Phenotypic and genotypic detection of metallo-B-lactamases producing *Pseudomonas aeruginosa*: A study done in two university hospitals

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Background
Metallo-beta-lactamase (MBL) producing *Pseudomonas aeruginosa* has emerged as a threat to hospital-infection control due to its multidrug resistance, but the laboratory detection of these strains is not well defined.

Objectives
Screening for MBL production from *P. aeruginosa* isolates from different clinical specimens using different phenotypic and genotyping techniques. In addition, we aim to apply a PCR test for genetic confirmation of MBL producers.

Patients and methods
We used EDTA-disk screen test and molecular diagnostic assay for the detection of MBL-producing *P. aeruginosa* isolated from different clinical specimens collected in Kasr Alainy and Fayoum University Hospitals. Using Clinical and Laboratory Standards Institute-disk methodology, inhibition-zone diameters were determined in tests with imipenem (IPM) and meropenem disks alone and in combination with 750 μg of EDTA. This test was compared with the MBL E-test. Detection for MBL-production genes (*blaIMP* and *blaVIM*) was done using PCR.

Results
Of the 50 clinical strains of IPM nonsusceptible *P. aeruginosa*, 32/50 (64.0%) were MBL positive using disk-diffusion methods, 26/50 (52.0%) were positive for MBL by E-test, while 17/50 (34.0%) were positive for MBL genes: 17/50 (34.0%) for *blaVIM* and 0/50 (0%) for *blaIMP*. The EDTA disk-screen test using IPM showed 100% sensitivity and 54.5% specificity for detecting MBLs in clinical strains. While E-test showed 100% sensitivity and 69.7% specificity.

Conclusion
The EDTA disk-screen test was simple to perform and to interpret and can easily be introduced into the workflow of a clinical laboratory. We recommend that all IPM nonsusceptible *P. aeruginosa* isolates be routinely screened for MBL production using the EDTA disk-screen test and PCR confirmation be performed at a regional laboratory.

Keywords:
E-test, imipenem, metallo-beta-lactamase, *Pseudomonas aeruginosa*

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Introduction

Pseudomonas aeruginosa is a clinically troublesome Gram-negative pathogen that causes a wide range of opportunistic infections and nosocomial outbreak [1]. *P. aeruginosa* infections are often difficult to eradicate, in part, this is due to high-level resistance to many antibiotics and disinfectants because of both intrinsic and acquired mechanisms [2]. *P. aeruginosa* infection is still an annoying problem in UTI among compromised patients [3].

Carbapenems are potent agents for chemotherapy of infectious diseases caused by *P. aeruginosa*, since the β-lactamases produced by this organism are generally ineffectual against carbapenems. However, a group of β-lactamases that hydrolyze carbapenems as well as other broad-spectrum β-lactamases has been found in *P. aeruginosa* and was identified as metallo-B-lactamases (MBL) [4].

Microorganisms that produce MBL may be resistant to carbapenems in the routine antimicrobial-susceptibility tests, however, many appear as sensitive and lead to misinterpretation of Aspartate Amino Transferase (AST) results. Supplementary tests are required to detect this new mechanism. Rapid and accurate identification is essential for appropriate therapeutic and preventive interventions.

Patients and methods

The present study was performed at both Kasr Alainy Hospital (Cairo University) and Fayoum

University Hospital over a period of 1-year duration. This study was performed after taking informed consent from the patients and after permission from the Ethical Committee of Faculty of Medicine of both Cairo and Fayoum Universities.

Fifty imipenem (IPM) nonsusceptible *P. aeruginosa* clinical isolates were collected from different body sites as follows: 27 (27/54%) were from urine culture, 12 (12/24%) were from sputum culture, and nine (9/18%) were from wound-swab culture, while only one (1/2%) clinical isolate was collected from ear-discharge culture and one blood culture.

Culture of urine samples was done on CLED (Oxoid cop.England), while other specimens were cultured on blood agar and MacConkey agar (Oxoid cop.England) and then incubated aerobically at 37°C overnight. *P. aeruginosa* was identified as oxidase positive, nonfermenter, and producing pyocyanin pigments [5].

Antibiotic-susceptibility testing

Antimicrobial-susceptibility testing was performed using the disk-diffusion method as described by the Clinical and Laboratory Standards Institute. Antimicrobial disks used were obtained from Mast Diagnostics, England. We used *Escherichia coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 as quality-control strains for disk diffusion.

Screening of metallo-beta-lactamase by both disk and E-test

Imipenem EDTA-disk methods

Half-Muller EDTA solution (Sigma Chemicals, St Louis, Missouri, USA) was prepared by dissolving 186.1 g of disodium EDTA dihydrate in 1000 ml of distilled water and its pH was adjusted to 8.0 by using NaOH. The mixture was then sterilized by autoclaving (cycle 20 min, temp. 120, and pressure 2 bar). Four micrograms from EDTA solution was poured on IPM disks to obtain a desired concentration of 750 μg per disk. The EDTA-impregnated antibiotic disks were dried immediately in an incubator and stored at −20°C in airtight vials until used. An overnight broth culture of test strain (opacity adjusted to 0.5 McFarland opacity standards) was inoculated on a plate of Mueller Hinton agar. One 10-μg IPM disk was placed on the agar plate, and the EDTA-impregnated IPM was also placed on the agar plate. The plate was incubated at 37°C for 16–18 h. An increase in the zone size of at least 7 mm around the IPM-EDTA disk compared with IPM disk without EDTA was recorded as MBL producing [6].

E-test (Biomatrieux)

E-test MBL strips consist of a double-sided seven-dilution range of IPM (4–256 μg/ml) and (1–64 μg/ml) overlaid with a constant gradient of EDTA. Individual colonies were picked from overnight agar plate and suspended in 0.85% saline to a turbidity of 0.5% McFarland's standard. The MIC endpoints were read where the inhibition ellipses intersected the strip. A reduction of IPM MIC = 3 twofold in the presence of EDTA was interpreted as being suggestive of MBL production.

Genotypic analysis (IMP and VIM genes)

Extraction of genomic DNA from *P. aeruginosa* was done using the Gene*JET* Genomic DNA Purification Kit (Fermentas) (Catalog No. K0721). Amplification of IMP and VIM genes using PCR [7]. Enzymatic amplification was performed using PCR Master Mix Kits (Fermentas [3206 Tower Oaks BLVD Rockville, Maryland, USA]) (Catalog No. K1081). Primers used for detection of MBL genes were purchased from Sigma-Aldrich Co. (St Louis, Missouri, USA). The sequence of the primers is shown in Table 1 [8].

Procedure of gene amplification

Duplex PCR amplification for the simultaneous detection of IMP and VIM MBL genes was carried out on a Thermal Cycler 9700 instrument (Hypied Express).

The master mix for the PCR was prepared as follows: 12.5-μm master mix, 3.5-μm RNase-free water, and 1 μm of forward and 1 μm of reverse for each primer. Finally, after dispensing 20 μm of the master mix in the individual amplification tubes, 5 μm of the extracted DNA was added in the corresponding tubes, the total volume being 25 μm [9].

The PCR cycle was with an initial denaturation step at 94°C for 2 min followed by 30 cycles of DNA denaturation at 94°C for 1 min, primer annealing at 54°C for 1 min, and primer extension at 72°C for one-and-a-half minutes with a holding temperature of 72°C for 5 min. After the last cycle, the PCR products were stored at 4°C. The PCR products were analyzed by electrophoresis with 1.5% agarose gels in TBE

Table 1 Primers used in the study

	Sequence	Amplicon
VIM2004A	5'-GTT TGG TCG CAT ATC GCA AC-3'	382 bp
VIM2004B	5'-AAT GCG CAG CAC CAG GAT AG-3'	
IMP-A	5'-GAA GGY GTT TAT GTT CAT AC-3'	587 bp
IMP-B	5'-GTA MGT TTC AAG AGT GAT GC-3'	

buffer. The gels were stained with ethidium bromide and the PCR products were visualized under ultraviolet light [9].

Statistical methodology

Data were statistically described in terms of mean ± SD, median and range, or frequencies (number of cases) and percentages when appropriate. Comparison of numerical variables between the study groups was done using Mann–Whitney *U* test for independent samples. For comparing categorical data, χ^2 test was performed. Exact test was used instead when the expected frequency is less than five. Agreement was calculated using kappa statistic.

P values less than 0.05 were considered statistically significant. All statistical calculations were done using computer program SPSS (Statistical Package for the Social Science; SPSS Inc., Chicago, Illinois, USA), version 15 for Microsoft Windows.

Results

The present study was conducted on 50 patients having IPM nonsusceptible *P. aeruginosa* in their samples. Patients were diagnosed and selected among cases admitted to Kasr Aliny Hospital (Cairo University) and Fayoum University Hospital.

Their age ranged from 4 months to 75 years and the mean age was 45. There were 37 (37/74%) males and 13 (13/26%) females.

Among the selected cases, there were 19 (19/38%) cases in ICU Department, nine (9/18%) were from Urology, nine (9/18%) were in Neurology, four (4/8%) were from surgery, three (3/6%) were from internal medicine, three (3/6%) were from chest, and one (1/2%) case was from the following departments: ENT, Gynecology, and Neonatology.

Out of 50 samples, the majority were obtained from the ischemic heart disease and were seven (7/14%) cases, followed by six (6/12%) cases with pneumonia, six (6/12%) cases with stroke, six (6/12%) UTI cases, three (3/6%) myocardial infarction cases, two (2/4%) diabetic cases, two (2/4%) diabetic ketoacidosis cases, two (2/4%) with wound infection, one (1/2%) with bladder stone, one (1/2%) with burn second degree, one (1/2%) with chronic calcular cholecystitis, one (1/2%) with bladder cancer, one (1/2%) with C5 fracture, one (1/2%) with lung cancer, one (1/2%) with cerebral hemorrhage, one (1/2%) case with epilepsy, one (1/2%) case with intestinal obstruction, one (1/2%) case with otitis media, one (1/2%) case showed paraparesis, one (1/2%) with retroperitoneal sarcoma, one (1/2%) case had septicemia, one (1/2%) case showed transverse myelitis, and one (1/2%) case with ureteric stones.

The 50 clinical isolates showed 100% resistance to the following drugs: IPM, meropenem, amikacin, and augmentin. However, five clinical isolates showed 5/10% sensitivity to ceftazidim, the remaining 45 (45/90%) isolates were resistant. In addition, there were 14 (14/28%) clinical isolates that were sensitive to ciprofloxacin and –36 (36/72%) were resistant. Finally, five (5/10%) clinical isolates were sensitive to cefepime and –45 (45/95%) were resistant.

The collected clinical isolates were subjected to the effect of addition of EDTA to the disks by using IPM EDTA disc-diffusion method and the results were as follows: 32 (32/64.0%) clinical isolates showing positive results, and the other eighteen samples were negative (18/36.0%).

The collected samples are also subjected to MIC using E-test and the results were as follows: –26 (26/52%) showed positive results, while the remaining 24 (24/48%) clinical isolates showed no change in the MIC result after addition of EDTA.

Table 2 shows that *VIM* gene was detected in 17 (17/34.0%) clinical isolates, while *IMP* gene was not detected in any of our selected clinical isolates (Fig. 1).

Among 17 positive samples in PCR, there were 16 samples positive by E-test that represents 61.5% from E-test and 94.1% from PCR, and there was only one sample negative by E-test that represents 4.9% within E-test and 5.9% within PCR.

While 33 samples negative by PCR, there were 10 positives by E-test that represent 38.5% within E-test and 30.3% within PCR and there were 23 samples negative by E-test that represent 95.8% within E-test and 69.7% within PCR (Table 3).

Among 17 positive samples in PCR, there were 17 samples positive by disc-diffusion test that represents 53.1% from disc-diffusion test and 100.0% from PCR.

Table 2 PCR results for both *IMP* and *VIM* genes

Results of PCR	VIM gene		IMP gene	
	Frequency	Percent	Frequency	Percent
Positive	17	34.0	0	0
Negative	33	66.0	50	100
Total	50	100	50	100

Table 3 Relation between E-test and PCR

	PCR		Total
	VIM positive	VIM negative	
E-test			
Positive			
Count	16	10	26
% within E-test	61.5	38.5	100.0
% within PCR	94.1	30.3	52.0
Negative			
Count	1	23	24
% within E-test	4.2	95.8	100.0
% within PCR	5.9	69.7	48.0
Total			
Count	17	33	50
% within E-test	34.0	66.0	100.0
% within PCR	100.0	100.0	100.0
	Value	*P*	
Measure of agreement			
Kappa	0.556	<0.001	

P<0.05 was considered significant.

Table 4 Relation between PCR results and imipenem EDTA disc diffusion test

	PCR		Total
	VIM positive	VIM negative	
Disc diffusion			
Positive			
Count	17	15	32
% within disc diffusion	53.1	46.9	100.0
% within PCR	100.0	45.5	64.0
Negative			
Count	0	18	18
% within disc diffusion	0	100.0	100.0
% within PCR	0	54.5	36.0
Total			
Count	17	33	50
% within disc diffusion	34.0	66.0	100.0
% within PCR	100.0	100.0	100.0
	Value	*P*	
Measure of agreement			
Kappa	0.449	<0.001	

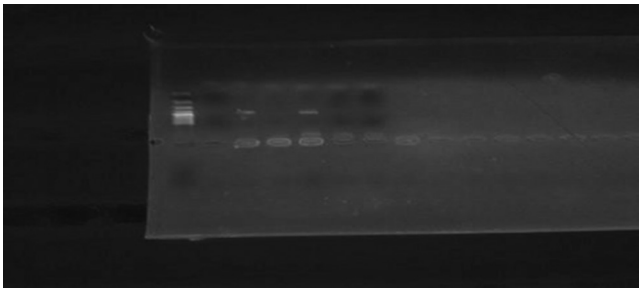
P<0.05 was considered significant.

While the 33 samples negative by PCR, there were 15 samples positive by disc-diffusion test that represent 94.9% within disc-diffusion test and 45.5% within PCR, and there were 18 samples negative by disc-diffusion test that represent 100.0% within disc-diffusion test and 54.5% within PCR as shown in Table 4.

Considering PCR is the gold standard for detection of *VIM* gene, EDTA disc- diffusion specificity was 54.5%, sensitivity was 100%, negative predictive value was 100%, positive predictive value was 53.1%, and accuracy was 70%.

In the E-test, specificity is 69.7%, sensitivity is 94.1%, negative predictive value is 95.8%, positive predictive value is 61.5%, and accuracy was 78%.

Figure 1



VIM gene in sample numbers (1) and (3) that appear at 382pb.

Discussion

P. aeruginosa is the leading cause of nosocomial infections. For treatment of these infections, carbapenems, especially IPM, are used. However, the prevalence of IPM resistance to *P. aeruginosa* has been increasing worldwide [4].

Resistance to carbapenems is due to impermeability via the loss of the OprD porin [10], the upregulation of an active-efflux-pump system of the cytoplasmic membrane [11], or the production of MBLs [12]. The presence of these mechanisms can lead to treatment failure in carbapenems therapy of *P. aeruginosa* infections.

The MBL enzymes efficiently hydrolyze all β-lactams, such as penicillins, cephalosporins, and carbapenems, except aztreonam *in vitro* [12]. These enzymes belong to Ambler class B and Bush group 3 and require divalent cations, usually zinc, as a cofactor for enzyme activity [13].

They are inhibited by metal chelators, such as EDTA, but are not affected by therapeutic β-lactamase inhibitors like sulbactam, tazobactam, or clavulanic acid [12].

Metallo-β-lactam genes are usually part of an integron structure and are carried on transferable plasmids, but can also be part of the chromosome [14]. Because of the integrin-associated gene cassettes, MBL-producing *P. aeruginosa* isolates are often resistant to different groups of antimicrobial agents, which can be transferred to various types of bacteria [15].

Several phenotypic methods are available for the detection of MBL-producing bacteria. All of these methods are based on the ability of metal chelators, such as EDTA and thiol-based compounds, to inhibit the activity of MBLs.

While most of these tests are technically demanding, expensive, time-consuming, and often subjective to interpret [16], the combined disk test using EDTA

with IPM is simple to perform and interpret and can be easily introduced into the workflow of a clinical laboratory. This test has been used in several studies where it produced excellent sensitivity and specificity for detecting MBL-producing *P. aeruginosa* strains [8].

In the present study, there were 32 (64.0%) samples MBL positive by IPM EDTA disk-diffusion method, which was much higher than a study done by Manoharan *et al.* [9], that detected 20 isolates positive from 61 samples with a percent of 32.7%. However, this was less than what Pitout *et al.*[8] detected; from 58 samples, there were 48 positive by disk-diffusion method with a percent of 85.7%.

The present study showed that the disk-diffusion method had a sensitivity of 100% and specificity 54.5%. This was in agreement with Franco *et al.* [17], who detected that the percent of positive isolates by disk diffusion was 76.8%, sensitivity was also 100%, but specificity was 32.7%.

In our study in all the 50 isolates, there were 52% positive by the E-test that was in agreement with Pitout *et al.* [8], who also reported 53.5% positive isolates by E-test from 56 isolates. While Manoharan *et al.*[9] reported 32.7% positive isolates among 60 isolates.

The sensitivity of E-test in our study was 100% and specificity was 94.1% that was similar to Franco *et al.*[17] who also reported 100% sensitivity of E-test, but the specificity was 30.2%.

In our study, we detected 34.1% positive isolates in the 50 isolates by PCR that was in agreement with Manoharan *et al.* [9], who also reported 31.2% positive isolates among 48 isolates. This was much higher than the study done by Franco *et al.* [17], who reported 19% positive *VIM* genes in seventy isolates.

In our study, we did not detect *blaIMP* gene and that was in agreement with other studies done by Manoharan *et al.* [9], Saderi *et al.* [18], and Franco *et al.* [17], who did not find *blaIMP* gene.

ICU stay increased the risk for acquisition of MBL-producing *P. aeruginosa* [19]. In our study, out of nineteen *P. aeruginosa* isolates from the ICU, seven (36.8%) were found to be MBL producers and the number of MBL producers from the ICU was statistically significant. These findings were consistent with the study by Varaiya *et al.* [20], who reported 20.8% MBL producers among *P. aeruginosa* isolates in the ICU.

In addition, similar to Fomda *et al.* [21], who detected eight (22%) out of 38 isolates from the ICU.

The maximum number of MBL-positive isolates were obtained from urine (52.9%) and the association was statistically significant, followed by sputum (29.4%) and then wound swab (17.6%). Hirakata *et al.*[22] reported in their study that the predominant source of isolation for MBL-positive *P. aeruginosa* was urinary tract (40.0%) followed by respiratory tract (18.8%) and abscesses, pus, and wounds (15%).

Hirakata *et al.*[7] suggested that malignancy is a risk factor for acquisition of MBL- producing *P. aeruginosa*. In their study, 53.8% MBL-producing *P. aeruginosa* were recovered from patients with malignancy. But in our study, only 5.8% of cases had malignant disease.

In all the 50 samples, a resistance to ceftazidime in this study (90%) was higher than the study done by Japoni *et al.* [23], which was 84.3%. In our study, resistance to amikacin (100%) and ciprofloxacin (72%), was the same when compared with the Japoni *et al.*[23] study (92.9 and 72.9%, respectively).

Possible reasons for the variety of antibiotic-resistance rates in the different studies were not understood, but it may reflect the amount of antibiotics used in various settings.

In our study, MBL producers by PCR showed in all the seventeen samples, they showed 100% resistance to the following: IPM, meropenem, ceftriaxone, amikacin, piperacillin, and augmentin, but showed 100% sensitivity to polymyxin B. This was in agreement with Saderi *et al.* [18], in their study, MBL producers showed very high resistance to all antimicrobials, except polymyxin B compared with nonproducers. In contrast to Fomda *et al.*[21] who reported that 9.1% MBL-positive isolates were sensitive to amikacin and ciprofloxacin, 18.1% were sensitive to piperacillin/tazobactum. All MBL-producing *P. aeruginosa* isolates (100%) were sensitive to polymyxin B.

The unique problem with MBLs is their unrivaled broad-spectrum resistance profile. In addition, in many cases, the MBL genes may be located on plasmids with genes encoding other antibiotic-resistance determinants. These MBL-positive strains are usually resistant to ß-lactams, aminoglycosides, and fluoroquinolones. However, they usually remain susceptible to polymyxins [24].

All the isolates in our study were sensitive to polymyxin B. In the absence of therapeutic MBL inhibitors, polymyxins have shown to be effective in the treatment of MDR *P. aeruginosa* infections. It has been claimed that polymyxins are not as toxic as previously thought [25]. However, they should not be used in monotherapy.

A combination therapy must be preferred. An aminoglycoside or a fluoroquinolone molecule that may have retained some activity against the isolate may be chosen substantiated by rapid determination of its MIC levels for the isolate. In addition, rifampicin may be an interesting agent for treating multidrug-resistant *P. aeruginosa* infections [24].

While E-test showed 100% sensitivity and 69.7% specificity, the EDTA disk-screen test is simple to perform and to interpret and can easily be introduced into the workflow of a clinical laboratory.

We recommend that all IPM nonsusceptible *P. aeruginosa* isolates be routinely screened for MBL production using the EDTA disk-screen test and PCR confirmation be performed at a regional laboratory.

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Conflicts of interest
There are no conflicts of interest.

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