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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}KPC, ^{bla}NDM-1, ^{bla}IMP, ^{bla}VIM, ^{bla}SIM, ^{bla}SPM, ^{bla}GIM, 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}VIM 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.^[1] In India, up to 40% *P. aeruginosa* isolates are shown to be carbapenem resistant.^[2] 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) genes. The molecular mechanism of carbapenem resistance in *P. aeruginosa* has been studied in very few studies restricted to North and South India.^[2,3]

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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 ^{bla}KPC, ^{bla}NDM-1, ^{bla}IMP, ^{bla}VIM, ^{bla}SIM, ^{bla}SPM, ^{bla}GIM, and ^{bla}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 µg), cefepime (30 µg), piperacillin-tazobactam (100 µg/10 µg), ticarcillin-clavulanate (75 µg/10 µg), imipenem (10 µg), meropenem (10 µg), doripenem (10 µg), amikacin (30 µg), gentamicin (10 µg), netilmicin (30 µg), tobramycin (10 µg), ciprofloxacin (5 µg), and levofloxacin (5 µg) (HiMedia Laboratories Pvt. Ltd., Mumbai, Maharashtra, India). The antimicrobial susceptibility was interpreted as per the Clinical and Laboratory Standards Institute 2017 guidelines using *P. aeruginosa* ATCC strain 27853 as quality control strain.^[4] In addition, imipenem and meropenem minimum inhibitory concentration was determined by agar dilution and E-test (HiMedia Laboratories Pvt. Ltd., Mumbai, Maharashtra, India).

Isolates with imipenem/meropenem/doripenem MIC ≥ 8 µg/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 µl of 0.5 M EDTA) and the imipenem disk (10 µg) 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 µl of 0.5 M EDTA).^[6] The zone of inhibition diameter with imipenem + EDTA disk ≥ 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 ^{bla}KPC, ^{bla}NDM-1, ^{bla}IMP, ^{bla}VIM, ^{bla}SIM, ^{bla}SPM, ^{bla}GIM, and ^{bla}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 ^{bla}IMP, VIM, GIM, SPM, and SIM and the second set of multiplex PCR detected ^{bla}NDM-1, OXA-48, and KPC. The PCR reactions consisted of a final reaction volume of 25 µl containing 5 µl of template DNA, 12.5 µl of 2X KAPA2G Fast Multiplex Master Mix, and 0.5 µl 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 × 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 CTT AAY TCT C	188
IMP family-R	CCA AAC YAC TAS GTT ATC T	
VIM family-F	GAT GGT GTT TGG TCG CAT A	390
VIM family-R	CGA ATG CGC AGC ACC AG	
GIM-1-F	TCG ACA CAC CTT GGT CTG AA	477
GIM-1-R	AAC TTC CAA CTT TGC CAT GC	
SPM-1A-F	AAA ATC TGG GTA CGC AAA CG	271
SPM-1A-R	ACA TTA TCC GCT GGA ACA GG	
SIM-1-F	TAC AAG GGA TTC GGC ATC G	571
SIM-1-R	TAA TGG CCT GTT CCC ATG TG	
NDM-1 F	ACC GCC TGG ACC GAT GAC CA	264
NDM-1 R	GCC AAA GTT GGG CGC GGT TG	
OXA-48-F	TTGGTGGCATCGATTATCGG	744
OXA-48-R	GAGCACTTCTTTGTGATGGC	
KPC-F	ATGTCACGTATCGCCGTCT	893
KPC-R	TTTTCAGAGCCTTACTGCC	

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 $\mu\text{g}/\text{ml}$). 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. Figures 1 and 2 depict the positive CDST and DDST, respectively.

Of 102 CRPA isolates, *bla*VIM 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 were 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 [Figure 3]. No CRPA isolates were found to possess *bla*IMP, *bla*KPC, and *bla*OXA-48. The distribution of various carbapenemase genes in 102 CRPA isolates

is provided in Table 2. Five isolates of CRPA that were positive for NDM-1 were sent for sequencing by Sanger's method to Eurofins Genomics India Pvt. Ltd., Bengaluru, Karnataka, India, and interpreted using BLAST software (NIH, USA), of which three were NDM-1 and two were found to be NDM-5. The sensitivity of DDST and CDST was found calculated taking the presence of any MBL gene as a reference test and was found to be 81.35% and 92.18%, respectively, as shown in Table 3.

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



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

Table 2: Distribution of various carbapenem-resistant genes in carbapenem-resistant *Pseudomonas aeruginosa* isolates ($n=102$)

Type of carbapenemase gene identified	Number of isolates (%), ($n=102$)	CDST+	CDST–	DDST+	DDST–
<i>bla</i> NDM-1	19 (18.62)	19	0	15	4
<i>bla</i> VIM	17 (16.62)	16	1	14	3
<i>bla</i> SIM	6 (5.88)	5	1	6	0
<i>bla</i> GIM	6 (5.88)	4	2	5	1
<i>bla</i> VIM+ <i>bla</i> NDM-1	6 (5.88)	6	0	5	1
<i>bla</i> VIM+ <i>bla</i> GIM	3 (2.94)	3	0	3	0
<i>bla</i> GIM+ <i>bla</i> NDM-1	2 (1.96)	2	0	2	0
<i>bla</i> GIM+ <i>bla</i> SPM	1 (0.98)	1	0	0	1
<i>bla</i> VIM+ <i>bla</i> GIM+ <i>bla</i> SIM	1 (0.98)	0	1	1	0
<i>bla</i> VIM+ <i>bla</i> SPM+ <i>bla</i> NDM-1	1 (0.98)	1	0	0	1
<i>bla</i> VIM+ <i>bla</i> GIM+ <i>bla</i> NDM-1	1 (0.98)	1	0	1	0
<i>bla</i> VIM+ <i>bla</i> GIM+ <i>bla</i> SPM	1 (0.98)	1	0	1	0
Total	64	59	5	53	11

CDST=Combined disk synergy test

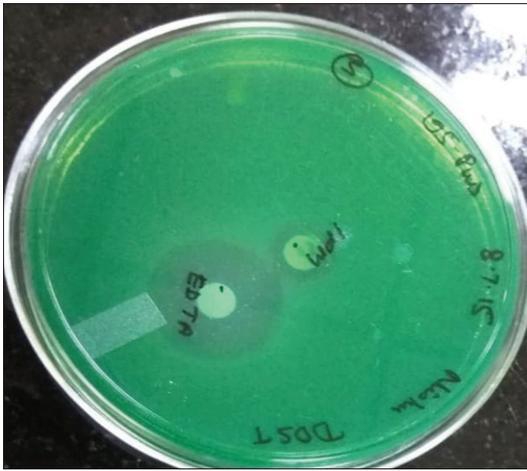


Figure 2: Isolate showing positive double-disk synergy test with enhanced zone of inhibition around ethylenediaminetetraacetic acid as compared to imipenem

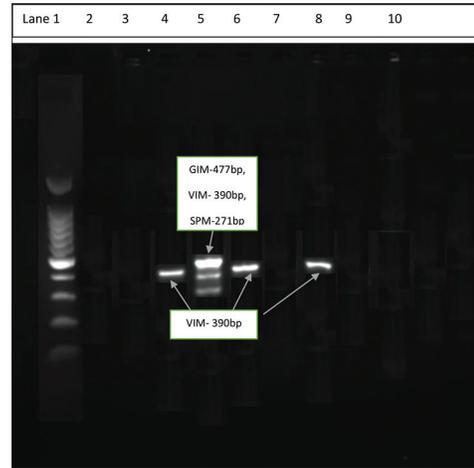


Figure 3: Multiplex polymerase chain reaction - Lane 1 showing 100 bp ladder; Lanes 4, 6, and 8 showing ^{bla}VIM-390 bp; and Lane 5 showing ^{bla}GIM + ^{bla}VIM + ^{bla}SPM

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. ^{bla}VIM was the most prevalent gene in CRPA (30/64, 46.87%), followed by ^{bla}NDM-1 (29/64, 45.31%). ^{bla}VIM and ^{bla}NDM 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, ^{bla}VIM and ^{bla}NDM were present in 23.1% and 17.3% of 156 CRPA isolates.^[15] In another study by Mohanam and Menon in 2017, ^{bla}VIM and ^{bla}NDM were present in 32% and 27% of 213 CRPA isolates.^[16] In a multicentric study from India, ^{bla}VIM and ^{bla}NDM 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 ^{bla}KPC, ^{bla}IMP, and ^{bla}OXA-48 were identified. ^{bla}KPC and ^{bla}OXA-48 were also absent in 156 CRPA isolates analyzed by Ellapan *et al.*^[15] In another multicentric study by Khurana *et al.*, ^{bla}KPC and ^{bla}OXA-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. ^{bla}VIM + ^{bla}NDM-1 was the most common combination (6/102, 5.88%), followed by ^{bla}VIM + ^{bla}GIM (3/102, 2.94%). The coproduction of ^{bla}VIM + ^{bla}NDM-1 had previously been described in 7.1% CRPA isolates by Ellapan *et al.*^[15] The coexistence of ^{bla}VIM + ^{bla}NDM-1 and ^{bla}KPC-2 and ^{bla}NDM-1 in CRPA isolates from India is also described previously by Paul *et al.*^[18,19]

No carbapenem-resistant gene was detected in 38 (38/102, 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 ^{bla}VIM.^[15]

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. Carbapenem-resistant and cephalosporin-susceptible *P. aeruginosa* is a rare phenotype. Previous carbapenem 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 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.

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Conflicts of interest

There are no conflicts of interest.

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