academicJournals

Vol. 15(8), pp. 278-283, 24 February, 2016
DOI: 10.5897/AJB2015.14822
Article Number: 4281D5B57342
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Detection of metallo-beta-lactamase producing *Pseudomonas aeruginosa* using a modified IMP-lysate assay

Olfa Sioud[1]*, Mariem Nasri[1], Mahjoub Aouni[1] and Maha Mastouri[1,2]

[1]Laboratoire des maladies transmissibles et des substances biologiquement actives Faculté de Pharmacie-5000-Monastir-Tunisie.
[2]Laboratoire de Microbiologie C H U Fattouma Bouguiba -5000- Monastir-Tunisie.

Received 27 June, 2015; Accepted 10 December, 2015

Since the increasing prevalence of carbapenem-resistant *Pseudomonas aeruginosa* spp., accurate detection of metallo-β-lactamase (MBL) such as bla_{VIM} type enzyme producing isolates became very important. However, phenotypic MBL detection methods previously reported are not highly sensitive or highly specific. This study aimed to evaluate the performance of a modified IMP-lysate test, the double-disk-synergy-test (DDST) and the combined-disk-test (CDT) for detecting MBL bla_{VIM} gene in *P. aeruginosa.* The reference technique was PCR molecular test. The study used 12 bla_{VIM2} producer isolates, 13 MBL-negative controls which included 4 imipenem-susceptible strains and 9 imipenem-resistant strains harbouring bla_{SHV-2a} genes collected from two Tunisian hospitals and *P. aeruginosa* ATCC27853 and *P. aeruginosa* COL-1 as negative and positive controls respectively. CDT showed 100% of sensitivity. The highest level of specificity was shown by IMP-lysate test (76.92%). To evaluate efficiencies of methods, the study noted that the highest Youden Index (YI) was shown by IMP-lysate method (0.7), followed by DDST (0.6) than CDT (0.2). Since MBL-Etest and PCR were expensive and not adaptable for extension use in clinical microbiology laboratories, a modified IMP-lysate MBL hydrolytic activity can be chosen by laboratories with limited resources as an inexpensive, simple, and accurate test to detect . *P. aeruginosa* producing bla_{VIM} enzyme.

Key words: Metallo-beta-lactamase, VIM, phenotypic detection, *pseudomonas aeruginosa*.

INTRODUCTION

The infections caused by multidrug-resistant *Pseudomonas aeruginosa* strains are becoming increasingly prevalent and now constitute a serious threat to public health worldwide (Oliver et al., 2015; Kaye and Pogue, 2015). Due to their stability against most β-lactamases, carbapenems such as imipenem have been

*Corresponding author. E-mail: olfasioud@yahoo.fr. Tel: +216 21 557 394. Fax: +216 73 460 678.

the drug of choice for treatment of infections caused by multidrug resistant *P. aeruginosa*. Nevertheless, the first isolate of *P. aeruginosa* with transferable imipenem resistance due to metallo-β-lactamase (MBL) production was reported in Japan in 1991 (Watanabe et al., 1991). Since then, it has been described from various parts of the world (Aubron et al., 2005; Mazzariol et al., 2011; Viedma et al., 2012;Sardelic et al., 2012) including Africa (Pitout et al., 2008; Jacobson et al., 2012; Touati et al., 2013) and Tunisia (Mansour et al., 2009; Hammami et al., 2010; Hammami et al., 2011; Ktari et al., 2011).

Treatment of infectious diseases caused by the carbapenem-resistant *P. aeruginosa* is becoming more challenging with each passing year. It have been associated with increased mortality and costs due to prolonged hospitalization and prolonged treatment with antibiotics (Liu et al., 2015). MBL producing isolates have an ability to spread and to hydrolyse virtually all β-lactam agents except monobactam (Maltezou, 2009). Detection of this resistance-phenotype by routine laboratories is essential to initiate adequate therapy and to implement proper infection control practices. However, no standardized phenotypic method is available, and previously reported phenotypic combined disk tests, are not highly sensitive or highly specific for the detection of MBL in *P. aeruginosa* (Peter et al., 2014). The most used phenotypic screening methods in clinical microbiology laboratories are double-disk-synergy-test (DDST), combined-disk-test (CDT) (Lee et al., 2001; Yong et al., 2002; Lee et al., 2003) and the current most widely accepted technique; the MBL E-test (AB Biodisk, Solna, Sweeden) (Tan et al., 2008). All of these phenotypic tests are based upon the ability of chelating agents, EDTA and thiol-based compounds, to inhibit the MBL activity (Andrade et al., 2007).

A big concern with using these methods is the direct bactericidal effect of EDTA on the test strain (Conejo et al., 2003; Tan et al., 2008) . To circumvent this problem, Tan et al. (2008) proposed an IMP-lysate assay that provides a simple, inexpensive and reproducible functional screen for MBL-producing *P. aeruginosa* (Tan et al., 2008). The IMP-lysate assay allows EDTA to interact with the carbapenamase *in vitro*, effectively reducing the concentration of EDTA that interacts with the growth of the indicator strain. However, the IMP-lysate test requires specific equipment, such as microcon concentration device (Millipore Bedford, MA) to concentrate the lysate which is not available in most laboratories. So, the study propose a modification in the technique (the study removed the lysate-concentration step with the microcon device, and the study incubated lysate solution with imipenem and EDTA before depositing it on the plate.) without reducing confidence of the assay. The aim of this study was to compare the performance of this modified IMP-lysate test, the DDST and the CDT for detecting MBL bla_{VIM} gene in *P. aeruginosa* by referring to the molecular test; the PCR.

MATERIALS AND METHODS

Clinical isolates

The *P. aeruginosa* strains used in this study were isolated from clinical specimens collected from separate patients hospitalized in different medical and surgical wards at Fattouma Bourguiba Hospital in Monastir, Tunisia and Tahar Sfar Hospital in Mahdia, Tunisia on variable periods of times (Table 1). Identification of the isolates to the species level was performed by Api 20 NE (Biomerieux, France) and by using matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) (AutoflexTM; Bruker Daltonics, Bremen, Germany) with the Flex control software (Bruker Daltonics) (Seng et al., 2009). A total of 12 bla_{VIM2} producer isolates were screened. MBL-negative controls included 4 imipenem-susceptible strains and 9 imipenem-resistant strains harbouring bla_{SHV-2a} genes. Bla_{VIM2} and bla_{SHV-2a} genes were detected by PCR. Susceptibilities of stains of antimicrobials were performed by the disk diffusion method in Mueller Hinton agar (Bio-Rad, Marnes-la-Coquette, France) according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2014). Two control strains were used; *P. aeruginosa* ATCC27853 as negative control (NC) and *P. aeruginosa* COL-1 carrying the bla VIM2 gene as positive control.

Combined disk tests (CDT) (method A)

Test organisms were inoculated onto plates of Mueller Hinton according to the guidelines of the CLSI. Two IMP (10 µg) disks (Bio-Rad, Marnes-la-Coquette, France) were placed in an agar plate, and 10 µl of a 0.5 M EDTA solution (pH8) was added to one of the IMP disks. After incubation overnight at 37°C, the inhibition zones of the IMP disks with and without EDTA were compared. A ≥ 7 mm increase in the zone diameter for IMP in the presence of EDTA was interpreted as a positive test result.

Double disk synergy tests (DDST) (method B)

Tests organisms were inoculated onto plates of Mueller-Hinton agar as described for the standard disk diffusion test (CLSI). An IMP (10 µg) disk was placed 20 mm (center to center) from a blank disk containing 5 µl of 0.5 M EDTA (pH 8). Enhancement of the zone of inhibition in the area between the two disks was considered positive for a MBL.

PCR amplification

DNA template preparation was performed as followed. The organisms were cultured on blood agar plate (90 mm diameter) and incubated for 24 h at 37°C. Half of the overnight culture was inoculated into 600 µl of distilled water and vortexed. The cells were lysed by heating them at 100°C for 10 min and cellular debris was removed by centrifugation at 13000 r/min for 10 min. The superntent was used as a source of template for amplification. Standard PCR analysis was used for detection of VIM MBL and SHV Extended-spectrum β-lactamases (ESBL) genes. The specific primers used were: VIM-F: 5'-TGGTCTACATGACCGCGTCT3-3', VIM-R: 5'CGACTGAGCGATTTGTGTG-3', SHV-F: 5'-TTTATGGCGTTACCTTTGACC-3'and SHV-R: 5'-ATTTGTCGCTTCTTTACTCGC-3' (Yagi et al., 2000). Total bacterial DNA was purified with QUIAGEN Kit. Cycling parameters of standard PCR were as follows: an initial denaturation step for 15 min at 95°C, 35 cycles of 1 min at 95°C, 50s at 55°C and 1min at 72°C and a final incubation step for 7 min at 72°C. Positive PCR products were sequenced using BigDye® terminator chemistry on

Table 1. Evalation of IMP-lysate, combined-disk and double-synergy tests for detection of VIM MBL-producing isolates of *P.aeruginosa*.

Isolates	Date of isolation	Site of location	Phenotypic screening			Molecular screening	
	Day/ Month/ Year		Method A	Method B	Method C	VIMPCR	SHV 2a PCR
Ps 27	11/13/2007	ICU	Positive	Negative	Positive	Positive	Positive
Ps 30	6/7/2008	ICU	Positive	Positive	Negative	Positive	Negative
Ps 44	10/18/2008	ICU	Positive	Positive	Positive	Positive	Negative
Ps 48	12/12/2008	S	Positive	Positive	Positive	Positive	Positive
Ps 62	10/16/2009	S	Positive	Positive	Positive	Positive	Positive
Ps 63	10/16/2009	ICU	Positive	Positive	Positive	Positive	Positive
Ps 64	10/17/2009	ICU	Positive	Positive	Positive	Positive	Positive
Ps 65	10/22/2009	NN	Positive	Positive	Positive	Positive	Positive
Ps 66	5/11/2011	ENT	Positive	Positive	Positive	Positive	Positive
Ps 67	5/15/2011	Or	Positive	Positive	Positive	Positive	Positive
Ps 69	8/13/2008	ICU	Positive	Positive	Positive	Positive	Positive
Ps 70	8/18/2008	ICU	Positive	Positive	Positive	Positive	Positive
Ps 22	7/30/2005	ICU	Negative	Negative	Positive	Negative	Positive
Ps 28	6/4/2008	U	Positive	Negative	Negative	Negative	Positive
Ps 35	8/25/2008	ICU	Negative	Negative	Negative	Negative	Positive
Ps 50	1/23/2009	PNE	Negative	Negative	Negative	Negative	Positive
Ps 6	12/3/2004	ICU	Positive	Positive	Negative	Negative	Positive
Ps 11	1/5/2005	ICU	Positive	Positive	Negative	Negative	Positive
Ps 17	7/4/2005	ID	Positive	Negative	Negative	Negative	Positive
Ps 20	7/23/2005	ICU	Positive	Negative	Negative	Negative	Positive
Ps21	7/24/2005	ICU	Positive	Negative	Positive	Negative	Positive
Ps24	3/28/2006	ICU	Positive	Negative	Negative	Negative	Positive
Ps29	6/7/2008	ICU	Positive	Positive	Negative	Negative	Positive
Ps31	6/17/2008	ICU	Positive	Negative	Negative	Negative	Positive
Ps38	9/6/2008	ICU	Positive	Positive	Positive	Negative	Positive
PsCOL1	-	-	Positive	Positive	Positive	Positive	Negative
PsATCC27853	-	-	Negative	Negative	Negative	Negative	Negative

Ps, strains from Monastir hospital; Ps, strains from Mahdia hospital; ENT, ear,nose and throat; ICU, intensive care unit; NN, neonatal; S, surgery; Or, orthopedics; ID, I nfectious disease; U, urology; PNE, pneumology; Method A, CDT; Method B, DDST; Method C, Imp-lysate test.

an automated ABI 3730 sequencer (PE Applied Biosystems, Foster City, CA).

MBL hydrolytic activity test (method C)

Test organisms were inoculated onto blood agar plate (90 mm diameter) and incubated overnight at 37°C. Half of plate was resuspended in 1.5 ml sodium phosphate buffer (pH 7). The cells were subsequently lysed by cycling between (-20°C) and room temperature (RT) and a vortex (30 s) for a total of five times. The lysed cells were separated from the other debris by centrifugation (10 min, 3.200xg, 4°C) and filtration through a 0.22 µm syringe filter (Minisart, Sartorius Stedim Biotech, Goettingen, German). About 1 ml of filtrate was recuperated each time. Instead of concentrating the filtrate through a microcon device as described by Tan et al. (2008), it was divided equally into two tubes, one of which contained 25 µl of 0.5 M EDTA at pH 8. The two samples were incubated with 10 µg imipenem per ml for 20 min at 37°C and then 15 µl from each tube was transferred into 8 mm blank disks (spaced by 6 cm) in Mueller Hinton agar plates with indicator isolate *Escherichia coli* ATCC 25922 spread on the surface. On the same plate the study tested *P. aeruginosa* ATCC27853 (harboring no resistance mechanism) as a negative control and a blank disk with 15 µl of 10 µg imipenem per milliliter solution. The diameter of inhibition zones were measured after overnight incubation at 37°C. The IMP-lysate MBL hydrolytic activity assay was confirmed by comparing the sizes of zone inhibitions for three disks; the lysate-IMP disk impregnated with lysates from *P. aeruginosa* strain and IMP, the lysate-IMP-EDTA disk with the same lysates incubated with IMP and EDTA and the third IMP disk with imipenem solution. Negative and positive controls must be tested each time. The inhibition zone from the disk (IMP) provided a reference for comparison; it was about 20 mm (data not shown). The diminution of inhibition zone around lysate-IMP disk, compared to the IMP disk, proved the hydrolysis of IMP by the lysate solution. The increase of the zone around the lysate-IMP-EDTA disk compared to the lysate-IMP disk proved that the enzyme hydrolysing IMP was inhibited by EDTA was therefore a MBL.

Statistical method

The Youden's Index (YI)

The YI is the difference between the true positive rate and the false

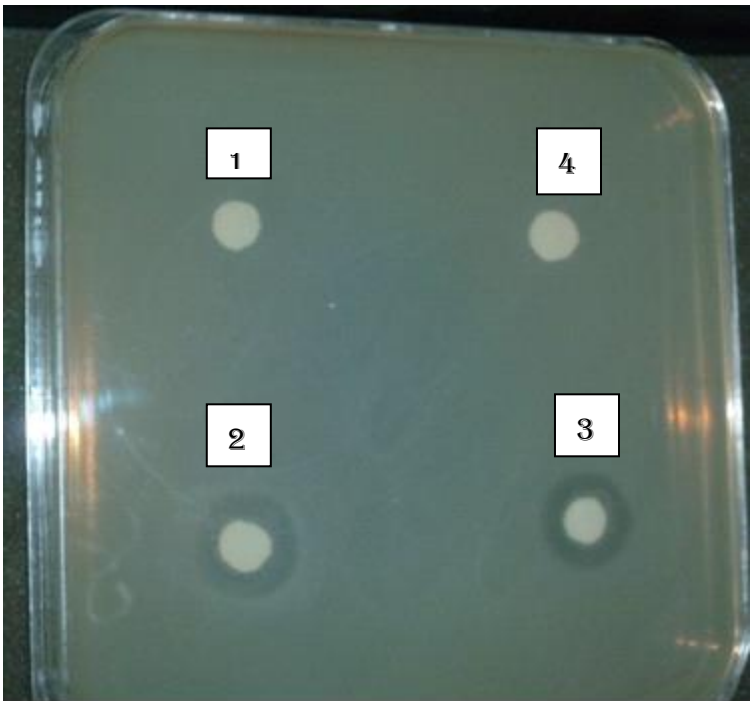

Figure 1. Results for IMP-lysate MBL hydrolytic activity test using disk 1 with lysates from *P.aeruginosa* COL-1 strain incubated with imipenem (IMP) at 10 µg per ml, disk 2 with the same lysates incubated with IMP and EDTA (0.5 M), disk 3 negative control with lysates from *P. aeruginosa* ATCC27853 incubated with IMP and disk 4 with EDTA (0.5) alone.

positive rate. It is a commonly used measure of overall diagnostic effectiveness. This index ranges between 0 and 1, with values close to 1 indicating that the biomarker's effectiveness is relatively large and values close to 0 indicating limited effectiveness (Schisterman et al., 2005) YI=sensibility+specificity-1.

RESULTS

The strains used in this study were unrelated; they were collected from two different hospitals, different medical and surgical wards, and at different time periods (Table 1). The positive control sample showed with method C no inhibition zone around disk (lysate-IMP) and 15 mm inhibition zone around disk (lysate-IMP-EDTA) (Figure 1). *P. aeruginosa* ATCC27853 sample (harbouring no resistance mechanism) showed, under the study experimental conditions, 16.87±1.76 inhibition zone around the disk (lysate-IMP).

Several tests were carried out on the negative control strain (*P. aeruginosa* ATCC27853). The lysate-IMP disk showed no upper than 5 mm decrease compared to the IMP disk, which represents the upper bound of MBL-negative strain and obviously the lower bound of MBL-positive strain. In fact, a decrease in the inhibition zone of >5 mm represents a strong argument for judging a positive strain by this assay. Disks impregnated with EDTA (0.5M) alone and lysate alone showed no inhibition zone with the indicator strain (Figure1).

Method C was compared to the two phenotypic methods considered in this study (method A and method B) and to the molecular method (PCR) as a reference method. Among the three phenotypic methods considered in this study, method A showed 100% of sensitivity. Method B and method C showed 91.66% of sensitivity. The highest level of specificity was shown by method C (76.92%), followed by method B (69.23%) than method A (23.07%) (Table 1). To evaluate efficiencies of methods, the study noted that the highest Youden Index (YI) was shown by method C (0.7), followed by method B (0.6) than method A (0.2).

DISCUSSION

There is an urgent need for an early detection of MBL-producing organisms for their prevention of their inter and intra hospital dissemination. Currently, the most widely accepted standardized MBL functional screen is the MBL E-test. However, this method suffers from high cost and unavailability of E-test strips. Both CDT and DDST have been reported to be reliable in screening for MBL production in *P. aeruginosa* (Yan et al., 2004). It has

been reported that CDT and DDST were simple, inexpensive phenotypic resources for detection of MBL that could be easily incorporated into the routines of clinical laboratories. In Tunisian clinical laboratories, for its high cost, the MBL E-test is not frequently used. Generally this test is used for research purposes and not for routine screening of MBL.

Although, MBL E-test has been evaluated to be a sensitive method for detection of MBL production in *P. aeruginosa* (Walsh et al., 2002). Qu et al. (2009) reported that several MBL-producing strains could not be detected by the MBL E-test. Several studies have been interested in evaluation and comparison of phenotypic tests (Yan et al., 2004; Picao et al., 2008; Qu et al., 2009). Variable results have been reported. Yan et al. (2004) found that the DDST was most sensitive for all bacterial species tested (Yan et al., 2004). However, one of the major disadvantages of the DDST is the subjective interpretation of results that depends upon the technician's expertise to discriminate true synergism from the intersection of inhibition zones (Picao et al., 2008; Qu et al., 2009). Other studies evaluated the CDT as most sensitive method (Berges et al., 2007; Picao et al., 2008). In this study, DDST was evaluated as more sensitive than CDT. Thus, it has been suggested that the selection of the best MBL screening method should be based on the isolated species, the local prevalence of MBL producers, and the ability of specialized technicians to correctly interpret MBL inhibition (Yan et al., 2004; Picao et al., 2008).

False detection of MBL in *P. aeruginosa* by E-test, CDT and DDST depend on EDTA susceptibility (Chu et al., 2005). It is known that EDTA may increase bacterial cell wall permeability and so it can increase the susceptibility of *P. aeruginosa* to various antimicrobials, including IMP (Ayres et al., 1999). Tan et al. (2008) proposed an IMP-lysate MBL assay that overcomes the major disadvantage of the three phenotypic methods; the direct bactericidal EDTA effect on the test strain (Tan et al., 2008). It provides a simple, inexpensive and reproducible functional screen for MBL-producing *P. aeruginosa*. However, that technique requires special equipment, specifically the Microcon Device. Due to the cost and the scarcity of the device, it is typically not available in laboratories. Thus, the study modified the technique to adapt it to laboratory's conditions. After the elimination of the step of enzyme-concentration from the original protocol, screening of MBL bla_{VIM} producing *P. aeruginosa* is still simple and easy. Importantly, EDTA still has little effect on the indicator strain.

In this study, method C showed 91.66% of sensitivity and 76.92% of specificity, and referring to Youden test, method C was more efficient than method A and B.

Conclusion

Since MBL-Etest and PCR were expensive and not adaptable for extension use in clinical microbiology laboratories, a modified IMP-lysate MBL hydrolytic activity can be chosen by laboratories with limited resources as an inexpensive, simple, and accurate test to detect *P. aeruginosa* producing bla_{VIM} type enzyme.

ACKNOWLEDGEMENTS

The authors would like to thank the leaders of the "Unité de Recherche en Maladies Infectieuses et Tropicales Emergentes (URMITE) Aix-Marseille France" for allowing them to identify strains by MALDI-TOF.

Conflict of interests

The author(s) did not declare any conflict of interest.

REFERENCES

Andrade SS, Picao RC, Campana EH, Nicoletti AG, Pignatari AC, Gales AC (2007). Influence of disk preparation on detection of metallo-beta-lactamase-producing isolates by the combined disk assay. J. Clin. Microbiol. 45:2058-2060

Aubron C, Poirel L, Fortineau N, Nicolas P, Collet L, Nordmann P (2005). Nosocomial spread of *Pseudomonas aeruginos*a isolates expressing the metallo-beta-lactamase VIM-2 in a hematology unit of a French hospital. Microb. Drug Resist. 11:254-259

Ayres HM, Furr JR, Russell AD (1999). Effect of permeabilizers on antibiotic sensitivity of *Pseudomonas aeruginosa*. Lett. Appl. Microbiol. 28:13-16

Berges L, Rodriguez-Villalobos H, Deplano A, Struelens MJ (2007). Prospective evaluation of imipenem/EDTA combined disc and Etest for detection of metallo-beta-lactamase-producing *Pseudomonas aeruginosa*. J. Antimicrob. Chemother. 59:812-813

Chu YW, Cheung TK, Ngan JY, Kam KM (2005). EDTA susceptibility leading to false detection of metallo-beta-lactamase in *Pseudomonas aeruginosa* by Etest and an imipenem-EDTA disk method. Int. J. Antimicrob. Agents 26:340-341

Conejo MC, Garcia I, Martinez-Martinez L, Picabea L, Pascual A (2003). Zinc eluted from siliconized latex urinary catheters decreases OprD expression, causing carbapenem resistance in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. 47:2313-2315

Hammami S, Boutiba-Ben B, I, Ghozzi R, Saidani M, Amine S, Ben RS (2011). Nosocomial outbreak of imipenem-resistant *Pseudomonas aeruginosa* producing VIM-2 metallo-beta-lactamase in a kidney transplantation unit. Diagn. Pathol. 6:106

Hammami S, Gautier V, Ghozzi R, Da CA, Ben-Redjeb S, Arlet G (2010). Diversity in VIM-2-encoding class 1 integrons and occasional bla_{SHV-2a} carriage in isolates of a persistent, multidrug-resistant *Pseudomonas aeruginosa* clone from Tunis. Clin. Microbiol. Infect. 16:189-193

Jacobson RK, Minenza N, Nicol M, Bamford C (2012). VIM-2 metallo-beta-lactamase-producing *Pseudomonas aeruginosa* causing an outbreak in South Africa. J. Antimicrob. Chemother. 67:1797-1798

Kaye KS, Pogue JM (2015). Infections Caused by Resistant Gram-Negative Bacteria: Epidemiology and Management. Pharmacotherapy 35:949-962.

Ktari S, Mnif B, Znazen A, Rekik M, Mezghani S, Mahjoubi-Rhimi F, Hammami A (2011). Diversity of beta-lactamases in Pseudomonas aeruginosa isolates producing metallo-beta-lactamase in two Tunisian hospitals. Microb. Drug Resist. 17:25-30

Lee K, Chong Y, Shin HB, Kim YA, Yong D, Yum JH (2001). Modified Hodge and EDTA-disk synergy tests to screen metallo-beta-lactamase-producing strains of Pseudomonas and Acinetobacter

species. Clin. Microbiol. Infect. 7:88-91
Lee K, Lim YS, Yong D, Yum JH, Chong Y (2003). Evaluation of the Hodge test and the imipenem-EDTA double-disk synergy test for differentiating metallo-beta-lactamase-producing isolates of *Pseudomonas* spp. and *Acinetobacter* spp. J. Clin. Microbiol. 41:4623-4629
Liu Q, Li X, Li W, Du X, He JQ, Tao C, Feng Y (2015). Influence of carbapenem resistance on mortality of patients with *Pseudomonas aeruginosa* infection: a meta-analysis. Sci. Rep. 5:11715
Maltezou HC (2009). Metallo-beta-lactamases in Gram-negative bacteria: introducing the era of pan-resistance? Int. J. Antimicrob. Agents 33:405-407
Mansour W, Poirel L, Bettaieb D, Bouallegue O, Boujaafar N, Nordmann P (2009). Metallo-beta-lactamase-producing *Pseudomonas aeruginosa* isolates in Tunisia. Diagn. Microbiol. Infect. Dis. 64:458-461
Mazzariol A, Mammina C, Koncan R, Di G, V, Di CP, Cipolla D, Corsello G, Cornaglia G (2011). A novel VIM-type metallo-beta-lactamase (VIM-14) in a *Pseudomonas aeruginosa* clinical isolate from a neonatal intensive care unit. Clin. Microbiol. Infect. 17:722-724
Oliver A, Mulet X, Lopez-Causape C, Juan C (2015). The increasing threat of *Pseudomonas aeruginosa* high-risk clones. Drug Resist. Updat. 21-22:41-59
Peter S, Lacher A, Marschal M, Holzl F, Buhl M, Autenrieth I, Kaase M, Willmann M (2014). Evaluation of phenotypic detection methods for metallo-beta-lactamases (MBLs) in clinical isolates of *Pseudomonas aeruginosa*. Eur. J. Clin. Microbiol. Infect. Dis. 33(7):1133-1141.
Picao RC, Andrade SS, Nicoletti AG, Campana EH, Moraes GC, Mendes RE, Gales AC (2008). Metallo-beta-lactamase detection: comparative evaluation of double-disk synergy versus combined disk tests for IMP-, GIM-, SIM-, SPM-, or VIM-producing isolates. J. Clin. Microbiol. 46:2028-2037
Pitout JD, Revathi G, Chow BL, Kabera B, Kariuki S, Nordmann P, Poirel L (2008). Metallo-beta-lactamase-producing *Pseudomonas aeruginosa* isolated from a large tertiary centre in Kenya. Clin Microbiol. Infect. 14:755-759.
Qu TT, Zhang JL, Wang J, Tao J, Yu YS, Chen YG, Zhou JY, Li LJ (2009). Evaluation of phenotypic tests for detection of metallo-beta-lactamase-producing *Pseudomonas aeruginosa* strains in China. J. Clin. Microbiol. 47:1136-1142.
Sardelic S, Bedenic B, Colinon-Dupuich C, Orhanovic S, Bosnjak Z, Plecko V, Cournoyer B, Rossolini GM (2012). Infrequent finding of metallo-beta-lactamase VIM-2 in carbapenem-resistant *Pseudomonas aeruginosa* strains from Croatia. Antimicrob. Agents Chemother. 56:2746-2749.
Schisterman EF, Perkins NJ, Liu A, Bondell H (2005). Optimal cut-point and its corresponding Youden Index to discriminate individuals using pooled blood samples. Epidemiology 16:73-81.
Seng P, Drancourt M, Gouriet F, La SB, Fournier PE, Rolain JM, Raoult D (2009). Ongoing revolution in bacteriology: routine identification of bacteria by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Clin. Infect. Dis. 49:543-551
Tan J, Pitout JD, Guttman DS (2008). New and sensitive assay for determining *Pseudomonas aeruginosa* metallo-beta-lactamase resistance to imipenem. J. Clin. Microbiol. 46:1870-1872
Touati M, Diene SM, Dekhil M, Djahoudi A, Racherache A, Rolain JM (2013). Dissemination of a class I integron carrying VIM-2 carbapenemase in *Pseudomonas aeruginosa* clinical isolates from a hospital intensive care unit in Annaba, Algeria. Antimicrob. Agents Chemother. 57:2426-2427
Viedma E, Juan C, Villa J, Barrado L, Orellana MA, Sanz F, Otero JR, Oliver A, Chaves F (2012). VIM-2-producing multidrug-resistant *Pseudomonas aeruginosa* ST175 clone, Spain. Emerg. Infect. Dis 18:1235-1241
Walsh TR, Bolmstrom A, Qwarnstrom A, Gales A (2002). Evaluation of a new Etest for detecting metallo-beta-lactamases in routine clinical testing. J. Clin. Microbiol. 40:2755-2759.
Watanabe M, Iyobe S, Inoue M, Mitsuhashi S (1991). Transferable imipenem resistance in Pseudomonas aeruginosa. Antimicrob. Agents Chemother. 35:147-151
Yagi T, Kurokawa H, Shibata N, Shibayama K, Arakawa Y (2000). A preliminary survey of extended-spectrum beta-lactamases (ESBLs) in clinical isolates of *Klebsiella pneumoniae* and Escherichia coli in Japan. FEMS Microbiol. Lett. 184:53-56
Yan JJ, Wu JJ, Tsai SH, Chuang CL (2004). Comparison of the double-disk, combined disk, and Etest methods for detecting metallo-beta-lactamases in gram-negative bacilli. Diagn. Microbiol. Infect. Dis. 49:5-11
Yong D, Lee K, Yum JH, Shin HB, Rossolini GM, Chong Y (2002). Imipenem-EDTA disk method for differentiation of metallo-beta-lactamase-producing clinical isolates of *Pseudomonas spp*. and *Acinetobacter spp*. J. Clin. Microbiol. 40:3798-3801.