Detection of carbapenemase-producing *Pseudomonas aeruginosa* by phenotypic and genotypic methods in a tertiary care hospital of East India

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**Abstract:**

**BACKGROUND:** Carbapenemase-producing *Pseudomonas aeruginosa* is a serious threat in hospital infection due to its multidrug resistance.

**AIM:** The aim of the study was to determine the frequency of carbapenem resistance in clinical isolates of *Pseudomonas aeruginosa* and detect the presence of carbapenemase enzymes in carbapenem-resistant *P. aeruginosa* (CRPA) isolates by phenotypic and genotypic methods.

**MATERIAL AND METHODS:** Double-disk synergy test (DDST) and combined disk synergy test (CDST) was performed in CRPA isolates and the prevalence of *bla*<sup>KPC</sup>, *bla*<sup>NDM-1</sup>, *bla*<sup>IMP</sup>, *bla*<sup>VIM</sup>, *bla*<sup>SIM</sup>, *bla*<sup>SPM</sup>, *bla*<sup>GIM</sup>, and *bla*OXA-48 was determined.

**RESULTS:** Of 559 isolates included in the study, a total of 102 isolates were resistant to carbapenem that accounted for overall 18.24% (102/559) prevalence. Of these 102 isolates, 89 (87.25%) isolates were positive by DDST and 95 (93.17%) isolates were positive by CDST. Of 102 CRPA isolates, *bla*<sup>VIM</sup> was detected in 30 isolates (30/102, 29.1%) followed by *bla*NDM-1 in 29 (29/102, 28.4%) isolates and *bla*SIM and *bla*GIM in 6 isolates each (6/102, 5.8%). A combination of two carbapenemase genes was detected in 12 isolates, with six (6/102, 5.88%) CRPA isolates harboring with both *bla*VIM and *bla*NDM-1 genes. Four isolates were found to harbor a combination of three carbapenem-resistant genes.

**CONCLUSION:** A high rate of carbapenemase production was observed in *P. aeruginosa*. Coproducers of multiple carbapenemases are also a cause of concern. An in-depth understanding of molecular mechanisms of resistance will be helpful in optimizing patient management and hospital infection control.

**Key words:** Carbapenemases, genotypic methods, phenotypic methods, *Pseudomonas aeruginosa*

**Introduction**

Carbapenem-resistant *Pseudomonas aeruginosa* (CRPA) is listed as an organism posing a serious threat by the Centers for Disease Control and Prevention. In India, up to 40% *P. aeruginosa* isolates are shown to be carbapenem resistant. Carbapenem resistance in *P. aeruginosa* has shown to be multifactorial, including production of carbapenemase, overexpression/overproduction of efflux pump, and porin loss. Carbapenemases in *P. aeruginosa* belong to three molecular classes, for example, class A (*bla*KPC), class B (*bla*IMP, *bla*VIM, *bla*NDM, *bla*SIM, *bla*GIM, and *bla*SPM), and class D (*bla*OXA-48). The molecular mechanism of carbapenem resistance in *P. aeruginosa* has been studied in very few studies restricted to North and South India.

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The aim of the present study was to determine the production of carbapenemase enzymes in CRPA isolates by phenotypic methods (double-disk synergy test [DDST] and combined disk synergy test [CDST]) and to determine the prevalence of \(\text{bla}_{\text{KPC}}, \text{bla}_{\text{NDM}-1}, \text{bla}_{\text{IMP}}, \text{bla}_{\text{VIM}}, \text{bla}_{\text{SPM}}, \text{bla}_{\text{GIM}}, \) and \(\text{bla}_{\text{OXA}-48}\). The susceptibility profile of \(P.\ aeruginosa\) to other antipseudomonal antibiotics was also investigated.

**Materials and Methods**

This study was conducted at the microbiology department of a tertiary care teaching hospital from January 2017 to May 2018. All consecutive nonduplicate isolates of \(P.\ aeruginosa\) from different clinical samples (blood, respiratory sample, pus, urine, sterile body fluids, and other samples) resistant to meropenem and/or imipenem by disk diffusion method were included in the study. The study was conducted after ethical clearance from the Institutional Ethics Committee (ref no-IEC/ AIIMS BBSR/PG Thesis/2017–18/3).

The initial isolation and identification of \(P.\ aeruginosa\) were carried out by standard bacteriological procedures. The following antimicrobials were tested by disk diffusion: ceftazidime (30 \(\mu\)g), cefepime (30 \(\mu\)g), piparicillin–tazobactam (100 \(\mu\)g/10 \(\mu\)g), ticarcillin–clavulanate (75 \(\mu\)g/10 \(\mu\)g), imipenem (10 \(\mu\)g), meropenem (10 \(\mu\)g), doripenem (10 \(\mu\)g), amikacin (30 \(\mu\)g), gentamicin (10 \(\mu\)g), netilmicin (30 \(\mu\)g), tobramycin (10 \(\mu\)g), ciprofloxacin (5 \(\mu\)g), and levofloxacin (5 \(\mu\)g) (HiMedia Laboratories Pvt. Ltd., Mumbai, Maharashtra, India). The antimicrobial susceptibility was interpreted as per the Clinical and Laboratory Standards Institute 2017 guidelines using the Clinical and Laboratory Standards Institute 2017 guidelines (KAPA Biosystems, Cape Town, South Africa). The first set of multiplex PCR detected \(\text{bla}_{\text{IMP}}, \text{bla}_{\text{VIM}}, \text{bla}_{\text{SPM}}, \text{bla}_{\text{GIM}}, \text{bla}_{\text{OXA}-48}\), and \(\text{bla}_{\text{KPC}}\). The PCR reactions consisted of a final reaction volume of 25 \(\mu\)l containing 5 \(\mu\)l of template DNA, 12.5 \(\mu\)l of 2X KAPA2G Fast Multiplex PCR Kit, and 0.5 \(\mu\)l of each oligonucleotide primer. Amplification was done with initial denaturation step at 95°C for 3 min, followed by denaturation at 95°C for 15 s, annealing at 60°C for 30 s, extension at 72°C for 30 s, and a final extension step at 72°C for 7 min. The product was visualized by 1.5% agarose gel electrophoresis, stained with ethidium bromide, and was viewed under automated gel documentation system (Syngene G: Box, Syngene, Cambridge, U.K.) using ultraviolet illumination. The primer sequences and the product sizes\(^{[7,8]}\) are given in Table 1.

**Statistical analysis**

The sensitivity and specificity of CDST and DDST were calculated by forming 2 \(\times\) 2 contingency tables and genotypic tests being considered as a gold standard.

| Table 1: Primers used for multiplex polymerase chain reaction for various carbapenemase genes with their product size |
|---------------------------------------------------------------|
| **Primer** | **Primer sequence (5’-3’)** | **Product size (bp)** |
| IMP family-F | GGA ATA GAG TGG TCT ATT AAY TCT C | 188 |
| IMP family-RCCA | AAC YAC TAS GTT ATC T | |
| VIM family-F | GAT GGT TGG TCG CAT A | 390 |
| VIM family-RGGA | ATG CCG ACC AGC ACC AG | |
| GIM-1-F | TCG ACA CAC CTT GGT CTG AA | 477 |
| GIM-1-R | AAC TTC CAA CTT TTC CAT GC | |
| SPM-1A-F | AAA ATC TGG GTA CCG AAA CG | 271 |
| SPM-1A-R | ACA TTA TCC GCT GGA ACA AG | |
| SIM-1-F | TAC AAG GGA TGC GGC ATG G | 571 |
| SIM-1-R | TAA TGG CCT GTC GTC ATG T | |
| NDM-1 F | ACC GCC TGG ACC CAT GAC CA | 264 |
| NDM-1 R | GCC AAA GGT GGC GCC GCT T | |
| OXA-48-F | TTGGTGGGCAGTGGTGCCTC | 744 |
| OXA-48-R | GAGGCCTTCTTTTGTGATGCC | |
| KPC-F | ATGTCACTGTATCGGCCTC | 893 |
| KPC-R | GTCAGCGGCTTACTGCC | |

Isolates with imipenem/meropenem/doripenem MIC \(\geq 8 \mu\)g/ml by agar dilution and E-test were screened for carbapenemase production by DDST and CDST by the following methods.

- **Double-disk synergy test:**\(^{[5]}\) As described by Lee et al., this test was carried out using ethylenediaminetetraacetic acid (EDTA) disk (10 \(\mu\)l of 0.5 M EDTA) and the imipenem disk (10 \(\mu\)g) using the standard method.\(^{[5]}\) The production of large synergistic inhibitory zones was interpreted as test positive for metallo-beta-lactamase (MBL)

- **Combined disk synergy test:**\(^{[6]}\) As described by Yong et al., this test was carried out using two imipenem commercial disks with and without EDTA (10 \(\mu\)l of 0.5 M EDTA).\(^{[6]}\) The zone of inhibition diameter with imipenem + EDTA disk \(\geq 7\) mm than the imipenem disk alone was considered positive for MBL production.

**Molecular detection of carbapenemase gene**

Multiplex polymerase chain reaction (PCR) for the presence of \(\text{bla}_{\text{KPC}}, \text{bla}_{\text{NDM}-1}, \text{bla}_{\text{IMP}}, \text{bla}_{\text{VIM}}, \text{bla}_{\text{SPM}}, \text{bla}_{\text{GIM}}, \text{bla}_{\text{OXA}-48}\) was carried out according to the method published by Ellington et al.\(^{[7]}\) and Kumarasamy et al.\(^{[8]}\) Briefly, DNA extraction from \(P.\ aeruginosa\) isolates was carried out using QIAamp DNA Mini kit spin-column method (QIAGEN, Hilden, Germany) as per manufacturer’s instruction. Two sets of multiplex PCR were done using KAPA2G Fast Multiplex PCR Kit (2X) as per manufacturer’s instructions (Kapa Biosystems, Cape Town, South Africa). The first set of multiplex PCR detected \(\text{bla}_{\text{IMP}}, \text{bla}_{\text{VIM}}, \text{bla}_{\text{SPM}}, \text{bla}_{\text{GIM}}, \text{bla}_{\text{OXA}-48}\), and \(\text{bla}_{\text{KPC}}\). The PCR reactions consisted of a final reaction volume of 25 \(\mu\)l containing 5 \(\mu\)l of template DNA, 12.5 \(\mu\)l of 2X KAPA2G Fast Multiplex Master Mix, and 0.5 \(\mu\)l of each oligonucleotide primer. Amplification was done with initial denaturation step at 95°C for 3 min, followed by denaturation at 95°C for 15 s, annealing at 60°C for 30 s, extension at 72°C for 30 s, and a final extension step at 72°C for 7 min. The product was visualized by 1.5% agarose gel electrophoresis, stained with ethidium bromide, and was viewed under automated gel documentation system (Syngene G: Box, Syngene, Cambridge, U.K.) using ultraviolet illumination. The primer sequences and the product sizes\(^{[7,8]}\) are given in Table 1.
Results

A total of 559 consecutive nonduplicate isolates of *P. aeruginosa* were recovered from various clinical samples received in the department of microbiology during the study period (January 2017–May 2018). Of 559 isolates of *P. aeruginosa*, a total of 102 isolates (102/559, 18.2%) were resistant to carbapenem (MIC ≥8 µg/ml). Of these 102 isolates, 19 (19/102, 18.6%) were from various intensive care units, 24 (24/102, 23.5%) were from various outpatient departments, and rest 59 (59/102, 57.8%) were from various admitted patients. CRPA was isolated from pus (38/102, 37.2%), followed by urine (31/102, 30.3%), respiratory samples (25/102, 24.5%), blood (3/102, 2.9%), and other samples (5/102, 4.9%), respectively. Among the various antipseudomonal antibiotics tested in CRPA isolates, the maximum susceptibility was observed for piperacillin–tazobactam (17/102, 16.6%), followed by ceftazidime (12/102, 11.7%) and aminoglycosides (8/102, 7.8%). Ticarcillin–clavulanate with a percentage susceptibility of 2.9% (3/102) was found to be least susceptible. All CRPA isolates were found to be resistant to ciprofloxacin and levofloxacin. Of 102 CRPA isolates, CDST and DDST were positive in 95 (93.1%) and 89 (87.2%), respectively, as shown in Table 2.

![Figure 1: Isolate showing positive carbapenemase-producing *Pseudomonas aeruginosa* test with ≥ 7 mm increase in the zone of inhibition of imipenem + ethylenediaminetetraacetic acid as compared to imipenem alone](image)

Discussion

Carbapenem resistance was noted in 18.2% *P. aeruginosa* isolates in our study. In the Indian Council of Medical Research scoping document, the prevalence of CRPA isolates across Indian hospitals is shown to be 42%–47% in the years 2014 and 2015.[9] In a recent single-center study from North India, the prevalence of CRPA is shown to be 72.5% in admitted patients.[10] The relatively lower prevalence of CRPA in our study could be due to

| Type of carbapenem-resistant gene identified | Number of isolates (N=102) | CDST+ | CDST– | DDST+ | DDST– |
|---------------------------------------------|-----------------------------|-------|-------|-------|-------|
| blaNDM-1                                    | 19 (18.62)                  | 19    | 0     | 15    | 4     |
| blaVIM                                      | 17 (16.62)                  | 16    | 1     | 14    | 3     |
| blaSIM                                      | 6 (5.88)                    | 5     | 1     | 6     | 0     |
| blaGIM                                      | 6 (5.88)                    | 4     | 2     | 5     | 1     |
| blaVIM+blaNDM-1                             | 6 (5.88)                    | 6     | 0     | 5     | 1     |
| blaVIM+blaSIM                               | 3 (2.94)                    | 3     | 0     | 3     | 0     |
| blaVIM+blaGIM                              | 2 (1.96)                    | 2     | 0     | 2     | 0     |
| blaGIM+blaSPM                              | 1 (0.98)                    | 1     | 0     | 0     | 1     |
| blaVIM+blaGIM+blaSIM                       | 1 (0.98)                    | 0     | 1     | 1     | 0     |
| blaVIM+blaSPM+blaNDM-1                      | 1 (0.98)                    | 1     | 0     | 0     | 1     |
| blaVIM+blaGIM+blaNDM-1                      | 1 (0.98)                    | 1     | 0     | 1     | 0     |
| blaVIM+blaSPM+blaGIM+blaNDM-1               | 1 (0.98)                    | 1     | 0     | 1     | 0     |

Table 2: Distribution of various carbapenem-resistant genes in carbapenem-resistant *Pseudomonas aeruginosa* isolates (N=102)
Table 3: Comparison of different phenotypic test in metallo-beta-lactamase-producing Pseudomonas aeruginosa (n=102)

| Type of test | Positive (%) | Negative (%) | Sensitivity (%) |
|--------------|--------------|--------------|----------------|
| DDST test    | 89 (87.25)   | 13 (12.75)   | 81.35          |
| CDST test    | 95 (93.17)   | 7 (6.83)     | 92.18          |

DDST=Double-disk synergy test, CDST=Combined disk synergy test

the higher proportion of isolates being included from outpatient settings.

In our study, the sensitivity of DDST and CDST was found to be 81.35% and 92.18%, respectively (taking the presence of any MBL gene as a reference test). The sensitivity of CDST for MBL detection was shown to be 86.7% and specificity of 51.1% in a previous study by Peter et al. in 2014.[11] The higher sensitivity of CDST compared to DDST, as observed in our study, is also described previously.[12] In the present study, the prevalence of MBL was 15.74% by phenotypic method (CDST), whereas the prevalence by genotypic method for MBL was found as 10.19%. The concordance rate between CDST and genotypic method of MBL detection in our study was found to be 92.18%. The overall rate of concordance between phenotypic and genotypic tests for the detection of MBL was 98% in a study by Kazi et al.[13]

In our study, 62.74% CRPA isolates had at least one carbapenemase gene. blαVIM was the most prevalent gene in CRPA (30/64, 46.87%), followed by blαNDM-1 (29/64, 45.31%). blαVIM and blαNDM are shown to be the most prevalent carbapenemase gene in the majority of Indian studies.[2,3,12,14] In the study by Ellapan et al. in 2018, blαVIM and blαNDM were present in 23.1% and 17.3% of 156 CRPA isolates.[15] In another study by Mohanam and Menon in 2017, blαVIM and blαNDM were present in 32% and 27% of 213 CRPA isolates.[16] In a multicentric study from India, blαVIM and blαNDM were the most frequent carbapenemases in CRPA isolates with regional differences in the carbapenemase profile across the study sites.[2] The incidence of CRPA has increased worldwide, and they pose a potential risk for therapeutic failure with the empirical treatments currently in place. In this study, no blαKPC, blαIMP, and blαOXA-48 were identified. blαKPC and blαOXA-48 were also absent in 156 CRPA isolates analyzed by Ellapan et al.[19] In another multicentric study by Khurana et al., blαKPC and blαOXA-1 were present in 43% and 56% of CRPA isolates from North India but were absent in CRPA isolates collected from South India.[17] In the present study, 15 CRPA isolates had coexistence of two or three carbapenem-resistant genes. blαVIM + blαNDM-1 was the most common combination (6/102, 5.88%), followed by blαVIM + blαGIM (3/102, 2.94%). The coproduction of blαVIM + blαNDM-1 had previously been described in 7.1% CRPA isolates by Ellapan et al.[15] The coexistence of blαVIM + blαNDM-1 and blαKPC-2 and blαNDM-1 in CRPA isolates from India is also described previously by Paul et al.[18,19]

No carbapenem-resistant gene was detected in 38 (3/102, 37.25%) CRPA isolates in our study. Other carbapenem mechanisms such as efflux pump and porin loss could have contributed for carbapenemase resistance, which were not studied in the present study. Loss of OprD porin and overexpression of mexA gene is also found in studies from India either as a standalone mechanism or in association with the presence of blαVIM.[19]

In our study, fluoroquinolones did not have any activity against CRPA isolates. Polymyxin B and colistin were found to be susceptible in all isolates of P. aeruginosa. There are recent studies of colistin resistance in <5% P. aeruginosa isolates from India, which warrant judicious use of this last-resort antibiotic.[14] Among
beta-lactam and beta-lactamase inhibitor combination, the maximum susceptibility was observed for piperacillin–tazobactam (16.67%), followed by ceftazidime with 11.76% and 2.94% for ticarcillin–clavulanate which was found to be least susceptible. Carbenapenem-resistant and cephalosporin-susceptible P. aeruginosa is a rare phenotype. Previous carbenapenem therapy and decreased OprD expression and efflux system overexpression are cited as possible mechanisms of this phenotype. The clinical utility in this group is, however, debatable.[20]

**Conclusion**

A high rate of carbenapenem production was observed in *P. aeruginosa*. Coproducers of multiple carbenapenemases are also a cause of concern. An in-depth understanding of molecular mechanisms of resistance will be helpful in optimizing patient management and hospital infection control.

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**Conflicts of interest**

There are no conflicts of interest.

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