KPC and VIM (4.7%), two strains carried both NDM and OXA (0.7%) and one strain carried both KPC and OXA (0.3%) resistance genes. Shankar et al.,<sup>36</sup> observed that 60% of the isolates co-produced *bla*<sub>SHV</sub>, *bla*<sub>TEM</sub> and *bla*<sub>CTX-M-15</sub>, *bla*<sub>OXA48</sub>-like was the predominant carbapenemase gene produced in 71% followed by co-production of *bla*<sub>NDM</sub> and *bla*<sub>OXA48</sub> in 11 per cent, *bla*<sub>NDM</sub> in 7% and *bla*<sub>KPC</sub> 3% and four isolates did not produce any of the carbapenemases genes tested.

Bhaskar et al.<sup>37</sup> studied 165 carbapenem resistant *K. pneumoniae* isolates. In their study, 9.7% were positive for *bla*<sub>NDM-1</sub> and these isolates were also found to be positive for one or more *bla* genes. Co-carriage of AmpC in ESBL and carbapenem resistant isolates were 7.8% and 3.6%, respectively and were negative for *bla*<sub>KPC</sub> genes. Han et al.<sup>38</sup> reported the KPC-2 gene as the most commonly detected followed by the CTX-M9 and OmpK.<sup>36</sup> However, in their study, IPM-4, NDM-1 and OXA-48 were not detected.

The epidemiology of carbapenem resistant *K. pneumoniae* was studied by Lau et al.<sup>39</sup> using molecular method. This study included 63 carbapenem resistant strains of *K. pneumoniae*. Carbapenemase genes were detected in 55 isolates, with *bla*<sub>OXA-48</sub> (63.5%) as the predominant carbapenemase gene, followed by *bla*<sub>NDM</sub> (36.5%). Alizadeh et al.<sup>40</sup> reported that *bla*<sub>VIM-1</sub> as the most prevalent gene followed by *bla*<sub>IMP-1</sub> and *bla*<sub>NDM-1</sub>. This study suggested that PCR could be considered a suitable tool for the rapid identification of strains with high epidemic potential that may be required for local outbreak studies. Li et al.<sup>41</sup> studied antimicrobial susceptibility profile, molecular characteristics, plasmid and integron-associated analysis, genetic environments of *bla*<sub>KPC-2</sub> and *bla*<sub>NDM-1</sub> of 66 strains of carbapenem-resistant *K. pneumoniae* isolated from BSIs. In their study, 3 strains were identified co-carrying *bla*<sub>NDM</sub> and *bla*<sub>IMP</sub> genes, including two isolates with *bla*<sub>NDM-1</sub> and *bla*<sub>IMP-4</sub> and one with *bla*<sub>NDM-5</sub> and *bla*<sub>IMP-4</sub>.

Kazi et al.<sup>42</sup> from Mumbai, reported that predominance of *bla*<sub>NDM1</sub> gene in their study by using multiplex PCR. These authors concluded that PCR is important for rapid detection of genes responsible for drug resistance and also highlighted the importance of implementation

of strict infection control measures and contact precautions to prevent spreading of *bla*<sub>NDM1</sub> mediated resistance in health-care setup. Bhatia et al.<sup>43</sup> studied 29 MDR *K. pneumoniae* isolates obtained from various clinical samples. These authors reported carbapenem resistance in 27 isolates. Carbapenem resistance was mainly encoded by OXA-48-like genes (21/27 [77.8%]) and all isolates had a varied arsenal of resistance genes to different antibiotic classes. Bhatia et al. highlighted the continuous need for genomic surveillance of MDR bacteria for developing treatment guidelines based on integrating phenotypic and molecular methods. In the study of Bhatt et al.<sup>44</sup> Out of 150 phenotypically confirmed carbapenemase-producing isolates, *bla*<sub>NDM</sub> gene was found in 85, *bla*<sub>VIM</sub> in 32, and *bla*<sub>IMP</sub> in 22 isolates. *bla*<sub>OXA-48</sub> and *bla*<sub>KPC</sub> genes were not found in any isolate. Moreover, there were 19 isolates, in which no gene was detected.

## CONCLUSION

In this study, carbapenem resistance was confirmed in 60 (25%) out of 240 *K. pneumoniae* isolated from different clinical specimens was done with polymerase chain reaction *bla*<sub>KPC</sub>, *bla*<sub>IMP</sub>, and *bla*<sub>NDM</sub> was the genes found to be associated with carbapenem resistance *K. pneumoniae*. As carbapenem resistance was high, this study highlights the importance of strict compliance with infection prevention and control measures, rational use of antimicrobials, and active surveillance for the presence of carbapenemase.

*K. pneumoniae* increases resistance to the carbapenem group of antimicrobials by producing carbapenemase. For the reservoir of resistance, carbapenemase non-producing isolates act to MBL and KPC production, which were associated with increased mortality and morbidity and can spread within healthcare settings and communities. Simultaneous detection of these resistance patterns of MBL and KPC-producing *Klebsiella* isolates can be controlled by early detection and antibiotic policies by curtailing the injudicious use of antibiotics. The cross-transmission of multidrug-resistant organisms would prevent the emergence and implementation of antimicrobial stewardship.

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## 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

This study was approved by the Institutional Ethics Committee, Malwanal University, Indore, India, with reference number MU/Research/EC/Ph.D/2018/13.

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