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Detection of Metallo Beta Lactamases Production in *Pseudomonas Aeruginosa* by Phenotypic Methods from Clinical Isolates in a Tertiary Care Hospital

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Abstract: ***Introduction**: Pseudomonas aeruginosa causes infections majorly of skin, bone, heart, eye, and septicemia. It is a primary contributor to persistent pulmonary infections in children and young people with cystic fibrosis. In the 1980s, carbapenems were created and are utilized as a last option for treating severe infections caused by multidrug - resistant Gram - negative bacilli. Resistance to carbapenems was observed in 1990s. The primary factor contributing to resistance is the synthesis of metallo beta - lactamases (MBL), which hydrolyze all the beta - lactams, including carbapenems. This study aimed to identify Pseudomonas aeruginosa from clinical specimen and its antibiotic susceptibility pattern, also to detect metallo - beta - lactamase production by phenotypic methods, which would assist clinicians to treat patients and improves the curable rate of patients. **Materials & Methods**: It is a prospective study done on various clinical samples received to central laboratory in department of microbiology, BMCRC Ballari. A total of 100 clinical samples of Pseudomonas aeruginosa were isolated and identified based on gram staining, colony morphology, preliminary tests and biochemical reaction as per standard microbiological procedures followed by Antibiotic susceptibility testing was carried out by using the Kirby - Bauer disc diffusion method on Mueller - Hinton agar and interpreted according to the Clinical Laboratory Standards Institute guidelines (CLSI) of 2023. Later Detection of Metallo - Beta - Lactamases was performed by using the Combined Disk Test, Double Disk Synergy Test, and MBL - E Test. **Results**: This study examined a total of 100 isolated Pseudomonas aeruginosa were identified from clinical specimen for the development of metallo beta - lactamases. From the 100 samples analyzed, 14 (14%) exhibited resistance to Imipenem, with 11 (11%) identified as MBL producers and the remaining as non - MBL producers. MBL production in CDST and E - test was 9 (81.8%) and 8 (72.7%), respectively and it was 7 (63.6%) in case of DDST. **Conclusion**: The current study found a relatively high prevalence of Pseudomonas MBL producers with resistance to imipenem. A significant degree of resistance is observed with frequently utilized antibiotics, necessitating the usage of polymyxin in most instances due to the heightened susceptibility of Pseudomonas to polymyxin. Furthermore, our research endorses the utilization of E - tests, CDST, and DDST for the identification of Pseudomonas MBL producers in areas where PCR detection is impossible.***

Keywords: Metallo - beta lactamases, CDT, DDST, Multidrug Resistant, Pseudomonas aeruginosa.

1. Introduction

Pseudomonas aeruginosa is an aerobic, non - spore - forming, Gram - negative rod and motile by polar flagella. They are ubiquitous, predominantly saprophytic, inhabiting wet environments, water, and soil, with some acting as opportunistic pathogens in humans, primarily causing severe infections in those with immunocompromised individuals (1) and In healthy individuals, infection often arises from the breakdown or circumvention of the protective mechanisms afforded by the epidermis, as shown in burn victims, puncture wounds, intravenous drug users employing tainted needles, and ocular injuries associated with contaminated contact lenses. This may lead to infections of the skin, bone, heart, eye, and even septicaemia. It is a primary contributor to persistent pulmonary infections in children and young people with cystic fibrosis (2) .

In the 1980s, carbapenems were created and are utilized as a last option for treating severe infections caused by multidrug - resistant of Gram - negative bacilli (3) . Resistance to carbapenems was observed in the 1990s. Pseudomonas aeruginosa exhibits intrinsic resistance to numerous antimicrobial agents, attributable to the chromosomal expression of resistance genes, the production of antibiotic - degrading or inactivating enzymes such as extended - spectrum β - lactamases, carbapenemases, metallo - beta - lactamases, and AmpC - type cephalosporinases, as well as the presence of efflux pumps in the outer membranes and reduced cell wall permeability due to the loss of the OprD porin and modifications in the antibiotic - binding protein site (4) . The primary factor contributing to resistance is the synthesis of metallo beta - lactamases (MBL), which hydrolyze all beta - lactams, including carbapenems.

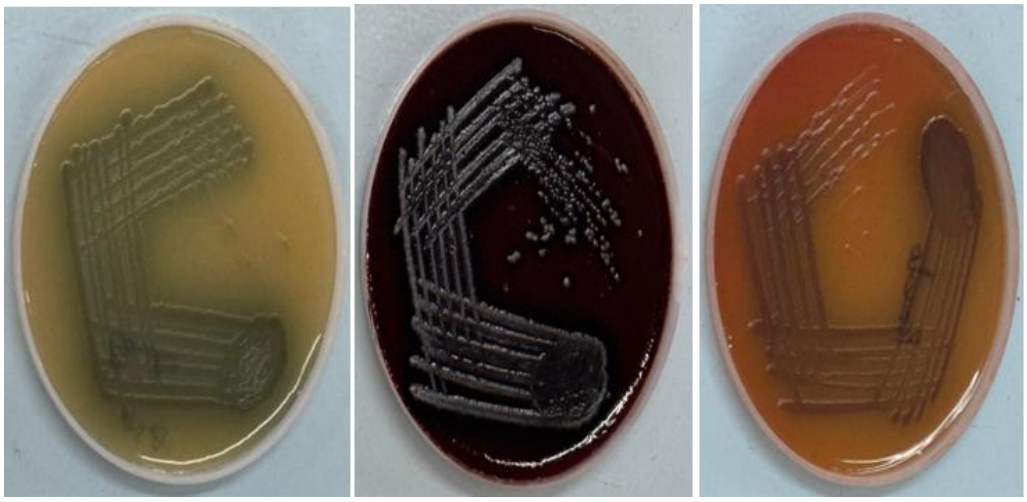
Multidrug - resistant of *Pseudomonas aeruginosa* strains causing infections are becoming more prevalent and causing a serious threat. Now days due to their increase in stability treatment for infections caused by multidrug resistant is becoming even more challenging. Therefore, this study mainly aimed to identify *Pseudomonas aeruginosa* from clinical specimen, and to interpret its antibiotic susceptibility pattern, and to detect metallo - beta - lactamase production by phenotypic methods, which would assist clinicians in improving the treatment and also the curable rate of patients.

2. Methodology

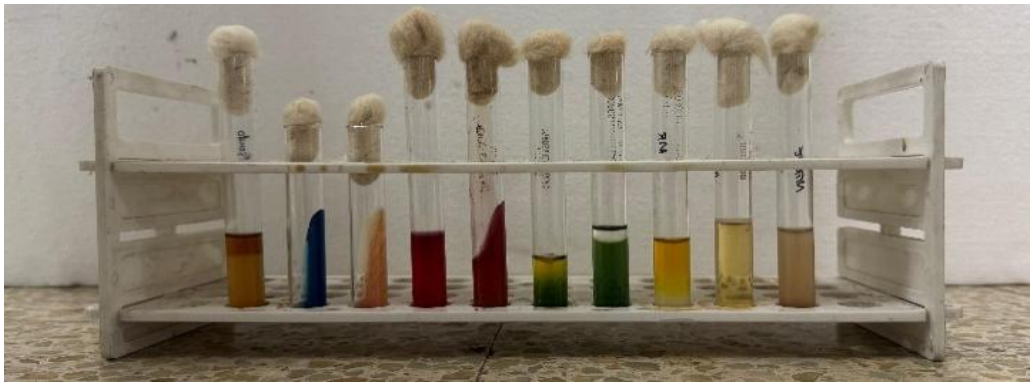
This was a prospective observational study conducted in the department of microbiology of a tertiary care hospital to detect metallo - beta - lactamase production. The isolates were collected over a period of one year. All samples were collected under strict aseptic precautions ensuing standard microbiology procedures and processed in the laboratory. All isolates of *Pseudomonas aeruginosa* were confirmed by preliminary and biochemical test from samples of inpatient departments were included in the study. Sample received in unsterile containers, any delay in transport and those samples from patients already on antimicrobial therapy were excluded from the study.

The collected samples were properly labelled and transferred safely to the microbiology laboratory with all the standard precautions. Without delaying Direct Gram's Staining was done for microscopic examination to observe pus cells and type of microbial flora followed by hanging drop motility testing was also done to detect motility of bacteria. All samples excluding blood were inoculated on nutrient agar, MacConkey agar and blood agar and was incubated for 18 to 24 hrs at 37 ° C. BHI broth was used only for blood culture, where a sample was inoculated and incubated at 37 °C for 48 hours. This broth was examined regularly for turbidity, and subcultured on blood agar and MacConkey agar. After incubation period based on the colony characteristics from culture media later processed by identification test to confirm pathogen.

Gram - negative isolates were identified by colony morphology. Preliminary tests and Biochemical reactions were performed as per standard microbiological procedures. Antibiotic susceptibility testing was carried out using the Kirby - Bauer discdiffusion method on Mueller - Hinton agar and interpreted according to the Clinical Laboratory Standards Institute guidelines of 2021. Detection of Metallo - Beta - Lactamases was performed using the Combined Disk Test, Double Disk Synergy Test, and MBL - E Test.



a) Nutrient agar b) Blood agar c) Mac conkey agar



d) Biochemical reactions

Figure 1 (a–d): showing *Pseudomonas aeruginosa* on Nutrient agar, Blood agar and Mac conkey agar. Biochemical reactions of *Pseudomonas aeruginosa*

Combined Disk Test

Test organisms were inoculated into Mueller Hinton agar plates, as per CLSI recommendations.0.5 M EDTA solution was prepared by dissolving 18.61 g in 100 mL of distilled water and correcting the pH to 8.0 using NaOH. The mixture underwent sterilization via autoclaving, two 10 µg Imipenem disks were placed on the plate, and suitable volumes of 10 µL of EDTA solution were introduced to one disk to achieve the target concentration (750 µg). The inhibition zones of the Imipenem and Imipenem - EDTA (Imp - EDTA) disks were analyzed by following 16–18 hours of incubation in ambient air at 35°C. In the combined disc test, an increase in inhibition zone of ≥ 7 mm with the Imipenem - EDTA disc indicated that the Imipenem disc alone was deemed MBL - positive.

Double Disk Synergy Test

The test strain was suspended to the turbidity of a McFarland no.0.5 standard and utilized to inoculate on Mueller–Hinton agar plate. Followed by drying process, later 10 - µg imipenem disc and a blank filter paper disc were placed 10 mm apart edge to edge. Subsequently, 10 µL of 0.5 M EDTA solution was added to the blank disc, yielding about 1.5 mg/disc. Following overnight incubation, the observation of an expanded zone of inhibition surrounding the blank disc was deemed indicative of a positive EDTA synergy test.

MBL E test (MIC test)

E - test MBL strips have a double - sided seven - dilution range of Imipenem IP (4–256 µg/mL) and IP (1–64 µg/mL) superimposed with a constant 36 gradient of EDTA. Single colonies were selected from overnight agar plates and suspended in 0.85% saline to achieve a turbidity of 0.5 McFarland standard. A sterile cotton swab was immersed in the inoculum suspension, and a inoculum was established on MHA plate. Excess moisture was permitted to be absorbed for about 15 minutes prior to the application of the E - test MBL strip. The plates were incubated for 16 to 18 hours at 37 degrees Celsius. The MIC endpoints were recorded at the intersections of the inhibition ellipses with the strip as shown in Figure 2. A two - fold drop in imipenem MIC=3 in the presence of EDTA was viewed as indicative of MBL formation.

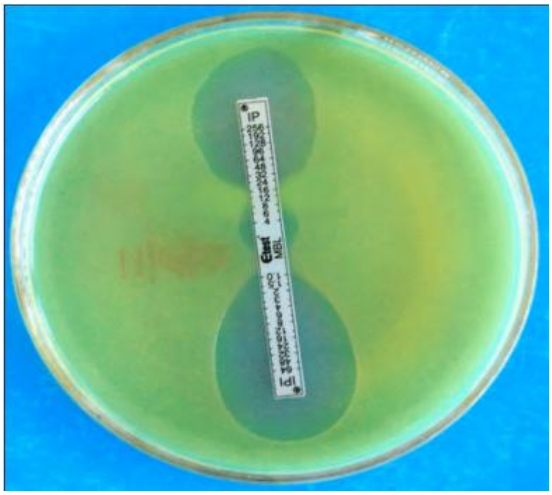


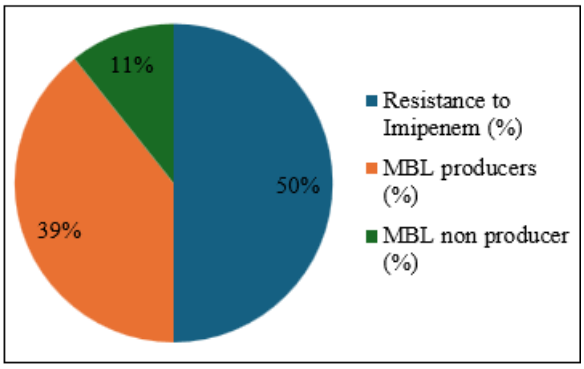
Figure 2: MBL E test showing two - fold drop in imipenem MIC=3 in the presence of EDTA indicating the MBL formation

3. Results

The present study examined a total 100 isolated *Pseudomonas aeruginosa* for the development of metallo beta - lactamases

Table 1: Distributing No. of cases by gender, susceptibility pattern, MBL and MBL non producers

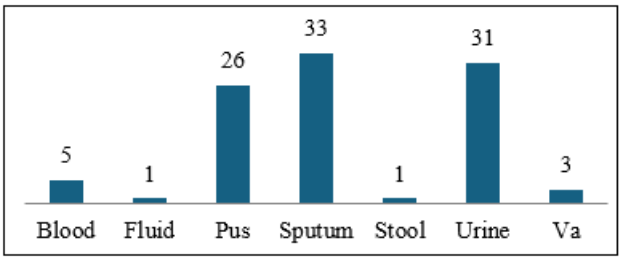
Gender	Sensitive to Imipenem (%)	Resistance to Imipenem (%)	MBL producers (%)	MBL non producer (%)	Total isolates (%)
Male	57 (57.0)	9 (9.0)	7 (7.0)	2 (2.0)	66 (66.0)
Female	29 (29.0)	5 (5.0)	4 (4.0)	1 (1.0)	34 (34.0)
Total	86 (86.0)	14 (14.0)	11 (11.0)	3 (3.0)	100 (100.0)



Out of 100 samples analysed, 14 exhibited resistance to Imipenem, with 11 identified as MBL producers and the remaining as non - MBL producers

Table 2: Number of *Pseudomonas aeruginosa* isolated from various clinical specimen

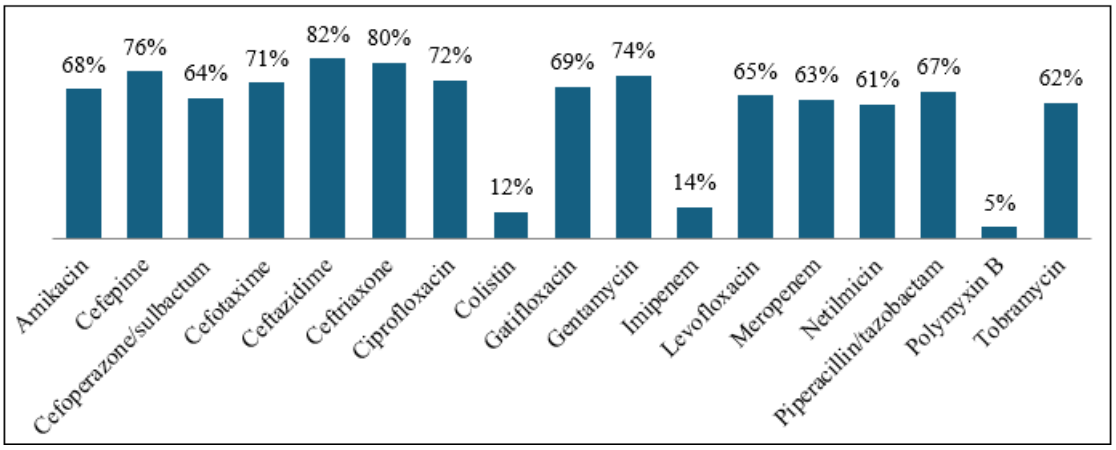
Specimen	Blood	Fluid	Pus	Sputum	Stool	Urine	Va
Frequency	5	1	26	33	1	31	3
Percentage	5.0	1.0	26.0	33.0	1.0	31.0	3.0



Majority of the specimens were sputum (33.0%), followed by urine (31.0%) and pus (26.0%)

Table 3: Resistance pattern of *P. aeruginosa*

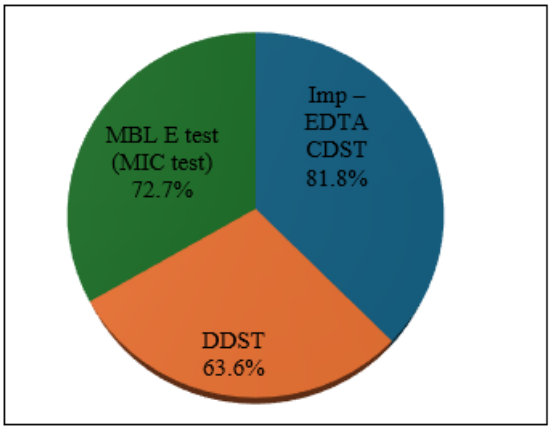
Antibiotic	Sensitive	Intermediate	Resistant
Amikacin	32 (32.0)	0 (0.0)	68 (68.0)
Cefepime	21 (21.0)	3 (3.0)	76 (76.0)
Cefotaxime	28 (28.0)	1 (1.0)	71 (71.0)
Ceftazidime	18 (18.0)	0 (0.0)	82 (82.0)
Ceftriaxone	20 (20.0)	0 (0.0)	80 (80.0)
Ciprofloxacin	27 (27.0)	1 (1.0)	72 (72.0)
Gentamycin	24 (24.0)	2 (2.0)	74 (74.0)
Imipenem	86 (86.0)	0.0	14 (14.0)
Levofloxacin	35 (35.0)	0 (0.0)	65 (65.0)
Meropenem	36 (36.0)	1 (1.0)	63 (63.0)
Piperacillin/tazobactam	31 (31.0)	2 (2.0)	67 (67.0)
Tobramycin	36 (36.0)	2 (2.0)	62 (62.0)
Colistin	88 (88.0)	0 (0.0)	12 (12.0)
Polymyxin B	95 (95.0)	0 (0.0)	5 (5.0)
Gatifloxacin	31 (31.0)	0 (0.0)	69 (69.0)
Cefoperazone/sulbactum	35 (35.0)	1 (1.0)	64 (64.0)
Netilmicin	39 (39.0)	0 (0.0)	61 (61.0)



The present investigation demonstrated the greatest resistance **to ceftazidime (82.0%), succeeded by ceftriaxone (80.0%) and cefepime (76.0%). Gentamicin (74.0%), Ciprofloxacin (72.0%), and Cefotaxime (71.0%)** exhibited significant resistance levels in this investigation (Table 3).

Table 4: MBL detection comparison by three different methods (n = 11)

Tests	Control	Number of MBL producers	Percentage Positivity (%)
Imp – EDTA CDST	Negative	9	81.8
DDST	Negative	7	63.6
MBL E test (MIC test)	Negative	8	72.7



Imipenem - resistant isolates for MBL production utilizing the combined disk synergy test (CDST), double - disk synergy test (DDST), and E - test. MBL production in CDST and E - test was 81.8% and 72.7%, respectively. CDST with Imipenem and EDTA, exhibiting a cut - off greater than 7 mm, effectively differentiated positive and negative outcomes. A significant drawback of DDST is its subjective interpretation. This study demonstrated that CDST was a sensitive method for detecting MBL than compared to DDST and E - test.

4. Discussion

Carbapenems are frequently employed as a last - resort antibiotic for treating infections caused by multidrug - resistant gram - negative bacilli, as they exhibit stability and are only affected by extended - spectrum and AmpC - β - lactamases. (5) However, there have been rising reports of resistance to these life - saving antimicrobials in Pseudomonas aeruginosa. (6, 7) Carbapenem resistance arises from diminished outer membrane permeability, enhanced efflux mechanisms, modifications of penicillin - binding proteins, and the synthesis of carbapenem - hydrolyzing enzymes, known as carbapenemases. The resistance arising from the synthesis of carbapenem - hydrolyzing enzymes, such as metallo - beta - lactamases (MBL), may be either chromosomally encoded or plasmid - mediated, hence presenting a risk of resistance dissemination by gene transfer among Gram - negative bacteria. (7) The presence of an MBL - positive isolate in a hospital environment is a therapeutic challenge and a significant concern for infection control management.

Even though MBL was first reported during the early 1960s, first plasmid - mediated MBL, IMP - 1 from *Pseudomonas aeruginosa* was reported from Japan during late 1991. (8) The first report of MBL - producing Pseudomonas aeruginosa in India occurred in 2002. (9) MBL isolation is critical in identifying the resistant strains and thereby provides the best treatment for those in need.

In this study the rate of isolation was more among males (66.0%) compared to females (34.0%). Similar findings were reported in the study conducted by Rakesh et al., where the male to female ratio was reported to be 1.56: 1. (10) Javiya et al., and Khan et al., also showed similar ratio in their studies. (11, 12) Thus a male preponderance is seen in the reporting of pseudomonas aeruginosa.

A diverse array of samples was collected for the examination of isolates. The predominant specimens were sputum (33.0%), followed by urine (31.0%) and pus (26.0%). In the research conducted by Radhika et al., wound swabs represented 40% of all specimens, followed by sputum at 18%, urine at 10%, and miscellaneous bodily fluids at 3%. (13) Wankhede et al., collected samples from specimens including wound swabs (44.11%) urine (25.29%), other body fluids (11.76%) and sputum (14%). (14) In the research by Arora et al., the predominant isolates were derived from urine (36.0%), followed by wound swabs (28%), blood (14%), sputum (10%), tracheal aspirate (8%), and other bodily fluids (4%). (15)

Considering the resistance to Imipenem in the current study, around 14.0% were resistant and 86.0% were sensitive.

Looking at the prevalence of MBL in the isolates, it was found that11.0% were MBL producers and 3.0% were MBL non producers. In a study conducted by Bashir et al., the prevalence of MBL in clinical isolates was found to be 11.66%. The MBL prevalence reported by Nagaveni et al., was 20.0%.

The current study showed highest resistance to ceftazidime (82.0%), followed by ceftriaxone (80.0%) and cefepime (76.0%). Gentamycin (74.0%), Ciprofloxacin (72.0%) and Cefotaxime (71.0%) also showed high levels of resistance in this study. Behera et al., found in their study that, higher resistance was seen with Cefotaxime (78.0%) and Ceftazidime (67.0%). Radhika et al., reported resistance in the order of Cefotaxime (71.6%) followed by Ceftazidime (55.0%). In the study conducted by Dwivedi et al., the resistance was found to be highest with Cefotaxime (90.0%) and Ceftazidime (85.0%) and in the Tavajjohi et al., study the resistance were 63.0% against Cefotaxime and 35.0% against Ceftazidime.

This study tested all Imipenem - resistant isolates for MBL production utilizing the combined disk synergy test (CDST), double - disk synergy test (DDST), and E - test. MBL production in CDST and E - test was 81.8% and 72.7%, respectively. CDST with Imipenem and EDTA, exhibiting a cut - off greater than 7 mm, effectively differentiated positive and negative outcomes. A significant drawback of DDST is its subjective interpretation. This study demonstrated that CDST was a sensitive method for detecting MBL, consistent with findings from Behera et al. study (CDST 88.8% and DDST 57.14%) (16) . The study conducted by P. Pandya et al., also showed similar results (CDST 96.3% and DDST 81.48%). (17) Studies conducted in other countries including Australia and Iran also showed almost similar findings. (18, 19) But few studies have demonstrated that DDST is more sensitive compared to CDST. (20) The MBL E - test was positive in 72.7% of the samples and this finding is similar to that of Behera et al. (16)

A limitation of the current study is the lack of PCR analysis to validate phenotypic methodologies. Consequently, MBLs have lately surfaced as a significant concern due to their ability to hydrolyse nearly all known beta - lactam antibiotics. The relevant genes are located on highly mobile components, facilitating their diffusion across other gram - negative bacteria. (21) The treatment of this multidrug - resistant bacterium is challenging due to the scarcity of accessible choices.

5. Conclusion

The current study found relatively high prevalence rate of *Pseudomonas aeruginosa* MBL producers with resistance to imipenem. Due to Increase in the resistant pattern of antibiotics can lead to increased morbidity, mortality and economic burden on patients. So it is necessary to detect MBL producing *Pseudomonas aeruginosa* by simple and effective methods. Therefore, our research endorses the utilization of E - tests, CDST, and DDST were the best identification methods for *Pseudomonas* MBL producers in areas where PCR detection is impossible. A significant degree of resistance is observed with frequently utilized antibiotics, necessitating the usage of polymyxin in most instances due to the heightened susceptibility of Pseudomonas to polymyxin. So this study is an effort to highlight the consequences and awareness of resistant pattern. Hence it is very important to detect the MBL earlier with available methods which help the clinicians to treat the patients intime with appropriate choose of antibiotic thereby can prevent the infection spread and also resistant strains for future.

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