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Occurrence of VIM-2 Metallo-ß- Lactamases in imipenem resistant and susceptible *Pseudomonas aeruginosa* clinical isolates from Egypt

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Different metallo-β-lactamases (MBL) have been increasingly recognized among imipenem (IMP)-resistant *Pseudomonas aeruginosa (P. aeruginosa)* isolates. The existence of MBL IMP-susceptible isolates has been lately reported bearing the risk of unnoticed spread in hospitals. Early detection of MBL-producing organisms is critical to stop their uncontrolled spread and to allow for the prompt use of appropriate antibiotic. The current study aimed to determine MBL frequency among IMP-resistant and susceptible *P. aeruginosa* isolates by phenotypic and molecular testing in a medical hospital setting in Cairo. A total of 50 P. *aeruginosa* isolates from Theodor Bilharz Research Institute (TBRI) hospital were identified and examined for the phenotypic expression of MBL by Imipenem-EDTA combined disk test (CDT) and the MBL E test. MBL coding genes, bla_{VIM-2} and $bla_{IMP-1,}$ were detected by PCR. Of all *P. aeruginosa* isolates, 41 (82%) were MBL producers by phenotypic methods while PCR assay detected VIM-2 type of MBL gene in 35 (70%) and all test isolates were negative for IMP-1 gene. Based on data from the combined use of the three methods, 32 (64%) were confirmed to be MBL producers. MBLs were detected in 64% (23/36) IMP-resistant, 58.3% (7/12) IMP-sensitive and 100% (2/2) IMP-intermediate isolates by both phenotypic tests and gene amplification of bla_{VIM-2}. All MBL carrying IMP-sensitive isolates had zone diameter between 16 to 20 mm by the Kirby-Bauer disk diffusion method. This study reports the occurrence of VIM-2 in IMP-susceptible *P. aeruginosa* isolates which may represent a risk for therapeutic failure. We propose that isolates having an IMP zone diameter ≤20mm should be screened for the presence of MBL. The CDT is an alternative phenotypic assay to detect MBL production in our population in circumstances where PCR is not a feasible option. The high prevalence of isolates possessing MBL activity in the present study represents an emerging threat of complete resistance to carbapenemes among *P. aeruginosa* in Egypt.

Key words: *Pseudomonas aeruginosa*, imipenem-resistance, metallo-β-lactamase (MBL) detection, VIM, imipenem (IMP).

INTRODUCTION

Pseudomonas aeruginosa acquire resistance to various antimicrobials by the production of beta lactamase enzymes like extended-spectrum beta lactamase (ESBL), AmpC and metallo β-lactamases (MBLs). (Manchanda and Singh, 2008) ESBLs are typically inhibitor-susceptible beta-lactamases that hydrolyze penicillins,

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cephalosporins and aztreonam and are encoded by mobile genes. AmpC β-lactamases preferentially hydrolyze cephalosporins and cephamycins and resist inhibition by clavulanate, sulbactam and tazobactam. MBLs hydrolyze carbapenems and other beta-lactams (Kumar et al., 2012).

Emergence of carbapenemases has been increasingly reported globally. Carbapenems, including imipenem and meropenem, are well-suited for the treatment of infections initiated by penicillin resistant or cephalosporin resistant gram negative bacilli; as these are stable to hydrolysis by most β-lactamases (extended spectrum and AmpC β-lactamases) (Khosravi et al., 2012 and Pereira et al., 2013). Carbapenemases are most often MBLs which are capable of hydrolyzing not only carbapenems, but also all β-lactam antibiotics except monobactams (aztreonam) (Livermore and Woodford, 2000; Walsh et al., 2005). MBLs are resistant to classical β-lactamase inhibitors, but are susceptible to EDTA and thiol-based compounds (Yong et al., 2002).

P. aeruginosa that are resistant to carbapenems have emerged and spread within hospital settings worldwide by modulating and combining different intrinsic mechanisms such as down regulation or loss of porins, especially OprD, overexpression of efflux pumps, substantial production of its chromosomal AmpC β-lactamase and target alterations (Lister et al., 2009).

Alternatively, *P. aeruginosa* may acquire genes encoding MBL and thus are considered the most clinically significant mechanism of carbapenem resistance in this species (Walsh, 2008; Picão et al., 2012). Genes encoding for MBL were shown to be carried on large transferable plasmids or were associated with transposons, allowing horizontal transfer of these MBL genes among different bacterial genera and species (Pereira et al., 2013; Fournier et al., 2013; Pitout et al., 2007). To date, five types of acquired MBL genes (IMP, VIM, SPM, GIM, and SIM) have been identified in Gram negative bacilli based on their divergent protein molecular structures (Franklin et al., 2006; Khosravi et al., 2011). While IMP and VIM variants have been reported worldwide, members of SPM, GIM, and SIM are restricted to certain geographical regions as in Malaysia (Khosravi et al., 2012; Ellington et al., 2007; Queenan and Bush, 2007).

MBLs, as thought earlier, are not just restricted to the carbapenem resistant strains, but some recent reports have argued about their presence in carbapenem susceptible *P. aeruginosa* clinical isolates that carried MBL-encoding genes (Pellegrino et al., 2008; Martins et al., 2007; Naas et al., 2011). Nevertheless, such findings are worrisome since carbapenem-susceptible MBL producers may act as reservoirs of such resistance determinants with potential risk for silent spread if such isolates are reported as sensitive without screening for the presence of MBLs (Picão et al., 2012).

Little is known about the occurrence of carbepenem resistance in *P. aeruginosa* in Egypt. Since early detection of MBL-producing organisms is critical to stop their uncontrolled spread and to allow for the prompt use of appropriate antibiotic, the current study aimed to determine MBL frequency among IMP-resistant and susceptible *P. aeruginosa* isolates in our institution by phenotypic and molecular testing.

MATERIALS AND METHODS

Clinical bacterial isolates

Fifty (50) *P. aeruginosa* clinical isolates were obtained prospectively from hospitalized and Intensive Care Unit (ICU) patients admitted to Theodor Bilharz Research Institute (TBRI) over the period of June 2009 to March 2010. *P. aeruginosa* isolates were identified by cultural characters and biochemical reactions using API 20 NE (bioMérieux, France). Bacterial strains were isolated from different clinical specimens including: urine (n=20), wound swab (n=16), sputum (n=7) and blood (n=7).

Antimicrobial susceptibility testing (AST)

Antimicrobial susceptibility testing was done by using commercially available disks (Mast Diagnostics, UK) in accordance with the CLSI guidelines (CLSI, 2010). Tested antibiotics were imipenem (IPM; 10 µg), meropenem (MEM; 10 µg), doripenem (DOR; 10 µg), amikacin (AK; 30 µg), tobramycin (TN; 10 µg), azteronam (ATM; 30 µg), ceftazidime (CAZ; 30 µg), pipracillin (PIP; 100 µg), pipracillin/ tazobactam (TZP; 100/10 µg), ciprofloxacin (CIP; 5 µg) and colistin (CO; 10 µg). All isolates were stored at -70°C as glycerol cultures. A carbapenem susceptible *P. aeruginosa* ATCC 27853 was used as negative control strain and VIM positive *P. aeruginosa* clinical isolate (Beaujon Hospital Paris, France) as positive control.

Phenotypic detection of MBL

All clinical isolates were inoculated on Mueller Hinton (MH) agar plates (MAST Diagnostics, UK) as recommended by the CLSI (2010) and screened for the presence of MBLs by two EDTA-based phenotypic methods; Imipenem (IMP)-EDTA combined disc test (CDT) and E test MBL (IP/IPI).

Imipenem (IMP)-EDTA combined disc test (CDT)

It was performed as described by Franklin et al. (2006) and Picảo et al. (2012). Two IPM disks (10 µg), one containing 10 µl of 0.1 M (292 µg) EDTA, were placed on the surface of on previously inoculated MHA plates 25 mm apart (center to center) and incubated at 35°C for 16 to 18 h. An increase in zone diameter by >4 mm around IPM-EDTA disk compared to that of IPM disk alone was considered positive for MBL production.

MBL E test (IP/IPI)

MBL E test (IP/IPI) (AB bioMérieux, Solna, Sweden) strips with a wide range concentrations of Imipenem (IP) (4-256 µg/ml) and Imipenem (1-64 µg/ml) in combination with a fixed concentration of EDTA (IPI) were applied on previously inoculated MHA plates and incubated over night at 35°C. Interpretation of results was carried out according to the manufacturer's instructions. The test was considered MBL positive when a) MIC ratio of IP/IPI in the presence of EDTA was greater than or equal to eight-fold (IP/IPI≥ 8) or by the presence of a phantom zone or a deformation of the imipenem

Table 1. Primers pairs used for PCR assay for detection of MBL genes as described by Franklin et al (2006).

Target gene	Primer pair	PCR product size (bp)	Reference
bla$_{IMP}$	F: 5' CTA CCG CAG CAG AGT CTT TG 3' R: 5' AAC CAG TTT TGC CTT ACC AT 3'	586	Senda et al. (1996)
bla$_{VIM}$	F: 5' AGT GGT GAG TAT CCG ACA G 3' R: 5' ATG AAA GTG CGT GGA GAC 3'	260	Tsakris et al. (2000)

ellipse. The MBL E test strip was also used to detect MICs for IMP simultaneously with MBLase detection. The MICs were interpreted as resistant, intermediate and sensitive as per the CLSI (2010) guidelines (Resistant: MIC ≥ 16 µg/ml, sensitive: MIC ≤ 4 µg/ml, intermediate: MIC 8 µg/ml).

Molecular detection of MBL

Polymerase chain reaction (PCR) testing was performed on all isolates to detect the presence of MBL. The primers used for the *bla*$_{IMP}$ and *bla*$_{VIM}$ genes are listed in Table 1. For each target gene, PCR amplification was carried out in a 50 µl reaction volume using a Thermal Cycler PTC-100^{Tm} (MJ Research, Inc). Test was performed as described by Franklin et al. (2006). The reaction mixture contained 10 mM Tris-Cl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, a 0.2 mM concentration of each deoxynucleoside triphosphate, a 0.2 µM concentration of each specific primer, and 1.35 U of AmpliTaq Gold DNA polymerase (Applied Bioystems). The master mix was spiked with template DNA from one to two colonies of pure culture, prior to amplification. For both PCRs, an initial 10 min denaturation step at 95°C was performed followed by 35 cycles of 45 s of denaturation at 94°C, 45 s of primer annealing at 56°C, and 50 s of primer extension at 72°C for blaIMP detection, or 35 cycles of 30 s of denaturation at 94°C, 40 s of primer annealing at 52°C, and 50 s of primer extension at 72°C for the blaVIM gene detection. Following the last cycle, an additional 7-min extension step was performed at 72°C, and the products were then held at 4°C. After agarose gel electrophoresis with ethidium bromide, the PCR products were analyzed under UV light.

Ethics

All the procedures were in accordance with the ethical standards of the responsible Theodor Bilharz Research Institute review board FWA 0001069 committee on human experimentation and with the Helsinki Declaration of 1975 that was revised in 2000.

Statistical analysis

Data were statistically described in terms of frequencies (number of cases) and relative frequencies (percentages). Agreement between the different studied techniques was done using kappa statistic. Accuracy was represented using the terms sensitivity, specificity, positive predictive value, and negative predictive value. A probability value (P value) less than 0.05 was considered statistically significant. All statistical calculations were done using computer programs Microsoft Excel 2007 (Microsoft Corporation, NY., USA), SPSS (Statistical Package for the Social Science; SPSS Inc., Chicago, IL, USA) version 15 for Microsoft Windows and Quick Calcs online calculators for scientists (Graph pad software Inc., San Diego, CA, USA).

RESULTS

Carbapenem susceptibility of the 50 studied *P. aeruginosa* isolates showed that 36/50 (72%) were IMP-resistant (zone diameter ≤ 13 mm), 12(24 %) were sensitive (zone diameter 16-20 mm) and 2 (4%) were intermediate (zone diameter 14-15mm) by disk diffusion test. MIC determination by E test confirmed the relative resistance and sensitivity to imipenem initially suggested by disc screening test (Resistant: MIC ≥16 µg/ml, Sensitive: MIC 4≤µg/ml, Intermediate: MIC 8 µg/ml). The resistance rates to meropenem and doripenem among the 36 IMP- resistant *P. aeruginosa* isolates was 94.4% and 97.2% respectively while resistance rates to the same antibiotics among the 12 IMP-sensitive isolates, was 83.3 and 100% respectively. All of the IMP- intermediate isolates were resistant to such carbapenems.

Analysis of the susceptibility patterns of the isolates against different antibiotics showed that 84% (42/50) were multi-drug resistant (MDR) with resistance to at least three drugs in the following classes: β-lactams, carbapenems, aminoglycosides and fluoroquinolones. Individually, 83.3% of both IMP- resistant and sensitive isolates and all of IMP-intermediate isolates were found to be MDR. Colistin revealed the highest efficiency (100%) against all the studied *P. aeruginosa* IMP-resistant, intermediate and sensitive isolates (Figure 1).

Of the 50 *P. aeruginosa* clinical isolates included in the study, 41 (82%) were MBL producers by both phenotypic tests used in the study namely; CDT and MBL Etest (Figure 2). PCR assay detected VIM-2 type of MBL among 35 (70%) of the isolates. All of the tested strains were negative for *bla* IMP gene. Thirty two (64%) of *P. aeruginosa* isolates were detected as MBL producers by phenotypic tests and PCR assay (Table 2 and Figure 3). Among the 36 IMP-resistant strains, 31 (86%) were detected as MBL producers by both CDT and MBL E test methods, while 23 (64%) were positive for MBLs by phenotypic tests and *bla*$_{VIM}$ amplification by PCR assay. The susceptibility pattern of IMP-resistant MBL-producing *P. aeruginosa* isolates is shown in Table 3. All of the 12 IMP sensitive isolates had zone diameters varying from 16-20 mm and 58.3% (7/12) of which were detected as MBL producers by the use of any of the three methods. All IMP- intermediate strains were found to be MBL producers by the combined use of all the three methods. Table 4 summarizes the sensitivity, specificity, PPV, NPV

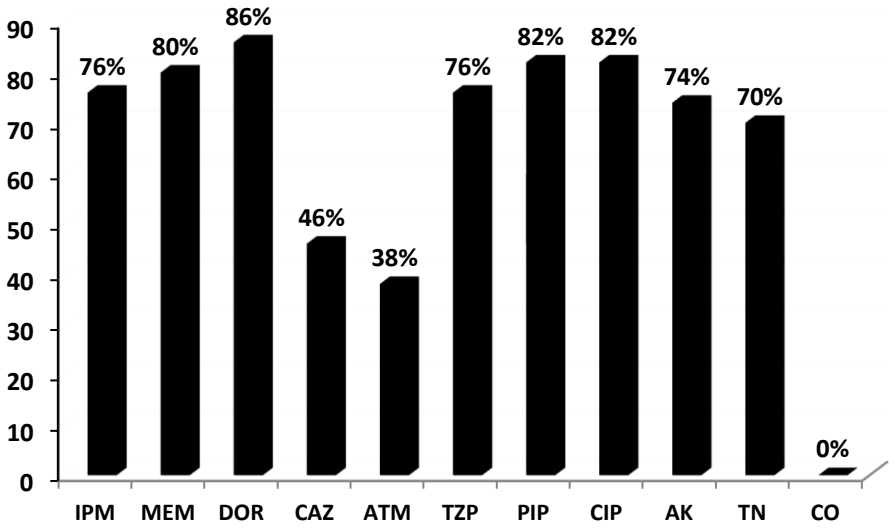

Figure 1. Drug resistance profile of 50 *P. aeruginosa* isolates to the respective antibiotics. IPM, imipenem; MEM, meropenem; DOR, doripenem; CAZ, ceftazidime; ATM, azteronam; TZP, pipracillin/tazobactam; PIP, pipracillin; CIP, ciprofloxacin; AK, amikacin; TN, tobramycin; CO, colistin.

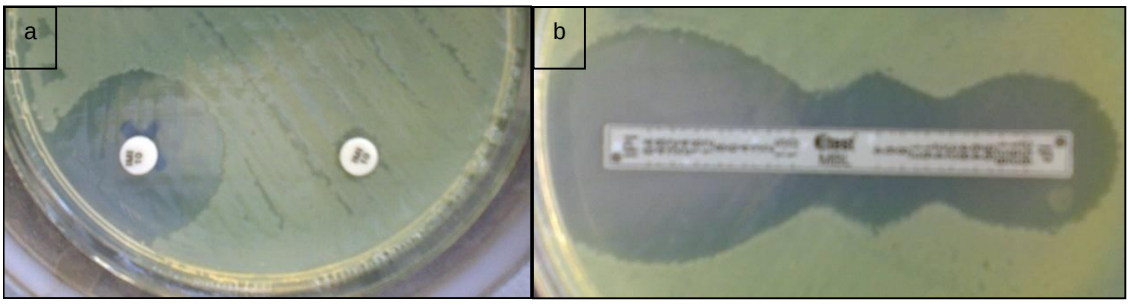

Figure 2. The phenotypic appearance of an MBL-producing *P. aeruginosa* isolates carrying *bla* $_{VIM}$ gene. **(a)** Combined-disk test using two IPM disks (10 µg), one containing 10 µl of 0.1 M (292 µg) EDTA, showing an increase in zone inhibition of >4 mm around the disk with EDTA. **(b)** MBL E Test (IP/IPI) showing deformed ellipse around strip.

and kappa values of the phenotypic tests for blaVIM gene according to the PCR results of the current study. CDT showed higher sensitivity (97.1%) than MBL E test (94.3%) with the same specificity of 46.6%. There was good agreement between both phenotypic methods and PCR.

DISCUSSION

The emergence of resistance to carbapenems mediated by MBL-producing P. aeruginosa is a cause for concern because of the ongoing spread and limited therapeutic options of this problematic organism (Crespo et al., 2004). With increasing reports of MBLs in carbapenem-susceptible isolates, we intended to determine MBL frequency in IMP- resistant and susceptible *P. aeruginosa* isolates by phenotypic and molecular testing as well as search for screening criteria for MBLs to select them out in IMP- susceptible P. aeruginosa isolates as has been urgently required (Franklin et al, 2006).

The prolonged use of imipenem/meropenem for the treatment of nosocomial infections can favor the development of resistance to carbepenems as well as other antibiotics (Lagatolla et al., 2004). In the current study, a high rate (72%) of IMP-resistance among *P. aeruginosa* isolates was revealed; such alarming result is higher than previous Egyptian reports (11.9 and 56%) (Ahmed et al., 2007; Risk and Zaki, 2007) as well as reports noted from Turkey and Tunisia in 30.8 and 37.6% respectively (Altoparlak et al., 2005; Lamia et al., 2007). In Japan, Fritsche et al. (2005) observed that the rate of resistance to carbapenems was increased from 19.3 in 1998 to 38% in 2002. A remarkable high rate of MDR isolates possessing resistance to IMP among the included *P. aeruginosa* was detected. Prior studies have shown that not only the

Table 2. Metallo-b-lactamases detection in the 50 studied *P. aeruginosa* isolates.

Test	IMP- resistente (Number = 36)	IMP-intermediate (Number = 2)	IMP-sensitive (Number = 12)	Total (Number = 50)
CDT	31(86%)	2 (100%)	9 (75%)	42 (84%)
MBL E test	32 (88.9%)	2 (100%)	7 (58.3%)	41(82%)
PCR	23(64%)	2 (100%)	10 (83.3%)	35 (70%)

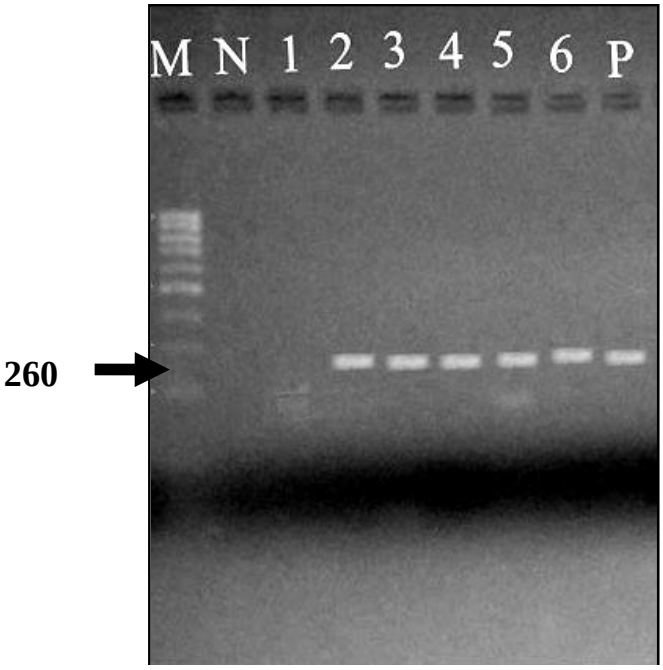

Figure 3. Agarose gel electrophoresis of PCR products of MBL-producing *P. aeruginosa* isolates carrying *bla* $_{VIM}$ gene (260 bp). Lane1 (M): molecular weight marker (1000bp). Lane (2) N: negative control. Lane (3): MBL non-producing isolate. Lanes (4-8): MBL producing isolates. Lane (9) P: positive control.

increased use of carbapenem, but also the prolonged exposure to colistin and quinolones independently could act as important contributors to the acquisition of MDR *P.* aeruginosa **(**Toraman et al., 2004; Mentzelopoulos et al., 2007). In the studied *P. aeruginosa* isolates that had MDR phenotype, colistin was the most effective antimicrobial. Franco et al. (2010) reported that colistin appeared to be the most active antimicrobial agent despite of its renal toxicity.

Production of MBL by *Pseudomonas spp.* and other gram negative bacteria has tremendous therapeutic consequences, since these organisms also carry other MDR genes (Khosravi et al., 2012; Viedma et al., 2013). Detection of acquired MBL production is a crucial step towards large scale monitoring of these emerging resistant determinants. Though there are several screening methods recommended for the detection of MBL production, no single test when used alone is specific for these enzymes.

Previous reports (Manoharam et al., 2010; Behera et al., 2008) have supported the present study findings and recommended the use of combined disk test (imipenem and imipenem plus EDTA disc diffusion screening test). The combined disk test has been validated against a PCR and has been found of good performance (sensitivity 97%, specificity 46.6% and NPV 87%). Such method is simple to perform, and the materials used are cheap, nontoxic, and easily accessible, making it highly applicable to routine clinical laboratories. It may not be practically possible for all laboratories to perform the E test due to cost constraints and availability. In a Brazilian study, Marra et al. (2006) found a 69.6% false MBL detection rate with EDTA. Chu et al. (2005) also reported that methods using EDTA are highly sensitive but not specific.

Among the studied *P. aeruginosa* isolates, MBLs had

Table 3. Susceptibility patterns of IPM Resistant MBL-producing *P. aeruginosa* isolates.

Antibiotic	MBL-producing *P. aeruginosa* IPM resistant (number =23)		
	S number (%)	I number (%)	R number (%)
Meropenem	1(4.3)	0 (0)	22 (95.7)
Doripenem	0 (0)	0 (0)	23 (100)
Ceftazidime	7 (30.4)	6 (26.1)	10(43.5)
Azteronam	8 (34.8)	8 (34.8)	7 (30.4)
Pipracillin/Tazobactam	4(17.4)	0 (0)	19 (82.6)
Pipracillin	4 (17.4)	0 (0)	19 (82.6)
Ciprofloxacin	4 (17.4)	0 (0)	19 (82.6)
Amikacin	3 (13)	0(0)	20 (87)
Tobramycin	2 (8.7)	4 (17.4)	17 (73.9)
Colistin	23 (100)	0 (0)	0 (0)

S, sensitive; I, intermediate; R, resistant.

Table 4. Performance of phenotypic methods for detecting VIM enzyme.

Phenotypic methods		PCR for *bla* $_{VIM}$ (gold standard)						
		Positive number (35)	Negative number (15)	SN (%)	SP (%)	PPV (%)	NPV (%)	Kappa (agreement)
CDT	Positive	34	8	97.1	46.6	80.9	87.5	0.505*
No=42	Negative	1	7					
MBL E test	Positive	33	8	94.3	46.6	80.5	77.8	0.462*
No=41	Negative	2	7					

*Good agreement.

been detected in 82 and 70% by phenotypic tests and PCR assay respectively. However, there could be other resistance mechanisms involved, such as permeability mutations via the loss of porins or the up-regulation of efflux systems, which may be missed by the MBL PCR (Manoharam et al., 2010).

The prevalence of MBLs has been increasing significantly. They account for up to 40% of worldwide IMP - resistant *P. aeruginosa*; furthermore, enzyme types may vary by regional areas (Livermore, 2001). In the present study, 86% of the IMP-resistant *P. aeruginosa* isolates were MBL positive by both phenotypic tests, with 64% positive for VIM-2. It has been reported that VIM-2 producers have been identified in Europe, East Asia, South and North America and Middle East (Pitout et al., 2007; Risk and Zaki, 2007; Guerin et al., 2005). The *bla*$_{IMP-1}$ gene was not detected in our study, but it has previously been found in a previous Egyptian study (Risk and Zaki, 2007).

Among the studied IMP- resistant MBL-producing isolates, 34.5% were resistant to ceftazidime. Using ceftazidime resistance (MIC 8 mg/L) along with carbapenem resistance as criteria for MBL screening could reduce the number of isolates to be tested as well as the number of false-positive results in the MBL E test and IPM-EDTA test and increase the PPV (Buchunde et al, 2012). Aztreonam was the most effective antimicrobial after colistin among the studied IMP- resistant MBL-producing isolates, but 30.4% of such isolates were not susceptible to aztreonam, a profile which is suggesting the coexistence of another mechanism of resistance among these isolates, most importantly ESBL or the AmpC-type-β-lactamases (Franklin et al., 2006). Similar findings were also reported, by Renu et al. (2010). On the other hand, Franklin et al. (2006) found that 63% of IMP–resistant carrying MBL enzyme gram- negative isolates were sensitive to aztreonam. Toleman et al. (2005) and Renu et al. (2010) demonstrated that a large production of MBL genes are associated with one or more aminoglycoside- or B-lactam resistant genes, partially explaining MDR cases.

The occurrence MBL production is not restricted to the carbapenem resistant strains, but some recent reports have argued about their presence in carbapenem susceptible organisms (Franklin et al., 2006; Renu et al., 2010). They might be unrecognized as the MBL detection has not been routinely performed in most clinical microbiology laboratories (Picão et al., 2012). Such organisms often carry hidden MBL genes that remain currently unknown. As a consequence, these isolates will be able

to participate in horizontal MBL gene transfer with other gram-negative pathogens and contribute significantly to MBL-related outbreaks (Pitout et al., 2005). Among the studied IMP-susceptible isolates, 58.3% of which were proved to be MBL producers by both phenotypic testing and PCR assay, compared to higher rates varying from 30-88%, which have been reported by other workers (Franklin et al., 2006; Yan et al., 2004).

Identifying MBL-carrying isolates has been challenging due to the emergence of carbapenem-susceptible MBL carrying organisms which may be missed in daily laboratory practice, compromising the sensitivity of detection methods (Walsh et al., 2005). It has been suggested that screening only carbapenem resistant organisms, as is most often performed, might produce suboptimal results (Franklin et al., 2006). In the current study, two of the *bla* $_{VIM-2}$ -producing IMP-susceptible clinical isolate showed negative MBL Etest and one isolate carrying the same gene was negative for CDT. That observation raises the issue of although PCR assay is a more expensive and laborious approach; however the disadvantages might be outweighed by the prevention of horizontal interspecies spread of hidden MBLs, especially in regions where VIM producers are common (Peleg et al., 2005).

In the current study, 7 of IMP-susceptible isolates carrying MBLs had zone diameter in between 16-20 mm. We could not detect any IMP-sensitive with zone diameter ≥ 21 mm as an MBL producer, thus we recommended that all isolates with zone diameter ≤ 20 mm should be routinely screened for MBL production, compared to ≤ 23 mm that has been suggested by some workers (Franklin et al., 2006; Renu et al., 2010).

In conclusion, the high prevalence of isolates possessing MBL activity among the population study represents an emerging threat of complete resistance to carbapenems among *P. aeruginosa* in Egypt. VIM-2 type of MBL was detected in IMP-susceptible *P. aeruginosa* isolates; a finding which may represent a risk for therapeutic failure since carbapenem-susceptible MBL producers may act as reservoirs of such resistance determinants with potential risk for silent spread if such isolates are reported as sensitive without screening for the presence of MBLs. Isolates having an IMP zone diameter ≤ 20 mm should be screened for the presence of MBL. The CDT is an alternative phenotypic assay to detect MBL production in our population in circumstances where PCR is not a feasible option.

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