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Phenotypic Detection and Molecular Characterization of Metalo-Beta-lactamases in Carbapenem Resistant *Pseudomonas Aeruginosa* in Zagazig University Hospitals

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ABSTRACT

Background: *Pseudomonas aeruginosa* (*P. aeruginosa*) is an opportunistic nosocomial pathogen that acquires resistance to conventional anti-pseudomonal antibiotics especially carbapenems. Due to the existing lack of choices, treating individuals infected with MBL-resistant pseudomonas is difficult.

Objective: The aim of the present study was to evaluate the performance of different phenotypic methods for the detection of metallo-beta-lactamase (MBL) producing *P. areuginosa* in comparison with polymerase chain reaction (PCR) for MBLs genes. **Patients and methods:** This study was conducted two hundred and twenty-five (225) samples obtained in ICUs in Zagazig University Hospitals, 80 samples were *P. aeruginosa* positive of age group between 39-70 years, including 50 males and 30 females.

Results: The antimicrobial resistant pattern of *P. aeruginosa* isolates by disc diffusion method. Among the 80 *P. aeruginosa* isolates, all isolates were resistant to CAZ and CTX. While 75 isolates (93.8%) were resistant to IPM, MEM and TBZ. All cases were negative regarding the presence of IMP gene. VIM gene was positive in 48% of cases. While NDM-1 gene was positive in 60% of cases. Both VIM and NDM-1 genes were positive in 29.3% of cases**.** CDST is more sensitive, while DDST with both concentration of EDTA is less sensitive but more specific. Sensitivity, specificity, PPV and NPV of DDST (taking the presence of any MBL gene as a reference test were 96.6%, 94.1%, 98.2% and 88.9% respectively. **Conclusion:** The ICU was a highly dispersed location for MBL-producing *P. aeruginosa*, which is one of the main sources of resistance to carbapenem and other antimicrobial drugs.

Keywords: Carbapenem Resistant, *Ps. Aeruginosa*; Metalo-Beta-lactamases.

INTRODUCTION

As an opportunistic infection, *Pseudomonas aeruginosa* frequently exhibits multiple drug resistance. Because there are few effective treatments for this disease, carbapenem-resistant Pseudomonas aeruginosa poses a serious threat to public health [(1)]. Hospital isolates often have multidrug resistance mechanisms because they survive in the hospital environment as a means of survival. As a result, the number of antibiotics available for therapy is restricted. When treating severe pseudomonas infections, carbapenems are the recommended antibiotic [(2)].

There are several reasons why carbapenems are resistant to them, but the primary one is the synthesis of MBL enzymes, which are broad-spectrum enzymes that can hydrolyze the majority of beta-lactam antibiotics without being inhibited by sulbactam or clavulanic acid, the two common beta-lactamase inhibitors [(3)]. There are two groups of enzymes that hydrolyze carbapenem that have been identified: MBLs and serine enzymes with SBLs. It was known that there were three different kinds of MBL genes: NDM-1 (New Delhi MBLs), VIM (Verona integrin-encoded MBLs), and IMP (Imipenemase). The majority of the genes producing MBLs are found on integrons and plasmids, which facilitates the genetic elements' broad distribution [(4)].

Other non-molecular based approaches have been developed, but all rely on chelating chemicals such as thiol compounds, dipiclonic acid, and ethylenediaminetetraaccetic acid (EDTA) to limit carbapenemase activity. PCR is regarded the gold standard for detecting carbapenemase genes [(5)]. The aim of the present study was to evaluate the performance of different phenotypic methods for the detection of MBL producing *P. aeruginosa* in comparison with PCR for MBLs genes.

PATIENTS AND METHODS

This cross-sectional study was carried out in the Medical Microbiology and Immunology Department, and ICUs, Zagazig University Hospitals. This study was conducted on 80 hospitalized patients of all age groups; they were 50 males and 30 females of age group between 39-70 years.

Inclusion criteria: Hospitalized patients admitted in ICUs were included for study criteria.

Exclusion criteria: Apparently healthy individuals admitted to ICU under observation without clinical manifestations of infections like fever etc.

Samples collection: Full history including name, age, sex and cause of admission were taken from ICU staff doctors. In addition, history of antimicrobial administration, underlying disease and any previous medical history. Sputum samples were collected from inpatients with lower respiratory tract infections early in the morning in clear dry container after requesting the patients to rinse the mouth with water and then expectorate deep cough sputum. Urine samples were sterilely aspirated via the catheter lumen.

The new catheter was placed for a maximum of 30 minutes, it was clamped off above the port to enable the collection of an adequate volume of new urine. The catheter port was disinfected with 70% alcohol. A sterile 10 ml syringe was attached to the sampling port to aspirate urine. Then urine was collected and transported

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in a sterile container. Endotracheal aspirate were collected from patients in ICU by sterile suction catheter and part of the catheter contain large amount of aspirate was aseptically cut into a sterile cup. Using sterile cotton swabs inserted deeply into the depth of the infected lesions, pus from septic wounds was collected. Blood samples were withdrawn using strict aseptic technique after proper cleaning of the venipuncture. The recommended specimen volume of blood was: 5-10 ml of blood for adult and 1-5 ml of blood for children. Blood cultures should ideally be obtained as soon as feasible following a fever spike and before to the initiation of any antibiotic medication.

Culturing the samples: In order to identify the growth characteristics, each sample was streaked onto MacConkey, Nutrient, and Blood agar plates and incubated for 24 hours at 37 °C. The Pseudomonas aeruginosa pure isolates were placed on 1% nutritional agar slant and refrigerated at 4 °C [6].

Identification of P. aeruginosa:

1- **Colony morphology:** On nutrient agar colonies are smooth, mucoid, large translucent 3 mm in diameter. MacConkey agar showed pale yellow non-lactose fermenting colonies. It causes hemolysis on blood agar.
2- **Characteristic smell:** They had sweet grape-like odour.
3- **Exopigment production:** *P. aeruginosa* on nutrient agar can secrete a variety of exopigments including, pyocyanin (blue), pyoverdine (yellow green fluorescent), pyorubine (red) and pyomelanine (dark brown).
4- **Gram stain smear:** Appear as gram negative bacilli, non- capsulated arranged in short chains.
5- **Oxidase test:** was done by touching the colony with oxidase detection strip and wait for up to 5 seconds. A deep violet colour indicate a positive reaction.
6- **Other Biochemical reactions:** *P. aeruginosa* have characteristic biochemical reactions. Negative indole, methyl red, Voges-Proskauer & urease. Positive citrate, catalase and motility test. Non-fermentive to any sugar with red slant and red button triple sugar iron (TSI) [7].

Antibiotic susceptibility testing:

For testing the sensitivity of the isolated strains by disc diffusion technique, the used antibiotic discs were: amikacine (30µg), aztreonam (30µg), cefepime (30µg), imipenem (10µg), gentamicin (10µg), levofloxacin (5µg), ceftazidime (30µg), ciprofloxacin (5µg), piperacillin/tazobactam (110µg), meropenem (10µg) and cefotaxime (30µg). All isolated strains of *P. aeruginosa* were evaluated for antibiotic susceptibility using (Kirby Bauer technique) disc diffusion method.

Detection of metallo-B-lactamase activity

All *Pseudomonas aeruginosa* IMP were used as a screening test to identify MBL-producing P. aeruginosa. Metallo-B-lactamases require zinc to function properly. So, these approaches rely on the capacity of metal chelators, such as EDTA, to block the activity of MBL enzymes.

a- Combined disk synergy test: The tested isolate was cultured overnight on two Mullar Hinton (MH) agar plates (0.5 McFarland opacity standard). Each one has two 10 ug imipenem discs spaced 30 mm apart from centre to centre on infected plates. Ten microliters of 0.5 M and 0.1 M EDTA solution were applied to one of the imipenem discs in two plates. After overnight incubation, if the zone of inhibition of imipenem with EDTA discs in comparison with imipenem alone was greater than 7 mm, the test was pronounced positive [5].

b- Double disc synergy test: An overnight culture of the tested isolate (0.5 McFarland opacity standard) was seeded onto two MH agar plates. In each, a 10 ug imipenem disc and a sterile blank disc (Hi-media) were put 20 mm apart on infected plates. A 10 ul volume of 0.5 M and 0.1 M EDTA solution was added to the blank discs in two plates, respectively. After overnight incubation, the zone of inhibition surrounding imipenem disc spreads towards EDTA disc, which is a favourable outcome [5].

C- Agarose gel electrophoresis: The amplified PCR products were visualised using agarose gel electrophoresis, as described by **Lee *et al.*** [8]. 20 µl of the PCR amplification products were loaded into the wells. The DNA MW marker (100-1000) base pair (bp) was run in parallel. One well was kept foe negative control sample in each run to avoid false positive results. The electric current was adjusted to 90 volts for 45 minutes. The DNA would migrate toward the anode. After finishing the procedure, the gel was viewed and photographed over an ultraviolet transilluminator at 320 nm. DNA bands were compared to the DNA MW marker [9].

Ethical consideration: Zagazig Faculty of Medicine Ethics Committee gave its approval to this study. All participants gave written consents after receiving all information. The Helsinki Declaration was followed throughout the study's conduct.

Statistical analysis

Using SPSS version 26 database software programme, the gathered data were coded, input, displayed, and examined by a computer. Numbers and percentages were used to convey the data for qualitative factors, and mean and standard deviation were used for quantitative variables. The relationship between the row and column variables is determined using the Chi-square test (X^2). P-values were established at ≤ 0.05 and ≤ 0.001 for outcomes were considered significant.

RESULTS

The present results showed the age of the studied group ranged from 39 to 70 years old with the mean of 55.13 years. Regarding sex distribution and residence, 62.5% of them were males and 56.2% were from urban area (Table 1).

Table (1): Age, sex and residence of the studied group (n=80)

	Variables	Study group (n=80)
Age (years):	Mean± SD Range	55.13 ± 8.7 39 – 70
	Variables	**%**
Sex:	Male Female	62.5 37.5
Residence:	Rural Urban	43.7 56.2

Figure (1) showed the numbers and percentage of *P. aeruginosa* isolates from different clinical samples and different source of infections.

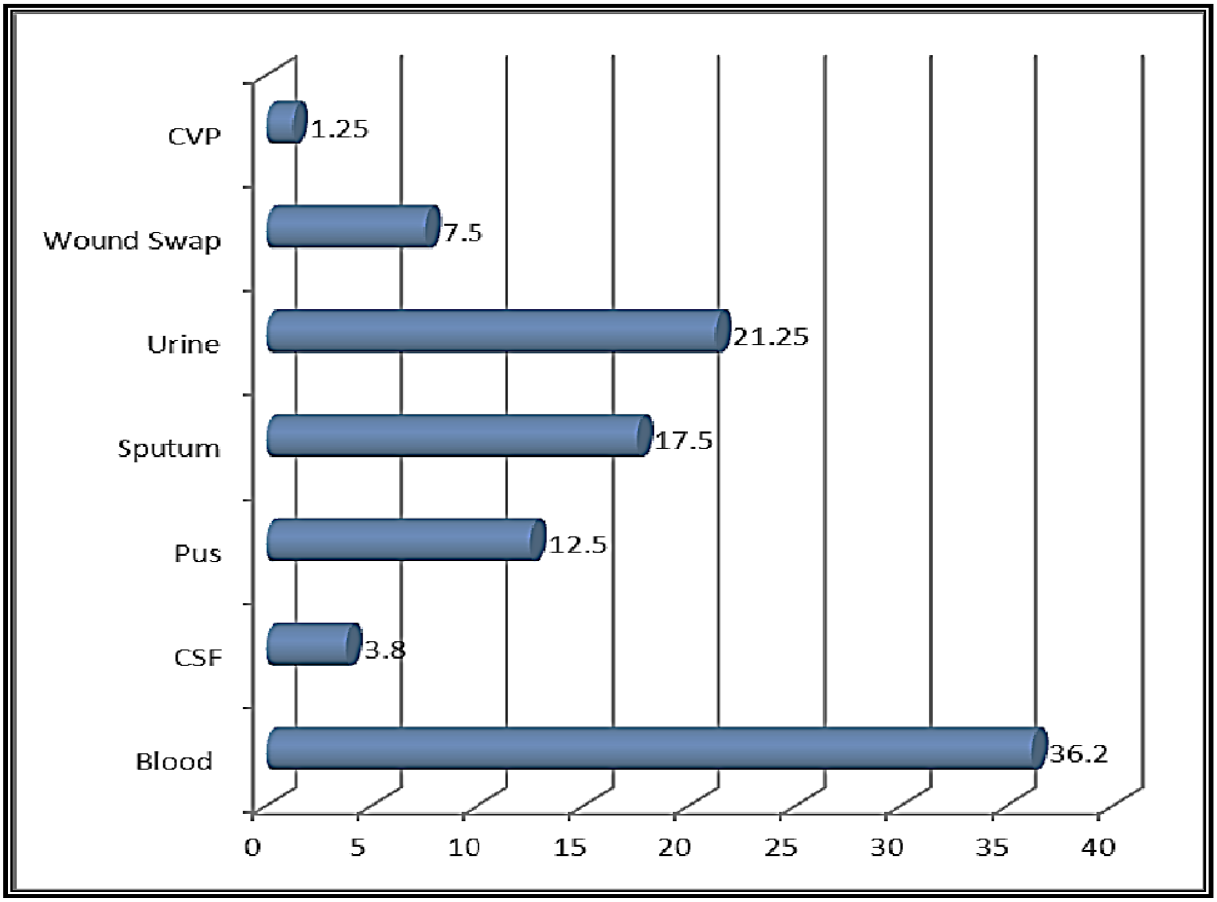

Figure (1): Distribution of different samples among the studied group (n80).

After isolation of *P. aeruginosa* from different samples, the antimicrobial susceptibility pattern of all *P. aeruginosa* isolates by disc diffusion method was performed. Out of 80 isolates, 75 were resistant to imipenem (Table).

Table (2): Distribution of Imipenem resistant *Pseudomonas areuginosa* strains in the studied group (n=80)

Sensitivity to Imipenem	Study group (n=80)	
	No	%
Pseudomonas areuginosa		
Sensitive	5	6.2
Resistant	75	93.8

The antimicrobial resistant pattern of *P. aeruginosa* isolates by disc diffusion method. Among the 80 *P. aeruginosa* isolates, all isolates were resistant to CAZ and CTX. While 75 isolates (93.8%) were resistant to IPM, MEM and TBZ (Table 3).

Table (3): Distribution of resistant rate of *Pseudomonas areuginosa* isolates regarding different antibiotics in the studied group (n=80)

Antibiotic Discs	Study group (n=80)	
	No of resistant isolate	%
Imipenem (IPM)	75	93.8
Meropenem (MEM)	75	93.8
Pipracillin/Tazobactam (TBZ)	75	93.8
Amikacin (AK)	78	97.5
Ciprofloxacin (CIP)	77	96.25
Azteronam (ATM)	77	96.25
Cefipim (FEB)	78	97.5
Gentamycin (CN)	79	98.75
Levofloxacin (LEV)	77	96.25
Ceftazidim (CAZ)	80	100.0
Cefotaxime (CTX)	80	100.0

positive in 62.7 % of isolates (Figure 2).

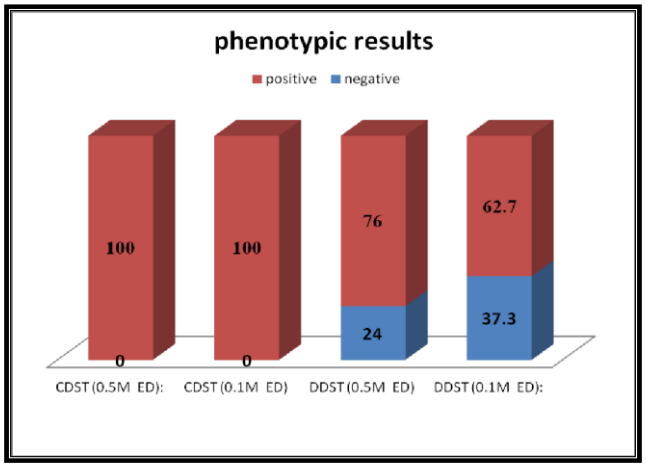

Fig. (2): Distribution of different phenotypes among the resistant group (n=75).

When evaluating imipenem resistant *Pseudomonas aeruginosa* isolates, all cases were negative regarding the presence of IMP gene. On the other hand, VIM gene was positive in 48% of cases. While NDM-1 gene was positive in 60% of cases. Both VIM and NDM-1 genes were positive in 29.3% of cases (Table 4).

Table (4): Distribution of different genes among *P. areuginosa* strains in the studied group (n=75)

Variables	Study group (n=75)	
	No	%
IMP:		
Negative	75	100.0
Positive	0	0.0
VIM:		
Negative	39	52.0
Positive	36	48.0
NDM-1:		
Negative	30	40.0
Positive	45	60.0
Both VIM and NDM-1		
Negative	53	70.7
Positive	22	29.3

Comparing the two phenotypic methods as screening test of MBL detection with the result of PCR as a gold standard, CDST was more sensitive (sensitivity = 100%), while DDST with both concentration of EDTA was less sensitive but more specific. Sensitivity, specificity, PPV and NPV of DDST (0.5M EDTA) (taking the presence of any MBL gene as a reference test) were 96.6%, 94.1%, 98.2% and 88.9% respectively (Table 5).

Table (5): Sensitivity, specificity, Positive Predictive Value (PPV) and Negative Predictive Value (NPV) of DDST 0.5 M EDTA (n=75).

Phenotypic DDST (0.5M EDTA)		PCR	
		Positive (n=58)	Negative (n=17)
Positive (n=57)		56	1
Negative (n =18)		2	16
Sensitivity	Specificity	PPV	NPV
96.6%	94.1%	98.2%	88.9%

Sensitivity, specificity, PPV and NPV of DDST (0.1M EDTA) were 79.3%, 94.1%, 97.8% and 57.1% respectively (Table 6).

Table (6): Sensitivity, specificity, Positive Predictive Value (PPV) and Negative Predictive Value (NPV) of DDST 0.1 M EDTA (n=75)

Phenotypic DDST (0.1M EDTA)		**PCR**	
		Positive (n=58)	**Negative (n=17)**
Positive (n=47)		46	1
Negative (n =28)		12	16
Sensitivity	**Specificity**	**PPV**	**NPV**
79.3%	94.1%	97.8%	57.1%

DISCUSSION

Pseudomonas aeruginosa produces MBLs, which are broad-spectrum enzymes that hydrolyze the majority of beta lactam antibiotics, with the exception of monobactams, and are frequently the cause of carbapenem resistance [(10)]. The worrying emergence of MBLs that produce *P. aeruginosa* in intensive care units is a result of the misuse of carbapenem antibiotics. Because wide spectrum antibiotics are often used in intensive care units, there is significant selection pressure. As a result, the competitive flora are eliminated, which leads to a rise in multidrug-resistant strains [(1)].

In this study, the age of the studied group ranged from 39 to 70 years old with the mean of 55.13 years. Regarding sex distribution and residence, 62.5% of them were males and 56.2% were from urban area. This is consistent with the findings of **Kali *et al.*** [(11)] who discovered that *P. aeruginosa* infections were more common in men (85.7%) and in young to middle-aged adult females aged 19 to 65 (75.5%). In a different investigation, **Ameen *et al.*** [(12)] discovered that the patients' mean age, from 1 month to 85 years, was 28.2 ±18 years when MBL-producing strains were identified. The MBL positive isolates were distributed by gender, with 56.8% of the isolates being male and 43.2% female. Also, **Hashem *et al.*** [(13)] found that the ratio of men to females was 1.8:1, with males making up 64.7% and females 35.3%. The study carried out by **Sheikh *et al.*** [(14)] revealed very identical results, indicating that out of the 369 confirmed *P. aeruginosa* isolates, 219 (59.3%) isolates were acquired from male participants and 150 (40.7%) isolates from female cases.

In the current study, about 36.2% of *P. aeruginosa* were isolated from blood sample followed by urine (21.25%), then sputum (17.5%), pus (12.5%) and wound (7.5%) and CSF samples represented 3.8% while swab represented 1.25%). These results are somewhat consistent with previous investigations in which *P. aeruginosa* was commonly identified to induce cutaneous infections with purpura and respiratory infections. According to **Kali *et al.*** [(11)] the most often obtained specimens were respiratory specimens (12.2%) and pus swabs (55.1%). However, **Ameen *et al.*** [(12)] discovered that endotracheal tube secretions (16.2%) was the least common source of MBL positive strain, with pus (43.29%) and wound swabs (21.6%) coming in second and third, respectively. According to **Hashem *et al.*** [(13)], wounds were the most prevalent kind of specimen, followed by pus swabs (43%) and sputum swabs (23%). Slight difference in percentages were seen in study carried out by **Wang and Wang** [(15)] reported that of among the various samples, *P. aeruginosa* isolated from blood tested positive for MBL in 57.8% of cases. *P. aeruginosa* isolated from pus was 54.5%, from sputum was 44.6%, from burned wound was 43.7% and from urine was 36.0% all tested positive in subsequent tests. This difference is due to different causes of admission and different duration of hospital stay. The resistant strains were acquired in part due to insufficient antiseptic procedures and poor cleanliness in the wards.

In the current study, the antimicrobial resistance pattern of *P. aeruginosa* isolates was determined using the disc diffusion method. All 80 isolates were resistant to CAZ and CTX. While, 75 (93.8%) isolates were resistant to IPM, MEM, and TBZ. This is consistent with the findings of **Abaza *et al.*** [(16)] and **Gaballah *et al.*** [(17)], who discovered that carbapenem resistance is also prevalent in Egypt.

The propagation of resistance within our isolates may have been facilitated by the presence of resistant genes on mobile genetic elements, in addition to the abuse of imipenem and meropenem in our hospital settings. According to **Corehtash *et al.*** [(18)], strong resistance patterns against amikacin (82%) and ciprofloxacin (93.7%) were seen for *P. aeruginosa* in a burn centre. Ipenem resistance pattern was found in 79.2% of the isolates. In a 2013 research at a burn medical centre in the northwest Iranian province of Gilan, the percentage of participants who were resistant to tested antibiotics was 57.5% for ceftazidime, 65% for ciprofloxacin, 67.5% for gentamycin, 87.5% for piperacillin, 90% for amikacin, and 97.5% for imipenem. The incidence of amikacin- and ciprofloxacin-resistant *P. aeruginosa* isolates was reported to be 52%, and 62.7% in the **Mirzaei *et al.*** [(18)] investigation. Additionally, 65% of the isolates of *P. aeruginosa* were imipenem resistant.

Antibiotics currently available to combat *P. aeruginosa* include carbapenems (imipenem and meropenem), aminoglycosides (gentamycin and amikacin), fluroquinolones (ciprofloxacin and levofloxacine), third and fourth generation cephalosporins (ceftazidime, cefoperazone, and cefepime), and antipseudomonal penicillins (piperacillin-tazobactam). Similar to previous study, ours showed a significant level of resistance to all beta-lactam antibiotics. Nevertheless, the majority of strains are vulnerable to aztreonam in contrast to all other beta-lactams. Our study found 96.25% resistance to aztreonam, but studies by Am**een *et al.*** [(12)] and **Basak *et al.*** [(20)] in India found 86.1% and 100% resistance, respectively, to aztreonam.

The present study showed that, when evaluating imipenem resistant *P. aeruginosa* isolates by phenotypic

methods, all cases were positive regarding CDST (0.5 M EDTA) and CDST (0.1 M EDTA). Regarding DDST (0.5 M EDTA) about 76.0 % of isolates were positive, while DDST (0.1 M EDTA) was positive in 62.7 % of isolates. This comes in agreement with **Kali *et al.*** [11] which found that positive findings were found in 100% of the strains in the CDST.

Currently, MBLs enzymes can be found using molecular techniques (PCR) and phenotypic testing like DDST and CDST. The gold standard test has a high specificity and sensitivity and is called PCR. However, both the expense and the technical limitations restrict its utilisation. Two phenotypic assays for chelating agent-based MBLs enzyme detection are CDST and DDST. Despite, its limited specificity, CDST is deemed adequate for screening due to its straightforward method and easy interpretation [21].

In our study, when evaluating imipenem resistant *Pseudomonas aeruginosa* isolates, all cases were negative regarding the presence of IMP gene. In the other hand, VIM gene was positive in 48% of cases, while NDM-1 gene was positive in 60% of cases. Both VIM and NDM-1 genes were positive in 29.3% of cases. This comes in agreement with **Basha *et al.*** [22] who found that PCR assays showed blaNDM-1 gene in majority of cases (90.9%) and blaVIM gene was present in (18.1%) of isolates. 9.0% of isolates carry both bla NDM-1 and blaVIM. Positive findings were not found for any of the isolates for the genes encoding IMP-type MβL. Furthermore, it was determined by **Diab *et al.*** [23] and **Zafer *et al.*** [24] that this gene (IMP) is absent. According to **Zafer *et al.*** [24] Egypt has a significant NDM-1 frequency in *P. aeruginosa*. These investigations have documented the co-existence of two MBL genes (NDM-1 and VIM) in isolates of *P. aeruginosa*. The VIM gene in P. aeruginosa is the most prevalent of multiple genes encoding the MBLs [25, 26]. A research from Egypt, in contrast to ours, found that no *P. aeruginosa* in that sample tested positive for the blaVIM gene [16].

In this work, comparing the sensitivity of CDST to PCR, the gold standard technique used in this work, it was 100% in both 0.5 M and 0.1 M EDTA concentrations. Similar results were reported by **Khosravi *et al.*** [27] and **Bora *et al.*** [28] who demonstrated 100% sensitivity of the CDST approach, suggesting that it would be a better regular screening strategy to take into consideration for the early identification of bacteria that produce MBL. The sensitivity of the CDST approach was found to be 92.2% in two earlier investigations by **Verma *et al.*** [5]**,** which is marginally lower than the results of the current study. The specificity of CDST in their study was 0%, as all isolates were found to be positive with no negative isolates**.** In other study that was conducted by **Ranjan *et al.*** [29], the specificity of CDST was 90.5%. They reported that temperature, aeration, pH, and media thickness are some of the parameters that restrict phenotypic techniques like CDST and DDST. Diffusion, however, affects the synergy between IPM and IPM + EDTA disc. To show a synergy, EDTA has to diffuse towards the IPM disc and reach a concentration with strong chelating activity. This might account for the variations in these phenotypic techniques' outcomes.

The current findings showed that, DDST (0.5M EDTA) demonstrated a sensitivity and specificity of 96.6% and 94.1% respectively when any MBL gene was present as a reference test. Almost, similar outcomes were attained by **Khosravi *et al.*** [27], which detected that the sensitivity of DDST was 100% and a specificity was 96.6%, which is slightly higher than our result. **Ranjan *et al.*** [29] conducted a research in India, which exhibited up to 70.8% sensitivity with a specificity of 100%, regarding the presence of any MBL genes.

In this work, CDST was more sensitive in detecting MBL generation than DDST, while the latter was more specific. This is in agreement with **Chand *et al.*** [30]**,** who revealed that the CDST is the most sensitive approach for detecting MBL generation. Various groups of researchers, including **Khosravi *et al.*** [27] and **Ranjan *et al.*** [29], discovered that DDST-IMP was more specific in detecting MBL generation than CDST-IMP.

CONCLUSION

One of the main factors contributing to resistance to carbapenem and other antimicrobial drugs is the extensively dispersed MBL-producing *Pseudomonas aeruginosa*, which is seen in intensive care units. The proliferation of MBL manufacturers can result in a therapeutic dead end if there are no new drugs developed in the future. While PCR is considered the gold standard for detecting the MBL gene, our study found that the DDST approach—which is easy to use, affordable, and suitable for any laboratory—is still the preferred method for phenotyping MBL-producing *P. aeruginosa* isolates.

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