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Phenotypic detection methods of metallo-β-lactamases -producing *Pseudomonas aeruginosa* strains isolated in urology ward from Skikda hospital Algeria

Fatma Zohra Mellouk* and Sameh Meradji

Laboratory of Biochemistry and Applied Microbiology, Department of Biochemistry, Badji Mokhtar-Annaba University, Algeria.

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Acquired metallo-β-lactamases (MβL) are emerging determinants of resistance in *Pseudomonas aeruginosa* . The objectives of this study were to phenotypically detect MβL in *P. aeruginosa* collected in urology ward from Skikda hospital Algeria. A total of seventeen *P. aeruginosa* isolates were identified using API 20NE and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALD-TOFMS). Antibiotic susceptibility was performed using disk diffusion method on Muller-Hinton agar. The minimum inhibitory concentrations (MIC) of imipenem were determined by Etest method. Positively screened isolates were further subjected to four different methods phenotypic; Modified Hodge test (MHT), Imipenem-EDTA combined disk test (CDT), Imipenem-EDTA double-disk synergy test (DDST) and new biochemical method Modified Carba NP test (MCNP). Out of 32 (45.71%) isolates were resistant to imipenem; 20 (62,5%) isolates were MβL producing, 5 (15.62%) were carbapenemase class A or D producing, and 7 (21.87%) isolates were detected as negative test. Rapid detection of MβL-producing *P. aeruginosa* may help inappropriate antimicrobial therapy and avoid the development and dissemination of these strains. Thus far, the validation of a simple and accurate MβL detection method such as CDT, DDST and MCNP test, can be easily incorporated into the daily routine of a clinical laboratory.

Key words: Phenotypic detection, *Pseudomonas aeruginosa*, Metallo-β-lactamases.

INTRODUCTION

Pseudomonas aeruginosa is a well-known isolate in hospital settings, and has been frequently associated with nosocomial outbreaks among susceptible patients (Paterson, 2006). Owing to its persistence in the hospital environment, as a survival strategy an array of multidrug resistance mechanisms are often seen in such hospital isolates (Walsh et al., 2005).

In recent years, Algeria has been considered among the countries that reported high rates of antimicrobial resistance in *P. aeruginosa*. For this, carbapenems antibiotics are among the best choices for the treatment of infections caused by multi-drug-resistant *P. aeruginosa* isolates in our hospital and another region in the world especially with imipenem and ceftazidim, which are

*Corresponding author. E-mail: melloukzahra@hotmail.fr.

considered to be the drugs of choice. However, resistance to this novel antibiotic is increasing worldwide (Touati et al., 2013; Sefraoui et al., 2014; Hammami et al., 2011). Among the various antimicrobial resistance mechanisms, the production of carbapenemase is one of the most important mechanisms by which *P. aeruginosa* acquires carbapenem resistance. Many carbapenemases have been identified in *P. aeruginosa*, including (1) KPC and GES variants of Ambler class A, (2) IMP-, VIM-, SPM-, GIM-, NDM-, and FIM-type metallo-β-lactamases (MβLs) of Ambler class B, and (3) OXA variant enzymes of Ambler class D (Poirel et al., 2010; El et al., 2011).

Metallo-β-lactamase activity has emerged as one of the most feared resistance mechanisms because of the ability of MβLs to hydrolyze virtually all β-lactam agents, including carbapenems. However, MβLs are unable to hydrolyze monobactams because their genes are carried on highly mobile elements. The prevalence of MβL-producing *P. aeruginosa* causing nosocomial infections has been increasing worldwide (Walsh, 2010). Therefore, early detection and identification of MβL-producing organisms is of crucial importance for the prevention of nosocomial dissemination through appropriate treatment, as well as the implementation of infection control measures (Cornaglia et al., 2011). Several phenotypic methods are available for the detection of MβL-producing *P. aeruginosa*. These tests include:

(1) Modified hodge test (MHT): The advantage of MHT is that different carbapenemase classes can be recognized in a single plate, and the disadvantage cannot discriminate between various classes of carbapenemases, and leads to false positives for Amp C (chromosomal β-lactamases) and Extended spectrum beta lactamases (ESBL) isolates.
(2) Imipenem-EDTA combined disk test (CDT),
(3) Imipenem-EDTA double-disk synergy test (DDST), this two methods incorporating the use of metal chelating agents, such as ethylenediaminetetraacetic acid (EDTA) which are capable of blocking MβL activity and have been developed to detect MβL-producing organisms,
(4) The new biochemical method Modified Carba NP (MCNP): The advantages of the MCNP test are the rapid detection of various classes of carbapenemases (class A: KPC, class B: MBL and class D: OXA types) using a single protocol.

The aim of this study was to detect MβL-producing *P. aeruginosa* isolates from urology ward using different phenotypic methods currently in use (MHT, CDT, DDST and MCNP test).

MATERIALS AND METHODS

Bacterial isolates

Seventeen non-repeat *P. aeruginosa* were isolated from urine in urology ward from Skikda hospital Algeria, between April 2014 and April 2016. These isolates were identified using API 20NE and matrix-assisted laser desorption and ionization time-of-flight mass spectrometry (MALDI-TOF MS) method (Microflex; Bruker Daltonics) as previously described (Seng et al., 2009).

Antibiotic susceptibility testing

Antibiotic susceptibility testing for all the collected samples was done by the disk diffusion method on Muller-Hinton agar according to the Antibiogram Committee of the Société Française de Microbiologie (CA-SFM) (www.sfm-microbiologie.org). The following antibiotics were tested: aztreonam (30 µg), ceftazidim (30 µg), Cefepim (30 µg), ticarcillin/clavulanic acid (75 µg + 10 µg), ticarcillin (75 µg), piperacillin (30 µg), imipenem (10 µg), amikacin (30 µg), gentamicin (10 µg), tobramycin (10 µg), nitilmicine (10 µg), nalidixic acid (30 µg), ciprofloxacin (5 µg) and colistin (50 µg). In addition, the minimum inhibitory concentrations (MIC) of imipenem was determined by the Etest method (bioMérieux)

Phenotypic detection of metallo-β-lactamases

Phenotypic detection of MβL-producing was performed using:

Modified hodge test (MHT)

The MHT was performed as follows, Muller Hinton agar was inoculated with a 0.5 Mc Farland suspension of an *E. coli* strain wild ATCC 25922. An imipenem disk is placed in the center of the plates, and the isolates to be tested are seeded from the disk to the periphery of the plates. After a night of incubation at 37°C, the deformation of the inhibition diameter at the intersection between a streak and the culture of *E. coli* indicates the production of a carbapenemase which hydrolyses imipenem by the isolate tested (Lee et al., 2010).

Imipenem-EDTA combined disk test (CDT)

The CDT was performed as described by Yong et al. (2002). Test organisms were inoculated onto plates with Mueller Hinton agar, and two 10 µg imipenem disks were placed on the plate, and appropriate amounts of 10 µL of EDTA solution were added to one of them to obtain the desired concentration (750 µg). The inhibition zones of the imipenem and imipenem-EDTA disks were compared after 16 to 18 h of incubation in air at 35°C. If the increase in inhibition zone with the imipenem and EDTA disk was ≥ 7 mm than the imipenem disk alone, it was considered as positive test (Yong et al., 2002).

Imipenem-EDTA double disk synergy test (DDST)

The DDST was performed as described by Lee et al. (2003). Test organisms were inoculated onto plates with Mueller Hinton agar, an imipenem (10 µg) disk was placed 20 mm centre to centre from a blank disk containing 10 µl of 0.5 M EDTA (750 µg). Enhancement of the zone of inhibition in the area between imipenem and the EDTA disk in comparison with the zone of inhibition on the far side of the drug was interpreted as a positive result (Lee et al., 2003)

Modified carba NP test (MCNP)

The MCNP test was performed as follows. One inoculation loop (10

µl) of the tested strain, directly recovered from a Mueller Hinton agar plate, was resuspended in 200 µL of 0.02% CTAB (Sigma-Aldrich Chimie, Saint-Quentin-Fallavier, France) and vortexed for 1 to 2 min. Subsequently, 100 µL of the bacterial suspension was mixed with 100 µL of diluted phenol red solution (2 mL of phenol red (Sigma-Aldrich) solution 0.5% (wt/vol) with 16.6 mL of distilled water) containing 0.1 mM ZnSO4 (pH 7.5) in the first tube, tube 1, used as negative control and a diluted phenol red solution containing 0.1 mM ZnSO4 (pH 7.5) supplemented with 6 mg/mL of commercially available imipenem in the second tube, tube 2. Tubes 1 and 2 were vortexed, then incubated at 37°C for a maximum of 2 h. Carbapenemase activity was revealed when negative control and the test solutions, respectively, were red vs. yellow or red vs. orange. In contrast, both solutions remained red in the case of non carbapenemase producers (Bakour et al., 2015). *P.aeruginosa* ATCC 27853 was used as the negative control and *P.aeruginosa* VIM-4, VIM-2 and *Acinetobacter baumannii NDM-1, OXA-23* and *Klebsiella pneumoniae OXA-48, KPC* were used as the positive control for all methods.

RESULTS

Of the 70 isolates of *P. aeruginosa,* 37 (52.85%) were resistant to aztreonam, 45 (64.28%) to ceftazidim, 32 (45.71%) to cefepim, 40 (57.14%) to ticarcillin, 65 (92.85%) to ticarcillin/clavulanic acid, 27 (38.57%) to piperacilin, 32 (45.71%) to imipenem, 18 (25.71%) to amikacin, 40 (57.14%) to gentamicin, 33 (47.14%) to tobramicin, 27(38.57%) to nitilmicin, 49 (70 %) to nalidixic acid, 23 (32.85%) to ciprofloxacin, and all isolates were susceptible to colistin (Table 1).

The MIC of imipenem by E test was determined for all isolates imipenem resistant by the disk, and a total of 32 (45.71%) were found to be resistant to imipenem. All imipenem resistant isolates (32 isolates) were tested for MβL-producing by phenotypic methods detection. The first test MHT showed that 25 of 32 isolates gave positive result , the second test DDST showed 20 of 32 isolates gave positive result, similar result with CDT, and 25 of 32 isolates with the new test Modified Carba NP(Table 2) .

The globel result of the 4 phenotypic methods used (MHT + DDST + CDT + MCNP) showed positive result of 20 (62.5%) isolates N (15,16,18, 27, 32, 33, 34, 35, 36, 38, 39, 41, 58, 60, 62, 63, 64, 65, 66, 68) ,which shows the presence of carbapenemase class B (th metallo-β-lactamases), similar to those shown by VIM-4 ,VIM-2, and NDM-1-producing control strains (class B) (Table 2 and Figures 1 to 3). For the other 5 (15,62 %) isolates N (17, 31, 48, 61, 70), the 4 phenotypic methods showed the positive result of MHT + MCNP, and negative result of CDT + DDST, which shows the presence of other class of carbapenemases class A or class D, similar to those shown by KPC (class A) and OXA-48 , OXA-23(class D) producing control strains (Table 2).

The remaining 7 (21,87 %) imipenem-resistant *P. aeruginosa* isolates N (1, 14, 21, 59, 67, 37, 69) were negative for all methods tested (Table 2). Thus, the carbapenem resistance phenotype of the latter isolates may be attributed to other resistance mechanisms, such as porin loss, increased efflux, and AmpC over expression (imipenem resistant phenotypic negative)

DISCUSSION

Rapid and accurate detection of carbapenemase producing *P. aeruginosa* is crucial to implementing timely appropriate treatment and infection control procedures (Peter et al., 2014). Phenotypic tests, like MHT, DDST, CDT and MCNP test, represent cost-effective tools in clinical laboratories for a first-line detection of carbapenemase resistance mechanisms.

DDST, CDT and MCNP test are highly sensitive screening tests for the exclusion of either carbapenemase producing *P. aeruginosa* or MβL, and appears to be a simple accurate and inexpensive methods for the detection of carbapenemase and it could be easily implemented in a routine laboratory through its inclusion in a standard disk diffusion panel.

In this study, among the tested phenotypic assays, we found important differences in terms of sensitivity and specificity. The MHT worked well for the detection of carbapenemase, while it was not able to consistently recognize MβLs. In addition, it has been reported that high levels of expression of AmpC coupled with decreased permeability may be interpreted as carbapenem hydrolyzing enzyme, and therefore may yield false positive results (Birgy et al., 2012; Doyle et al., 2012).

The successful detection of MβLs was mainly achieved by DDST, CDT and MCNP test. Such results were in accordance with Lee et al. (2003), Khosravi et al. (2012), Bartolini *et al*. (2014), and Anwar et al. (2016) who reported that DDST and CDT are acceptable method for MβL detection. The study of Bakour et al. (2015) revealed higher prevalence rate of MβL-producers by MCNP test.

The higher prevalence of resistance to aztreonam, ceftazidim, cefepime, piperacilin, ciprofl oxacin, gentamicin and amikacin observed in this study isolates is consistent with the results of Machado et al. (2011) and Chand et al. (2016). Also, the present study demonstrates the presence of high level resistance to imipenem (32 isolates 45,71%) from urology ward in the hospital (Table 1). Frequency of carbapenem resistance was observed in a study conducted by Pobiega et al. (2016).

This study showed that MβL production is an important cause of imipenem resistance among *P. aeruginosa* isolated from our hospital setting as 62.5% of the imipenem resistant isolates were MβL positive by phenotypic tests MHT, DDST, CDT and MCNP test, and especially with DDST, CDT and MCNP test . Pitout et al. (2005) reported that 46% of their *P. aeruginosa* clinical isolates were MβL positive using phenotypic methods (DDST). Heinrichs et al. (2015) reported that 16 of their

Table 1. Antimicrobial susceptibility in *P. aeruginosa* clinical isolates.

ATB			Years			
			2014	2015	2016	Total
B-lactamines	ATM	N	7	12	18	37
		Percentage	10	17.14	25.71	52.85
	CZ	N	9	16	20	45
		Percentage	12.85	22.85	28.57	64.28
	FEP	N	4	16	12	32
		Percentage	5.71	22.85	17.14	45.71
	TTC	N	5	14	21	40
		Percentage	7.14	20	30	57.14
	TIC	N	15	20	30	65
		Percentage	21.42	28.57	42.85	92.85
	PIP	N	5	12	10	27
		Percentage	7.14	17.14	14.28	38.57
	IPM	N	3	14	15	32
		Percentage	4.28	20	21.42	45.71
Aminoglycosides	AK	N	2	9	7	18
		Percentage	2.85	12.85	10	25.71
	GN	N	9	14	17	40
		Percentage	12.85	20	24.28	57.14
	TOB	N	8	12	13	33
		Percentage	11.42	17.14	18.57	47.14
	NT	N	4	14	9	27
		Percentage	5.71	20	12.85	38.57
Quinolones	AN	N	9	21	19	49
		Percentage	12.85	30	27	70
	CIP	N	2	11	10	23
		Percentage	2.85	15.71	14.28	32.85
Other	CI	N	0	0	0	0
		Percentage				

ATB, antibiotic; ATM , aztreonam; CZ , ceftazidim; FEP, Cefepim; TTC, ticarcillin/clavulanic acid; TIC, ticarcillin;, PIP, piperacillin; IPM, imipenem; AK, amikacin; GN, gentamicin; TOB, tobramicin; NT, nitilmicine; AN, nalidixic acid; CIP, ciprofloxacin; CI, colistin; N, number; %, porcentage.

P. aeruginosa clinical isolates were MβL positive using MCNP test, the study of Bakour et al. (2015) showed that the advantages of the MCNP test are the detection of different carbapenemase types from *Enterobacteriaceae*, *Pseudomonas* and *Acinetobacter* species using a single protocol, as well as the short time to results.

The most notable of the acquired MβLs, the IMP- and VIM-type enzymes, were first detected (Watanabe et al., 1991; Lauretti et al., 1999). Thereafter, many additional types of acquired have been reported, including the SPM-, GIM-, SIM-, KHM-, NDM-, AIM-, DIM-, SMB-, TMB-, and FIM-type enzymes(Wachino et al., 2011; El et al., 2012)

Table 2. Results for the detection of carbapenemase or metallo-β-lactamase (MBL)-producing *Pseudomonas aeruginosa* by using four different methods phenotypic.

Methods/Isolates			IMP disk	MIC IMP mg/L	MHT	DDST	CDT	MCNP	CP
Control strains CP	Class B	*P. aeruginosa* VIM-4	R	16	+	+	+	+	VIM-4
		P. aeruginosa VIM-2	R	16	+	+	+	+	VIM-2
		A. baumannii NDM-1	R	16	+	+	+	+	*NDM-1*
	Class D	*A. baumannii OXA-23*	R	16	+	-	-	+	*OXA-23*
		K. pneumoniae OXA-48	R	16	+	-	-	+	*OXA-48*
	Class A	*K. pneumoniae KPC*	R	16	+	-	-	+	*KPC*
Control negative		*P. aeruginosa* ATCC 27853	S	0	-	-	-	-	-
1			R	16	-	-	-	-	-
14			R	16	-	-	-	-	-
15			R	16	+	+	+	+	MBL
16			R	16	+	+	+	+	MBL
17			R	16	+	-	-	+	class A or D
18			R	16	+	+	+	+	MBL
21			R	16	-	-	-	-	-
27			R	16	+	+	+	+	MBL
31			R	16	+	-	-	+	class A or D
32			R	16	+	+	+	+	MBL
33			R	16	+	+	+	+	MBL
34			R	16	+	+	+	+	MBL
35			R	16	+	+	+	+	MBL
36			R	16	+	+	+	+	MBL
37			R	16	-	-	-	-	-
38			R	16	+	+	+	+	MBL
39			R	16	+	+	+	+	MBL
41			R	16	+	+	+	+	MBL
48			R	16	+	-	-	+	class A or D
58			R	16	+	+	+	+	MBL
59			R	16	-	-	-	-	-
60			R	16	+	+	+	+	MBL
61			R	16	+	-	-	+	class A or D
62			R	16	+	+	+	+	MBL
63			R	16	+	+	+	+	MBL

Table 2. Contd.

64	R	16	+	+	+	+	MBL
65	R	16	+	+	+	+	MBL
66	R	16	+	+	+	+	MBL
67	R	16	-	-	-	-	-
68	R	16	+	+	+	+	MBL
69	R	16	-	-	-	-	-
70	R	16	+	-	-	+	Class A or D

IMP: imipenem, MIC: minimum inhibitory concentrations, MHT: Modified Hodge test, CDT: Imipenem-EDTA combined disk test , DDST: Imipenem-EDTA double-disk synergy test , MCNP: Modified Carba NP, CP: carbapenemase producing, MBL: metallo-β-lactamases.

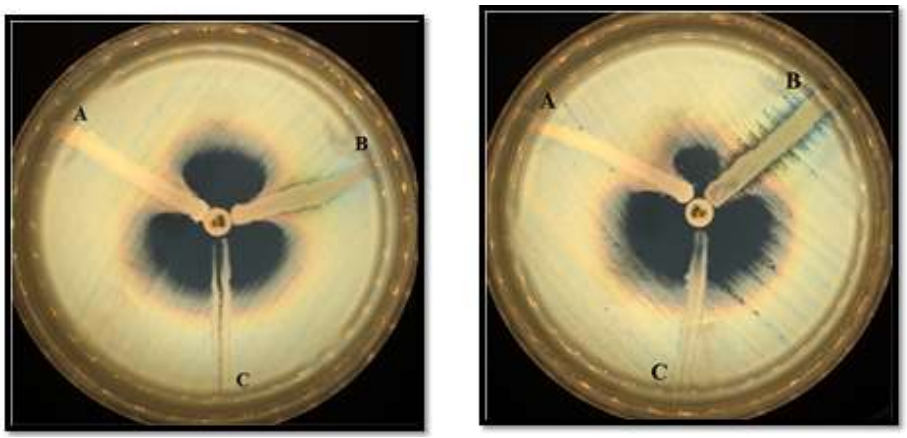

Figure 1. Phenotypic detection of metallo-beta-lactamases N 15, 16 by Modified Hodge test.
A: Positive control (carbapenemase producing *Klebsiella pneumoniae*), B: Strain tested, C: Negative control (carbapenemase non producing *Klebsiella pneumoniae*).

and have been detected with increasing frequency worldwide and frequently implicated in serious nosocomial infections and outbreaks (Maltezou, 2009).

In Algeria, there are frequent reports of MβL production in *P. aeruginosa* , namely with study of Touati et al. (2013) in Annaba and Sefraoui et al. (2014) in Oran, who indicate the spread of MβLs gene to different regions in Algeria. Also in the neighbouring countries, frequency of MβL production was observed in some African countries such as: Tunisia (Hammami et al., 2010; Ktari et al., 2011), Libya (Mathlouthi et al., 2015), Kenya (Pitout et al., 2008) and South Africa (Jacobson et al., 2012).

In the present study, 5 (15.62%) isolates shows the presence of other class of carbapenemases class A or class D, class A such as KPC in *P. aeruginosa* was first reported in Colombia and subsequently in Puerto Rico, Trinidad and Tobago, the United States, China and Iran

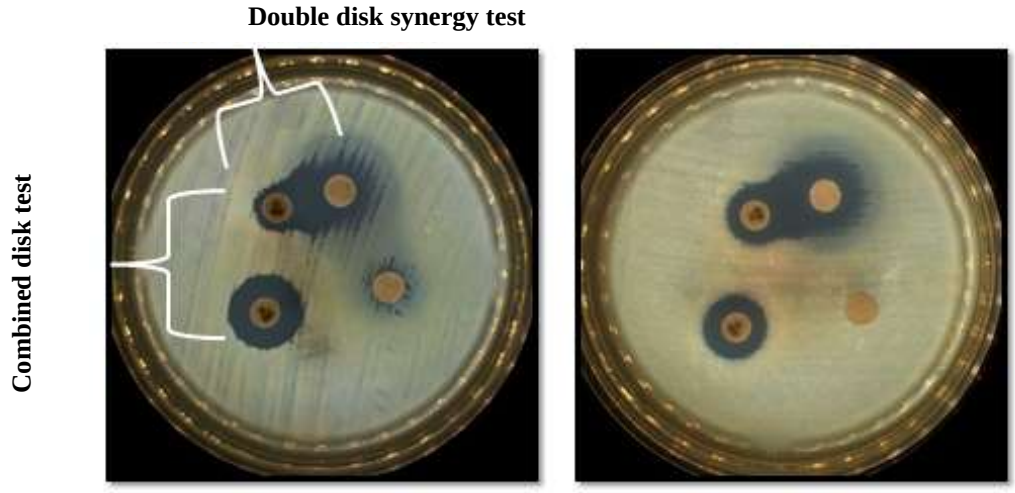

Figure 2. Phenotypic detection of metallo-beta-lactamases N 18, 34 by combined disk test and double disk synergy test.

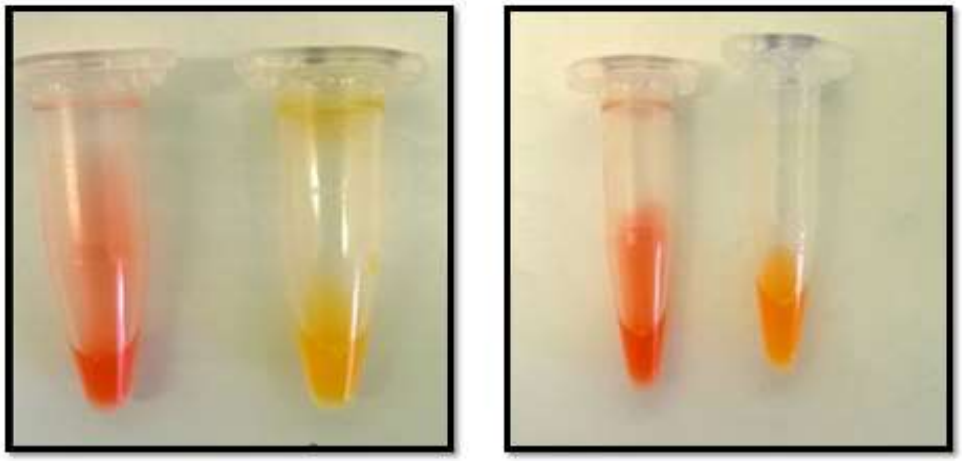

Figure 3. Phenotypic detection of metallo-beta-lactamases N 32, 33 by Modified Carba NP test.

(Falahat et al., 2016). For class D , to the best of the study knowledge, only OXA-40 has been detected in Spain and OXA-198 in Belgium (El et al., 2011).

Conclusion

This study clearly illustrated that MβL producing isolates of *P. aeruginosa* are important causes of imipenem resistance among this species isolated in urology ward from Skikda hospital. MβL-producing among imipenem-resistant isolates of *P. aeruginosa* is high and is an infection control issue. Early detection of MβL is of paramount importance for surveillance and control of antibiotic resistance, and must be routinely evaluated in all hospital settings. Simple phenotypic screening tests as the DDST, CDT and MCNP proved to be rapid and convenient tests for their detection in the clinical laboratory.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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