

Full Length Research Paper

## Phenotypic screening of metallo- $\beta$ -lactamase in multi-drug-resistant *Pseudomonas aeruginosa* using a combined disk diffusion method

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Clinical utilization of carbapenems remains under threat with the emergence of acquired carbapenemase-producing bacteria, particularly metallo- $\beta$ -lactamases (MBL). *Pseudomonas aeruginosa* which is an important opportunistic pathogen causing nosocomial infections is potentially resistant to different broad-spectrum antibiotics due to its ability to produce metallo- $\beta$ -lactamase (MBL). In the present 1 year study, 105 isolates of multidrug-resistant (MDR) *P. aeruginosa* were collected from Motahari, Milad and Taleghani hospital laboratories in Tehran. These isolates were screened by the disc diffusion and combined disc methods (imipenem + EDTA; meropenem + EDTA) to determine the capacity of producing MBL. The overall prevalence of MBL-positive isolates was 88.27% using imipenem and imipenem plus ethylene diamine tetra-acetic acid (EDTA) discs, meanwhile 92.73% of 55 randomly selected isolates evaluated phenotypically for the presence of an MBL, using meropenem and meropenem plus EDTA discs as well, were MBL positive. In the light of our results, the rapidly spreading resistance among bacterial populations due to the extensive use of antibiotics is a matter of concern for the optimal treatment of patients and the determination of MBL production of MDR *P. aeruginosa* strains using a simple, reliable and inexpensive testing method is essential in patients suffering from resistant infections.

**Keywords:** *Pseudomonas aeruginosa*, metallo- $\beta$ -lactamase (MBL), imipenem, meropenem, ethylene diamine tetra-acetic acid (EDTA), multidrug-resistant (MDR).

### INTRODUCTION

Carbapenems are  $\beta$ -lactam group of drugs that are often used as antibiotics of last resort for treating infection due to multiple-resistant Gram-negative bacilli. They are also stable even in response to extended-spectrum and  $\beta$ -lactamases. However, this scenario has changed with the emergence of metallo- $\beta$ -lactamase (MBL)-producing strains (Jesudason et al., 2005). Resistance to carbape-

nem is now of global concern and being observed more frequently among nonfermenting bacteria, such as *Pseudomonas aeruginosa*.

Multidrug-resistant (MDR) *P. aeruginosa* is responsible for most nosocomial infections in hospitalized patients (Shanthi and Sekar, 2009; Zavascki et al., 2010). These MDR pathogens are capable of producing enzymes that

can inactivate beta-lactams, such as metallo- $\beta$ -lactamase (MBL) that is responsible for a significant proportion of carbapenem resistance in these bacteria (Borgianni et al., 2010; Moya et al., 2009). These enzymes can hydrolyse all classes of  $\beta$ -lactam drugs and withstand neutralization by  $\beta$ -lactamase inhibitors (Wan Nor Amilah et al., 2012). MBLs are  $\beta$ -lactamase enzymes that possess metal ion(s) at their active sites.

These enzymes require zinc for their catalytic activity and are inhibited by metal chelators, such as ethylene diamine tetra-acetic acid (EDTA) (Livermore and Woodford, 2000). The genes responsible for the production of MBLs are typically part of an integron structure and are carried on transferable plasmids or can also be part of the chromosome (Wan Nor Amilah et al., 2012).

MBL-producing Gram-negative bacilli have been increasingly reported in Asia, Europe, Latin American and the United States (Chu et al., 2001; Lyobe et al., 2000; Kurokawa et al., 1999; Miriagou et al., 2003; Toleman et al., 2004). The proportion of carbapenem resistance attributed to MBLs has increased significantly as well; the presence of MBLs accounted for resistance in 43.9% of Brazilian and 39.1% of Italian imipenem-resistant *P. aeruginosa* isolates (Toleman et al., 2005). These percentages represent a dramatic escalation in the fraction of resistance caused by these enzymes.

With the increase in worldwide occurrence, types, and rate of dissemination, early detection of MBL isolates is critical. The benefits of early detection include timely implementation of strict infection control practices as well as clinical guidance regarding the potential risks for therapeutic failure. Although polymerase chain reaction (PCR) is highly accurate and reliable, its accessibility is often limited to reference laboratories (Franklin et al., 2006). Several non-molecular techniques have been studied, mostly taking advantage of the enzyme's zinc dependence by using chelating agents, such as EDTA to inhibit its activity (Bush and Fisher, 2011; Bush and Jacoby, 2010; Deshmukh et al., 2011).

Since the rapid spread of bacterial resistance due to the extensive use of antibiotics remains a matter of concern for the optimal treatment of patients, the evaluation and use of a simple, reliable and inexpensive testing method for screening of MBL-producers in routine laboratory has become necessary.

In this study, our aim was to determine the incidence of metallo- $\beta$ -lactamase (MBL) enzymes in the *P. aeruginosa* clinical isolates using a combined disk diffusion method.

## MATERIALS AND METHODS

### Bacterial isolates collection and characterization

In the present study, 105 *P. aeruginosa* clinical isolates were collected over a period of 1 year (January 2012 to January 2013) from Motahari, Milad and Taleghani hospital laboratories in Tehran. The samples were immediately transported in transport culture media under standard conditions to the central laboratory of the

Infection Research Center. *P. aeruginosa* ATCC27853 was used as the negative control. Determination of *P. aeruginosa* strains was confirmed by standard biochemical tests (Wan Nor Amilah et al., 2012).

### Antimicrobial susceptibility test

Antimicrobial susceptibility testing was performed on Mueller-Hinton agar plates with: Piperacillin (100  $\mu$ g), Ticarcillin (75  $\mu$ g), Carbenicillin (100  $\mu$ g), P-Tazobactam (110  $\mu$ g), Ceftazidime (30  $\mu$ g), Aztreonam (30  $\mu$ g), Imipenem (10  $\mu$ g), Meropenem (10  $\mu$ g), Colistin sulphate (10  $\mu$ g), Gentamicin (120  $\mu$ g), Tobramycin (10  $\mu$ g), Amikacin (30  $\mu$ g), Ciprofloxacin (5  $\mu$ g), Levofloxacin (5  $\mu$ g) and Norfloxacin (10  $\mu$ g) (Thermo Scientific™, USA) discs by disc diffusion method and interpreted as per Clinical and Laboratory Standards Institute (CLSI, 2012) recommendations. Despite the different definitions of MDR micro-organisms (Falagas et al., 2006), MDR was used in this study for isolates that were resistant to at least three classes of antibiotics.

### Phenotypic detection of MBL

Screening of MBL-producing isolates was performed using a combined disk diffusion method. The isolates were evaluated phenotypically for the presence of a metallo- $\beta$ -lactamase (MBL), using the metal chelating agent: EDTA (Vahdani et al., 2012). Identification of MBL activity was performed by carbapenem-EDTA combined disk method. Two imipenem or meropenem [IMP (10  $\mu$ g), MEM (10  $\mu$ g)] discs were applied to a Muller Hinton agar plate inoculated with a bacterial suspension of 0.5 McFarland turbidity standards, and 10  $\mu$ l of a sterile 0.5 M EDTA (pH 8.0) solution was applied to one disk. A sterile 6-mm filter paper disc to which 10  $\mu$ l of 0.5 M EDTA was applied was used to determine if EDTA alone might inhibit the growth of the test isolates. The plates were incubated at 35°C under ambient air for 18 h.

The zones of inhibition around the IMP and IMP-EDTA discs were measured for all 105 isolates, however the zones of inhibition around the MEM and MEM-EDTA discs were measured for 55 isolates selected randomly and zone increases of  $\geq 7$  mm in the presence of EDTA were noted and interpreted as indicative of an MBL phenotype on the basis of criteria described previously (Bashir et al., 2011; John and Balagurunathan, 2011; Mochon et al., 2011).

## RESULTS

In this study, 105 clinical isolates of *P. aeruginosa* were collected from three hospital laboratories in Tehran during 1 year.

All samples were MDR. Antimicrobial susceptibilities were determined according to the interpretative criteria of the CLSI guidelines (Table 1). High resistance to all antimicrobial drugs was observed except for Colistin (3.81%) and Gentamicin (67.62%).

The overall prevalence of MBL-positive isolates was 88.27% using IMP and IMP-EDTA discs, meanwhile 92.73% of 55 randomly selected isolates evaluated phenotypically for the presence of a metallo- $\beta$ -lactamase (MBL), using MEM and MEM-EDTA discs as well, were MBL positive.

Among 105 isolates, seven isolates showed different MBL producing pattern using IMP and IMP-EDTA or MEM

**Table 1.** Antibiotic resistance among multi- drug- resistant *Pseudomonas aeruginosa* isolated from 3 hospital laboratories in Tehran.

Antibiotic Group	Antibiotic	Sensitive number (%)	Intermediate number (%)	Resistant number (%)
Penicillins	Piperacillin	2(1.90%)	7(6.67%)	96(91.43%)
	Ticarcillin	-	1(0.95%)	104(99.05%)
	Carbenicillin	-	1(0.95%)	104(99.05%)
β-lactam/β-lactamase inhibitor combinations	P-Tazobactam	1(0.95%)	15(14.28%)	89(84.76%)
Cephems	Ceftazidime	11(10.48%)	3(2.86%)	91(86.67%)
Monobactams	Aztreonam	1(0.95%)	7(6.67%)	97(92.38%)
Carbapenems	Imipenem	-	-	105(100%)
	Meropenem	-	-	105(100%)
Lipopeptides	Colistin	101(96.19%)	-	4(3.81%)
	Gentamicin	10(9.52%)	24(22.86%)	71(67.62%)
Aminoglycosides	Tobramycin	3(2.86%)	1(0.95%)	101(96.19%)
	Amikacin	4(3.81%)	2(1.90%)	99(94.3%)
	Ciprofloxacin	1(0.95%)	2(1.90%)	102(97.4%)
Fluoroquinolones	Levofloxacin	2(1.90%)	1(0.95%)	102(97.4%)
	Norfloxacin	1(0.95%)	3(2.86%)	101(96.19%)

**Table 2.** Different MBL producing pattern using IMP and IMP-EDTA or MEM and MEM-EDTA discs among 7 *Pseudomonas aeruginosa* isolates.

Sample number	IMP+IMP-EDTA	MER+MER-EDTA
2	-	+
5	-	+
22	-	+
23	+	-
24	-	+
35	+	-
37	-	+

+, MBL positive; -, MBL negative

and MEM-EDTA discs (Table 2).

## DISCUSSION

Carbapenems were the effective antibiotics for MDR gram-negative bacteria infections, especially in high-risk hospital settings (Qing et al., 2012). However, resistance is the result of different mechanisms, such as MBL production by micro-organisms. These enzymes belong to Ambler class B β-lactamases based on their amino acid sequence homology and to group 3 according to the Bush classification based on their substrate profiles (imipenem hydrolysis) (Ambler, 1980; Bush et al., 1995). The genes responsible for MBL production may be chromosomal or plasmid mediated (Wan Nor Amilah et al., 2012). MBL enzymes are inhibited by EDTA

(Livermore and Woodford, 2000). The rapid detection of MBL positive isolates is necessary to control infection and to prevent their dissemination.

The increasing use of extended spectrum antibiotics such as carbapenems would provide the selective pressure for selection of these enzymes (Irfan et al., 2008). In many studies across the world, different resistance ranges (4-60%) have been reported towards carbapenems. For instance, the incidence of MBL production in *P. aeruginosa* has been reported to be 10-30% from a variety of clinical specimens across India (Deshpande et al., 2010). However, in a study by Lagatolla et al. (2004) in Italy, 70% MBL was found in *P. aeruginosa*. Our prevalence of MBL in *P. aeruginosa* does not correlate with other studies across the country. In a study by Vahdani et al. (2012), 38 % of MBL production was reported in *P. aeruginosa*, and in another

study performed in Ahwaz, 19.51% of *P. aeruginosa* strains isolated from burn patients were reported as MBL producers (Khosravi and Mihani, 2008), whereas in the study of Manoharan et al. (2010), in accordance with our results, 87.8% of *P. aeruginosa* isolates were MBL-positive, using a combined disk diffusion method. This variation reflects the different diagnostic methods and the different rates of antibiotics used in different hospitals.

Although CLSI has recommended IMP- EDTA for MBL investigation in *P. aeruginosa* (CLSI, 2012), as mentioned earlier, in our study, two *P. aeruginosa* isolates were MBL - positive using IMP and IMP- EDTA discs while these isolates were negative for MBL using MEM and MEM -EDTA discs; this contrast was observed for five other isolates, conversely. Thus, using different subclasses of carbapenems such as imipenem + EDTA and meropenem + EDTA together for investigating MBL-positive isolates by combined disk diffusion method, seems to be necessary.

The emergence of MBL-mediated resistance in our country, Iran, is a matter of concern for the treatment of patients (Vahdani et al., 2012). The phenotypic screening of resistance is an important step for epidemiological purposes and for developing policies for effective infection control measures in order to manage and prevent the spread of resistant strains. We suggest accurate surveillance, especially of MBL, in MDR *P. aeruginosa* isolates as an important step for optimal antibacterial treatment in the future.

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## REFERENCES

- Ambler RP (1980). The structure of  $\beta$ - lactamases. Phil. Trans. R. Soc. Lond. B. 289:321-331.
- Bashir D, Thokar MA, Fomda BA, Bashir G, Zahoor D, Ahmad S, Toboli S (2011). Detection of metallo-beta-lactamase (MBL) producing *Pseudomonas aeruginosa* at a tertiary care hospital in Kashmir. Afr. J. Microbiol. 5(2):164-172.
- Borgianni L, Prandi S, Salden L, Santella G, Hanson ND, Rossolini GM, Docquier JD (2011). Genetic context and biochemical characterization of the IMP-18 metallo-beta-lactamase identified in a *Pseudomonas aeruginosa* isolate from the United States. Antimicrob. Agents. Chemother. 55(1):140-145.
- Bush K, Fisher JF (2011). Epidemiological Expansion, Structural Studies and Clinical Challenges of New Beta-Lactamases from Gram-Negative Bacteria. Annu. Rev. Microbiol. 65(1):455-478.
- Bush K, Jacoby GA (2010). Updated functional classification of  $\beta$ -lactamases. Antimicrob. Agents. Chemother. 54(3):969-976.
- Bush K, Jacoby JA, Medeiros AA (1995). A functional classification scheme for  $\beta$ -lactamases and its correlation with molecular structure. Antimicrob. Agents. Chemother. 39:1211-1233.
- Chu YW, Afzal-Shah M, Houang ETS, Palepou MFI, Lyon DJ, Woodford N, Livermore DM (2001). IMP-4, a novel metallo- $\beta$ -lactamase from nosocomial *Acinetobacter* spp. collected in Hong Kong between 1994 and 1998. Antimicrob. Agents. Chemother. 45:710-714.
- CLSI (2012). Performance standards for antimicrobial susceptibility testing. Twentieth informational supplement, document M100-S20, Clinical and Laboratory Standards Institute, Wayne, PA, USA.
- Deshmukh DG, Damle AS, Bajaj JK, Bhakre JB, Patwardhan NS (2011). Metallo- $\beta$ -lactamase-producing clinical isolates from patients of a tertiary care hospital. J. Lab. Physicians 39(2):93-97.
- Deshpande P, Rodrigues C, Shetty A, Kapadia F, Hedge A, Soman R (2010). New Delhi metallo-beta-lactamase (NDM-1) in Enterobacteriaceae: Treatment options with carbapenems compromised. J. Assoc. Physicians India. 58:147-149.
- Falagas ME, Koletsi PK, Bliziotis LA (2006). The diversity of definitions of multidrug-resistant (MDR) and pandrug-resistant (PDR) *Acinetobacter baumannii* and *Pseudomonas aeruginosa*. J. Med. Microbiol. 55:1619-1629.
- Franklin C, Liolios L, Peleg AY (2006). Phenotypic detection of carbapenemsusceptible metallo- $\beta$ -lactamase-producing Gram-negative bacilli in the clinical laboratory. J. Clin. Microbiol. 44(9):3139-3144.
- Irfan S, Zafar A, Guhar D, Ahsan T, Hasan R (2008). Metallo-beta-lactamase-producing clinical isolates of *Acinetobacter* species and *Pseudomonas aeruginosa* from intensive care unit patients of a tertiary care hospital. Indian J. Med. Microbiol. 26(3):243-245.
- Iyobe S, Kusadokoro H, Ozaki J, Matsumura N, Minami S, Haruta S, Sawai T, O'Hara K (2000). Amino acid substitution in a variant of IMP-1 metallo-  $\beta$ -lactamase. Antimicrob. Agents. Chemother. 44:2023-2027.
- Jesudason MV, Kandathil AJ, Balaji V (2005). Comparison of two methods to detect carbapenemase & metallo- $\beta$ - lactamase production in clinical isolates. Indian J. Med. Res. 121:780-783.
- John S, Balagurunathan R (2011). Metallo beta lactamase producing *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. Indian J. Med. Microbiol. 29(3):302-304.
- Khosravi AD, Mihani F (2008). Detection of metallo-beta-lactamase-producing *Pseudomonas aeruginosa* strains isolated from burn patients in Ahwaz, Iran. Diagn. Microbiol. Infect. Dis. 60:125-128.
- Kurokawa H, Yagi T, Shibata N, Shibayama K, Arakawa Y (1999). Worldwide proliferation of carbapenem-resistant Gram-negative bacteria. Lancet 354:955.
- Lagatolla C, Tonin EA, Monti-Bragadin C, Dolzani L, Gombac F, Bearzi C, Edalucci E, Gionechetti F, Rossolini GM (2004). Endemic carbapenem-resistant *Pseudomonas aeruginosa* with acquired metallo- $\beta$ -lactamase determinants in European hospital. Emerg. Infect. Dis. 10(3):535-538.
- Livermore DM, Woodford N (2000). Carbapenemase: a problem in waiting? Curr. Opin. Microbiol. 3:489-495.
- Manoharan A, Chatterjee S, Mathai D (2010). Detection and characterization of metallo beta lactamases producing *Pseudomonas aeruginosa*. Indian. J. Med. Microbiol. 28(3):241-244.
- Miriagou V, Tzelepi E, Gianneli D, Tzouveleki LS (2003). *Escherichia coli* with a self-transferable, multiresistant plasmid coding for metallo- $\beta$ -lactamase VIM-1. Antimicrob. Agents Chemother. 47:395-397.
- Mochon AB, Garner OB, Hindler JA, Krogstad P, Ward KW, Lewinski JK, Rasheed MA, Anderson KF, Limbago BM, Humphries RM (2011). New Delhi Metallo- $\beta$ -Lactamase (NDM-1)-Producing *Klebsiella pneumoniae*: Case Report and Laboratory Detection Strategies. J. Clin. Microbiol. 49(4):1667-1670.
- Moya B, Dötsch A, Juan C, Blázquez J, Zamorano L, Haussler S, Oliver A (2009).  $\beta$ -lactam resistance response triggered by inactivation of a non-essential penicillin-binding protein. PLoS Pathog. 5(3):1-10.
- Qing Y, Wenxia S, Qingqing W, Xuejun C, Jianping L, Mingming Z (2012). Emergence of carbapenem-resistant *Enterobacter cloacae* carrying IMP-26 metallo- $\beta$ -lactamase in class 1 integron and TEM-104 extended-spectrum  $\beta$ -Lactamase in conjugative plasmid. Afr. J. Microbiol. 6(13):3186-3191.
- Shanthi M, Sekar U (2009). Multi-drug resistant *Pseudomonas aeruginosa* and *Acinetobacter baumannii* infections among hospitalized patients: risk factors and outcomes. J. Assoc. Physicians India 57:638-640.
- Toleman MA, Biedenbach D, Bennet DMC, Jones RN, Walsh TR (2005). Italian metallo- $\beta$ -lactamases: a national problem? Report

- from the SENTRY antimicrobial surveillance programme. *J. Antimicrob. Chemother.* 55:61-70.
- Toleman MA, Rolston K, Jones RN, Walsh TR (2004). Characterization of *bla*VIM-7 from *Pseudomonas aeruginosa* isolated in the United States: an evolutionary distinct metallo- $\beta$ -lactamase gene. *Antimicrob. Agents. Chemother.* 48:329-332.
- Vahdani M, Azimi L, Asghari B, Bazmi F, Rastegar Lari A (2012). Phenotypic screening of extended-spectrum  $\beta$ -lactamase and metallo- $\beta$ -lactamase in multidrug-resistant *pseudomonas aeruginosa* from infected burns. *Ann. Burns Fire Disasters* 25(2):78-81.
- Wan Nor Amilah WAW, Noor Izani NJ, Ng WK, Ashraful Haq J (2012). A simple screening test for the detection of metallo- $\beta$ -lactamase-producing *Pseudomonas aeruginosa* and *Acinetobacter* in a tertiary care hospital. *Trop. Biomed.* 29(4):588-597.
- Zavascki AP, Carvalhaes CG, Picão RC, Gales AC (2010). Multidrug-resistant *Pseudomonas aeruginosa* and *Acinetobacter baumannii*: resistance mechanisms and implications for therapy. *Expert. Rev. Anti. Infect. Ther.* 8(1):71-93.