ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES
Knowledge to Innovation

Vol 16, Issue 8, 2023

Online - 2455-3891
Print - 0974-2441

Research Article

COMPARISON OF DOUBLE-DISC SYNERGY, COMBINED DISC DIFFUSION, AND E TEST FOR METALLO-BETA-LACTAMASE DETECTION IN *PSEUDOMONAS AERUGINOSA* IN A TERTIARY CARE HOSPITAL

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Received: 04 March 2023, Revised and Accepted: 20 April 2023

ABSTRACT

Objective: *Pseudomonas aeruginosa* is an important opportunistic pathogen associated with nosocomial infections. The metallo-beta-lactamases (MBLs) are an important class of carbapenemases which are predominantly produced by *P. aeruginosa*. This study is aimed to study the prevalence of MBLs among imipenem (IMP)-resistant *P. aeruginosa* and to evaluate three different methods of screening and detecting MBLs produced by *P. aeruginosa*.

Methods: 100 isolates of *P. aeruginosa* were obtained from various clinical specimens, including pus, wound, sputum, urine, body fluids, and ET tips received at the laboratory from July 2021 to December 2021. Screening for MBL production among IMP-resistant *P. aeruginosa* was done by double-disc synergy test and combined disc test. Confirmation of MBL production was done by E-test.

Results: Of the 100 *P. aeruginosa* isolates, 16 were IMP resistant, of which 16 are MBL producers. Most of the samples were obtained from 50 to 60 years followed by 40–50 and 60–70 were pus (50%) followed by sputum (20%), urine (15%), and body fluids (5%). Of the 100 isolates, 66 were isolated from females and 34 from males. Pus samples showed most MBL-producing isolates, accounting for 60.5%. MBL producers accounted for 90% of IMP-resistant cases using combined disc method and 100% using MBL E-strip test. By comparison, double-disc synergy test (DDST) retrieved 45% of *Pseudomonas* MBL producers.

Conclusion: This study found moderate prevalence of *Pseudomonas* MBL producers (16/100). The study supports the use of combined disc-diffusion test and DDST for screening and confirming MBL producers of *P. aeruginosa* by E test where polymerase chain reaction is not available.

Keywords: *Pseudomonas aeruginosa*, Imipenem-resistance, Metallo-beta-lactamases.

 DOI: http://dx.doi.org/10.22159/ajpcr.2023v16i8.47652. Journal homepage: https://innovareacademics.in/journals/index.php/ajpcr

INTRODUCTION

Pseudomonas aeruginosa is an important opportunistic pathogen associated with nosocomial infections and prevalent among burn wounds, skin and soft-tissue infections, septicemia, pneumonia and cystic fibrosis, organ transplants, and acute leukemia. *P. aeruginosa* resistant to carbapenems was currently reported increasingly and it is mediated mainly by metallo-beta-lactamases (MBL) production [1,2]. Overuse of multiple antibiotics, comorbidities, prolonged intensive care unit stay, intervention procedures such as mechanical ventilation, indwelling catheterization, total parenteral nutrition, and immunocompromised status of patients were the predisposing factors for the emergence of drug resistance in *P. aeruginosa* [3].

The MBLs are an important class of carbapenemases which are predominantly produced by *P. aeruginosa*. They belong to Ambler's class B and Bush–Jacoby–Medeiros Group 3 which hydrolyze all beta-lactam agents by utilizing Zinc at active site for activity [3]. The first MBL was from *Bacillus cereus* in the 1960s, and since then, 18 MBLs have been described in different Gram-negative bacteria from different regions of the world such as Asia, Europe, Australia, South America, and North America [4]. They are the most troublesome beta-lactamases because of their ability to confer resistance to carbapenems and all the beta-lactams (with the exception of aztreonam) and usually to aminoglycosides and quinolones. The metallo-beta-enzymes (imipenem [IMP], VIM, Sao Paulo MBL, German imipenemase types) are the most clinically significant carbapenemases [5].

P. aeruginosa possessing MBLs comprises nearly 20% of all nosocomial isolates in many countries. In India, the prevalence of MBL production in *P. aeruginosa* differs from one region to another between 7% and 65% [6]. In India, only blaVIM and NDM-1 have been reported in *P. aeruginosa*. There are various phenotypic methods performed for screening of MBL production by *P. aeruginosa*. These tests include the double-disc synergy tests using ethylenediaminetetraacetic acid (EDTA) with IMP or ceftazidime (CAZ), 2-mercaptopropionic acid with CAZ or IMP, Hodge test, combined disc-diffusion test (CDDT) and using EDTA with CAZ or IMP, the MBL E test, and microdilution method using EDTA and 1,10-phenanthroline with IMP. The present study aimed to describe the prevalence of MBL producers among IMP-resistant *P. aeruginosa* in a tertiary care hospital, Trichy, India. The objective of the present study was to evaluate three different methods of screening and detecting MBL-producing *P. aeruginosa* such as CDDT, double-disc synergy tests (DDST), and confirmatory MBL E-strip test [7].

METHODS

A prospective hospital-based descriptive study was conducted at Trichy SRM Medical College Hospital and Research Center, Trichy, during the study period (July 2021–December 2021). 100 isolates of *P. aeruginosa* based on clinical significance as pathogen were studied for detection of prevalence and MBL-producing isolates.

Inclusion criteria

1. Isolates from all clinical samples
2. Patients of all ages and both sexes
3. Isolates showing resistance to IMP were only tested for the production of MBLs.

Exclusion criteria

1. Mixed growth of >3 types (contaminated samples)
2. Isolates from improperly collected samples
3. Isolates sensitive for third-generation cephalosporins were not tested for MBLs.

Methodology

All the samples were collected under aseptic precautions using standard procedures and microbiologically processed according to standard guidelines. Direct smears with Gram's stain were examined for the presence of pus cells, epithelial cells, and microbial flora. Gram-stained smears revealed Gram-negative bacteria and pus cells. Specimens were inoculated and incubated in nutrient agar, blood agar, and MacConkey agar for 16–24 h and observed for colony morphology, pigment, odor, hemolysis, and lactose fermentation, and the colonies are further identified by biochemical tests using standard methods [8].

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was done by Kirby–Bauer disc-diffusion method using commercially available discs procured from HiMedia, Mumbai, India. The diameter of the zone of inhibition was measured and interpreted as per clinical and laboratory standard institute (CLSI) guidelines 2021 [9]. The antibiotics discs used in the study were cotrimoxazole (25 mcg), ciprofloxacin (5 mcg), gentamycin (10 mcg), amikacin (30 mcg), CAZ (30 mcg), piperacillin/tazobactam (100/10 mcg), IMP (10 mcg), aztreonam (30 mcg), polymyxin (50 mcg), and colistin (10 mcg). *P. aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922 and *Enterococcus faecalis* ATCC 29212 were used as quality control strains used according to the CLSI guidelines.

Methods for detection of MBL production

IMP-EDTA CDST

The IMP-resistant isolates were subjected to MBL screening by CDDT. The 0.5 McFarland standardized isolates were inoculated onto Mueller–Hinton agar plates as per the CLSI recommendations. A 0.5 M EDTA solution was prepared by dissolving 18.61 g of EDTA in 100 mL distilled water and pH adjusted by NaoH. The prepared EDTA solution was autoclaved. Two discs of IMP (10 mcg) were placed in plate and 10 µL of EDTA solution was added to one of the IMP discs to obtain a concentration of 750 mcg. The zone of inhibition of both IPM and IPM-EDTA discs was compared after incubation in air for 16–18 h at 35°C. If there was an increase in zone of inhibition with IPM-EDTA disc of ≥7 mm than IPM disc alone, it was considered as MBL positive.

IMP DDST

The isolates that were resistant to IMP (10 mcg) were subjected to MBL detection by DDST. Isolates were inoculated onto Mueller–Hinton agar plates recommended as per the CLSI guidelines. An IMP disc (10 mcg) is placed 20 mm center to center from a blank disc containing 10 µL of 0.5 M EDTA (750 µg). If there was enhancement of zone of inhibition in area between IMP and EDTA than the far side of the drug, it was considered as MBL positive.

MBL E-test

The IMP-resistant 0.5 Mac Farland standardized isolates were inoculated onto Mueller–Hinton agar plate and once dried, an E-test MBL strip is applied onto plate which is then incubated at 37°C for 16–18 h to detect the presence of MBL. The E-test MBL strip containing double-sided seven-dilution range of IPM (4–256 µg/mL) and IPM (1–64 µg/mL) in combination with a fixed concentration of EDTA was used. A reduction in the IMP MIC in the presence of EDTA of greater than or equal to eight-fold (IP/IPI=8) is interpreted as indicating MBL activity.

The data were collected and entered in Excel sheet and statistically analyzed by SPSS version 26 with mean, median, and mode.

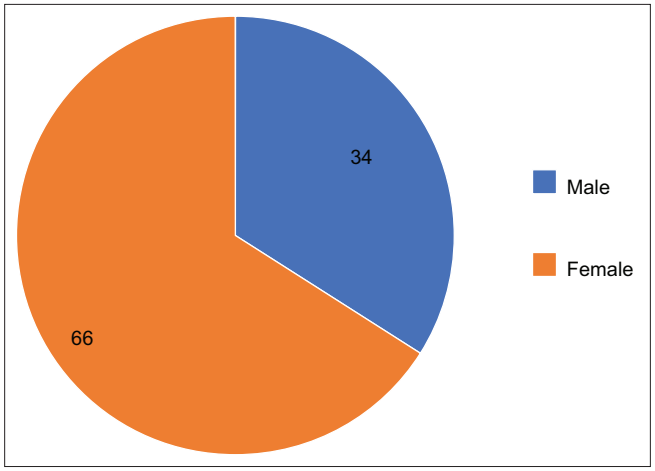

Fig. 1: Gender-wise distribution of isolates

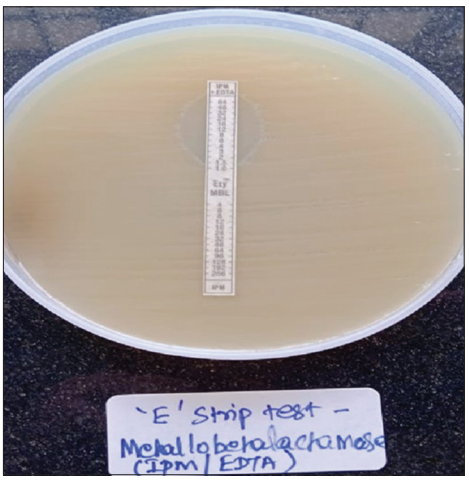

Fig. 2: Metallo-beta-lactamases E-strip test: MIC ratio of IP (Imipenem)/IPI (imipenem ethylenediaminetetraacetic acid) of >8 or >3 log2 dilution

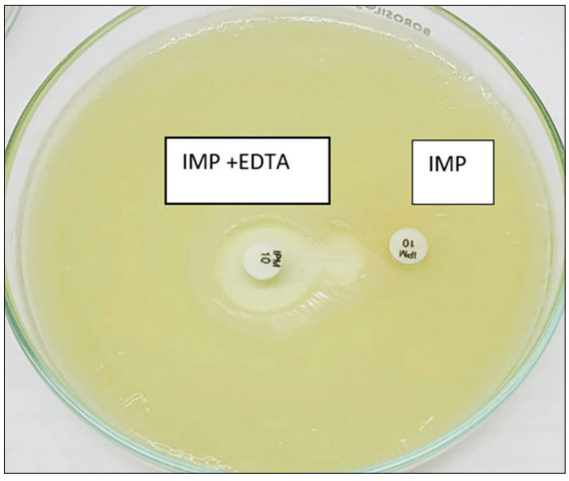

Fig. 3: Combined disc-diffusion test imipenem + ethylenediaminetetraacetic acid for metallo-beta-lactamases detection

RESULTS

100 isolates of *P. aeruginosa* obtained from various samples based on the clinical significance as pathogen were tested for detecting IMP

resistance and MBL production by phenotypic tests. The results were tabulated as follows.

Maximum isolates were in the age group 51–60 years (26%) followed by 41–50 years (25%) and the least number of isolates from 1 to 10 years (1%) and 81–90 years (1%).

Of the 100 *P. aeruginosa* isolates, 66 isolates were from females and 34 from males with male: female ratio 1:1.9.

Majority of isolates were from pus including swabs (55%) followed by sputum (25%), urine (10%), ET tip (3%), body fluids (5%), and blood (2%) in the present study.

Isolates were more sensitive to amikacin (95%) and piperacillin-tazobactam (91%) followed by ciprofloxacin (90%) and more resistant to imipenem (16%), followed by ceftazidime (15%) and cotrimoxazole (14%) in the present study.

In the present study, MBL producers accounted for 90% (n=14) of IMP-resistant cases (n=16) using combined disc method and 100% (n=16) using MBL E-strip test. And also, DDST retrieved 45% (n=7) of Pseudomonas MBL producers. MBL E-test was more sensitive than CDDT and DDST in the present study.

DISCUSSION

P. aeruginosa is a leading cause of health care-associated infections particularly multidrug-resistant isolates outbreaks. *Pseudomonas* infection is a cause of major concern for treating clinicians because of their multiple mechanisms for resistance which may be intrinsic as well as acquired. Acquired resistance was mainly due to carbapenemases particularly MBL in *P. aeruginosa*.

Moreover, the main problem arising with MBL is the development of unrivaled broad-spectrum resistance because of the location of MBL genes on plasmids which also contain genes encoding other antibiotic resistance determinants, i.e., aminoglycosides resistance genes. Mostly, these MBL-positive isolates are multidrug resistant including beta-lactams, aminoglycosides, and fluoroquinolones leaving the last treatment option to potentially toxic polymyxin B and colistin [10]. Therefore, various screening tests were employed to detect MBL production in IMP-resistant *P. aeruginosa* isolates for avoiding development and dissemination of multidrug-resistant strains with limited therapeutic options [11].

In the present study, the number of *P. aeruginosa* isolates was bigger in the age group of 20–60 years (81%). A similar observation was done in a study conducted by Srinivas *et al*. at Srikakulam [12] which showed that the isolation of *P. aeruginosa* was more common in the age group of 21–60 years (66.67%). In the present study, maximum isolation of *P. aeruginosa* was from females than males with male-to-female ratio 1:1.9 which was in discordance with a study done by Radhika *et al*. [13] at India, which reported a male-to-female ratio of 2:1.

In the present study, pus including swabs constituted 62.5% of all specimens, followed by sputum (12%), urine (12.5%), and other blood and body fluids (6.25%). This was in concordance with study done by Rashid *et al*. [14] where 55.1% of *P. aeruginosa* were from pus samples.

In the present study, *P. aeruginosa* isolates were more sensitive to amikacin (95%) and piperacillin-tazobactam (91%) followed by ciprofloxacin (90%) which was concordant with a study done at Egypt by Mahmoud *et al*., 2013 [15] in which out of 54 clinical isolates of *P. aeruginosa*, 48 (81%) were sensitive to amikacin.

In the present study, the resistance to IMP was 16% and CAZ (15%), and least resistance was to piperacillin-tazobactam (9%) which is in concordance with studies done at Srinagar by Bashir *et al*. from June 2007 to May 2008, [10] and Angadi *et al*., 2012 [16] showing an IMP resistance of 21.6% and 13.42%, respectively.

In the present study, the prevalence of MBL in clinical isolates was 16%. Similar observations were made by a study done by Bashir *et al*. at Srinagar [10] which reported a prevalence of 11.66%. In this study, we have used three different phenotypic methods of screening for MBL production. In the combined disc test using IMP and EDTA, with a cut-off >7 mm, the positive and negative results were more clearly interpreted. The subjective interpretation of result would be the major disadvantages of DDST in some instances.

In the present study, all IMP-resistant isolates were screened for MBL production using CDDT, DDST, and MBL E-test which showed 90%, 45%, and 100%, respectively, which was accordance with a study conducted at Gujarat Pandya *et al*., 2011 [17] which reported CDST

Table 1: Age-wise distribution of isolates

Age	Frequency (%)
1–10	1 (1)
11–20	2 (2)
21–30	15 (15)
31–40	15 (15)
41–50	25 (25)
51–60	26 (26)
61–70	10 (10)
71–80	5 (5)
81–90	1 (1)
Total	100 (100)

Table 2: Sample-wise distribution of isolates

Samples	Frequency (%)
Pus (including swabs)	55 (55)
Sputum	25 (25)
ET aspirate	3 (3)
Urine	10 (10)
Body fluids	5 (5)
Blood	2 (2)
Total	100 (100)

Table 3: Antimicrobial sensitivity pattern of *Pseudomonas aeruginosa*

Antibiotic	Number of isolates resistant	Number of isolates sensitive	Resistant (%)	Sensitive (%)
Ceftazidime	15	85	15	85
Cefepime	12	88	12	88
Imipenem	16	84	16	84
Aztreonam	10	90	10	90
Piperacillin tazobactam	9	91	9	91
Amikacin	5	95	5	95
Gentamycin	10	90	10	90
Ciprofloxacin	9	91	9	91
Cotrimoxazole	14	86	14	86
Polymyxin B	0	0	0	0

Table 4: Evaluation of three phenotypic tests for screening and detection of metallo-beta-lactamases isolates

Test	Control *Pseudomonas aeruginosa* ATCC 27853	Number of MBL producers (%)
IMP-EDTA CDDT	Negative	14 (90)
DDST test	Negative	7 (45)
MBL E-strip test	Negative	16 (100)

MBL: Metallo-beta-lactamases, CDDT: Combined disc-diffusion test, DDST: Double-disc synergy tests, IMP: Imipenem, EDTA: Ethylenediaminetetraacetic acid

(96.3%) and DDST (81.48%). The combined disc method using IMP + EDTA was found to be superior to DDST (using IMP-EDTA) in other published studies (Yan *et al*., 2004). In the present study, the IMP-EDTA CDDT and MBL E-test were 90% and 100%, which was in accordance with a study done at AIIMS, New Delhi, India, Behera *et al*., 2008 [6] who found that both combined disc and E-test were equally sensitive for MBL detection.

In the present study, MBL E-strip test was found to be more sensitive to detect MBL in *P. aeruginosa* but other conflicting reports are also found in other published studies by Yan *et al*. 2004 [18]. MBL E-test has been found to be insensitive to detect carbapenem-sensitive MBL-carrying organisms. In the present study, we screened only carbapenem-resistant *P. aeruginosa*. Among the 16 MBL-producing isolates, we found that all 14 isolates found to be MBL positive by CDDT were also positive with the E test and 7 which were positive by the combined and negative by DDST were found to be MBL positive by E test.

The MBL E-test strip using a combination of a beta-lactam substrate and a beta-lactam/MBL inhibitor is specially designed to detect as many clinically significant MBL as possible. The MBL E-test strip (IP-IPE) can detect MBL, both chromosomally and plasmid mediated, in aerobic and anaerobic bacteria [19]. This novel method could be employed by the clinical laboratories to closely monitor the production of MBL in various clinically significant bacteria and perform drug resistance surveillance [20].

CONCLUSION

The present study showed a (1) moderate prevalence (16%) of Pseudomonas MBL producers (16/100) with 100% polymyxin susceptibility, (2) the E-test is very sensitive than CDDT and DDST for the detection of MBL in *P. aeruginosa* since it detected all MBL producers (negative in CDT and DDST) but practically impossible for all laboratories to perform the E-test due to availability and cost constraints. Hence, routinely simple screening test like CDDT will be done for large-scale monitoring of these emerging-resistant determinants, and (3) even though polymerase chain reaction (PCR) remains the gold standard test to validate the results, it is not feasible to perform in routine microbiology laboratory [21].

Our study highlighted that all IMP/meropenem-resistant *P. aeruginosa* isolates should be routinely screened for MBL production using CDDT and DDST screen test and confirmed by MBL E-tests in regions where PCR detection cannot be performed but care should be taken when interpreting phenotypic tests results which are based on inhibitor synergy.

Limitations of study

1. Absence of a PCR analysis for the validation of phenotypical methods
2. It is a single-centered study
3. Sample size is also limited.

Recommendations

1. Continued health education and strict adherence of patients, staffs to infection control activities such as hand hygiene and personal protective equipment usage
2. Sterilization and disinfection of equipment and devices used in patient care and proper biomedical waste disposal were needed to avoid emergence of multidrug-resistant organisms.
3. Judicious antimicrobial therapy and formulating antibiotic policy in institution for avoiding the dissemination of these multidrug-resistant strains.
4. Strengthening of antimicrobial stewardship program by many antimicrobial surveillance studies at national level for close monitoring of drug resistance.

ACKNOWLEDGMENT

I sincerely thank my Professor and head and all faculties of the Department of Microbiology, Trichy SRM Medical College Hospital and research center, Irungalur, Trichy, for extending their help and guidance in conducting the microbiological analysis for the present study.

AUTHORS CONTRIBUTIONS

A. Anu Priya and D. Varshini conceptualized the study. D. Varshini and J. Lalithambigai performed the experiments and analyzed the data. and A. Anu Priya and D. Varshini wrote the manuscript.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

FUNDING

There is no external funding.

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