

**AN EVALUATION OF VARIOUS METHODS FOR THE DETECTION OF METALLO-B-LACTAMASE IN CLINICAL ISOLATES OF PSEUDOMONAS AERUGINOSA IN A TEACHING HOSPITAL OF RURAL GUJARAT, INDIA**Priyanka Davra<sup>1</sup>, Yagnesh Pandya<sup>2</sup>, Nimisha Shethwala<sup>3</sup>**HOW TO CITE THIS ARTICLE:**

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**ABSTRACT: BACKGROUND:** Metallo- $\beta$ -Lactamase (MBL) mediated resistance to various antibiotics is an emerging threat in Pseudomonas aeruginosa isolates. The present study was undertaken to detect MBL producing isolates of Pseudomonas aeruginosa from various clinical specimen by four different phenotypic methods. **OBJECTIVE:** To evaluate the various methods for the detection of Metallo- $\beta$ -Lactamase in clinical isolates of Pseudomonas aeruginosa in a teaching hospital of rural Gujarat – India. **STUDY SETTING:** Department of Microbiology, P.S. Medical College & Shree Krishna Hospital, Karamsad, Gujarat. **STUDY DESIGN:** Prospective observational. **MATERIALS AND METHODS:** Total 50 isolates of Pseudomonas aeruginosa isolated & identified from different clinical specimens as per the standard guidelines. Screening for MBL production was done by Imipenem-EDTA combined disc test, Imipenem-EDTA double disc synergy test (DDST), EDTA disc potentiation using four cephalosporins, MBL E-test. **RESULT:** Of 50 imipenem sensitive or resistant isolates, 24 were MBL positive, Of which 12(24%) were MBL positive by Imipenem-EDTA combined disc test and Imipenem-EDTA double disc synergy test (DDST), 2(4%) were MBL positive by EDTA disc potentiation using four cephalosporins and 19(63.33%) were MBL positive by MBL E-test method. **CONCLUSION:** Imipenem-EDTA combined disc test and Imipenem-EDTA double disc synergy test (DDST) are equally effective for MBL detection while MBL E-test is more effective for MBL detection but given the cost-constraints, combined disc test and DDST can be used as a convenient screening method in the clinical microbiology laboratory.

**KEYWORDS:** Imipenem-EDTA, Metallo- $\beta$ -Lactamase, Pseudomonas aeruginosa.

**INTRODUCTION:** Pseudomonas aeruginosa is one of the most important pathogens causing nosocomial infections; it is naturally resistant to many antimicrobial agents. It has a distinctive capacity to become resistant to many available antimicrobial agents via multiple mechanisms [1]. The worldwide spread of acquired metallo- $\beta$ -lactamases (MBLs) in clinically important pathogens, such as Pseudomonas spp., Acinetobacter spp., and members of the Enterobacteriaceae family, has become a great concern.[2,3]

MBL producing isolates are also associated with a higher morbidity and mortality.[4] These enzymes have the ability to hydrolyze the  $\beta$ -lactam chemical bond that distinguishes  $\beta$ -lactam antibiotics from other antibacterial agents, thereby rendering the molecules incapable of killing bacteria.

Today, over 890 unique  $\beta$ -lactamases have been identified in naturally occurring bacterial isolates.[5,6]

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These enzymes have been separated into groups, either according to the amino acid sequences of the enzymes or according to their inactivating properties for different classes of  $\beta$ -lactams. The molecular classification scheme divides  $\beta$ -lactamases into four classes based on the amino acid sequences of the proteins.<sup>[7,8]</sup>

The occurrence of an MBL positive isolate in a hospital environment poses not only a therapeutic problem, but is also a serious concern for infection control management. With the global increase in the occurrence and types of MBLs, early detection is crucial, the benefits of which include timely implementation of strict infection control practices and treatment with alternative antimicrobials.<sup>[9]</sup>

As a result of being difficult to detect, such organisms pose significant risks, particularly due to their role in unnoticed spread within institutions and their ability to participate in horizontal MBL gene transfer with other pathogens in the hospital.<sup>[9]</sup>

Therefore, early detection of MBL-producing organisms is of crucial importance for prevention of their inter- and intra- hospital dissemination, not only in institutions with high prevalence of such isolates but also in those in which phenotypes of resistance have never been detected. Various criteria for screening for MBL production in *Pseudomonas aeruginosa* have been suggested.<sup>[10]</sup>

Currently, no standardized method for MBL detection has been proposed and despite PCR being highly accurate and reliable, its accessibility is often limited to reference laboratories <sup>[11]</sup>. Several non-molecular techniques have been studied, all taking advantage of the enzyme's zinc dependence by using chelating agents, such as EDTA or 2 mercaptopropionic acid, to inhibit its activity.<sup>[11]</sup>

The aim of this study was to evaluate the accuracy of phenotypic tests for screening for MBL-producing isolates among *Pseudomonas aeruginosa* isolates.

**MATERIALS AND METHODS:** The study was conducted at the Shree Krishna Hospital located in Karamsad, The hospital has an advanced Central Diagnostic Laboratory which is well equipped and has been accredited by National Accreditation Board of Laboratories for Testing and calibration.

**Bacterial Isolates:** 50 non-duplicate *Pseudomonas aeruginosa* strains isolated from various clinical specimens collected during the period of 3rd June 2013 to 27<sup>th</sup> August 2013. The isolates were identified as *Pseudomonas aeruginosa* by conventional methods as well as by Vitek- 2 system (bioMerieux-France).

**Antibiotic susceptibility Testing**<sup>[12]</sup>: Antimicrobial susceptibility of all the isolates was performed by Kirby-Bauer disc diffusion method as per CLSI guidelines.

**Following antibiotic tested for pseudomonas Aeruginosa:** Levofloxacin (5 $\mu$ g), Imipenem (10 $\mu$ g), Meropenem (10 $\mu$ g), Ceftazidime (30 $\mu$ g), Gentamicin (10 $\mu$ g), Tobramycin (10 $\mu$ g), Piperacilin (100 $\mu$ g), Amikacin (30 $\mu$ g), Cefepime (30 $\mu$ g), Ciprofloxacin (5 $\mu$ g), Piperacillin-taxobactam (100/10 $\mu$ g), Ticracilin (75 $\mu$ g), Colistin, Ofloxacin (5 $\mu$ g), Norfloxacin (10 $\mu$ g), Nitrofurantion (300 $\mu$ g) from Hi-Media Laboratories. Antibiotic susceptibility of all the isolates was also done by the VITEK 2 advanced expert system.

**Metallo- $\beta$ -Lactamase Screening:** Clinical isolates of *Pseudomonas aeruginosa* were collected and confirmed. All the confirmed *Pseudomonas aeruginosa* isolates were tested for Metallo- $\beta$ -Lactamase producer by the following methods:

**Imipenem(IMP)-EDTA combined disc Test:** To identify Metallo- $\beta$ -Lactamase (MBL) production in these isolates, we used IMP-EDTA combined disc test developed by Yong D et al.<sup>[13]</sup>

**METHODS:** The strains were adjusted to the McFarland 0.5 standard and inoculated on to plates with Mueller Hinton agar as recommended by the CLSI.<sup>[14]</sup>

A 10 $\mu$ g imipenem disc and an imipenem-EDTA (750 $\mu$ g) disc were placed on Mueller Hinton agar. Another disc containing only 750 $\mu$ g EDTA was also placed as a control. Incubate the plates for overnight.<sup>[13]</sup> To make 0.5 M EDTA solution 186.1g of disodium EDTA was dissolved in 1000 ml of distilled water and pH was adjusted to 8.0 by using NaOH.

The mixture was then sterilized by autoclaving. EDTA-imipenem discs were prepared by adding EDTA solution to 10- $\mu$ g-imipenem discs to obtain a concentration of 750  $\mu$ g. The discs were dried immediately in an incubator and stored at 4 $^{\circ}$ C or -20 $^{\circ}$ C in an air tight vial. (color plate-1).

**Interpretation:** After overnight incubation, the established zone diameter difference of  $\geq 7$  mm between imipenem disc and imipenem plus EDTA was interpreted as MBL positive.<sup>[13]</sup>

**Imipenem-EDTA double disc synergy test (DDST):** We used Imipenem-EDTA double disc synergy test developed by Lee et al.<sup>[15]</sup>

**METHODS:** The strains were adjusted to the McFarland 0.5 standard and inoculated on to plates with Mueller Hinton agar as recommended by the CLSI.<sup>[14]</sup>

(10 $\mu$ g) imipenem and imipenem-EDTA(750 $\mu$ g) disc was placed 20 mm centre to centre from EDTA(750 $\mu$ g) discs on Mueller Hinton agar. Incubate the plates for overnight. <sup>[15]</sup> (colour plate-2).

**Interpretation:** After overnight incubation, Enhancement of the zone of inhibition in the area between imipenem and the EDTA disc and the zone of enhancement in the area between imipenem-EDTA (750 $\mu$ g) and the EDTA disc in comparison with the zone of inhibition on the far side of the drug was interpreted as a positive result.<sup>[15]</sup>

**EDTA disc potentiation using ceftazidime, ceftizoxime, cefepime, and Cefotaxime:** We used EDTA disc potentiation with four cephalosporins (ceftazidime, ceftizoxime, cefepime, and cefotaxime).

**METHODS:** The strains were adjusted to the McFarland 0.5 standard and inoculated on to plates with Mueller Hinton agar as recommended by the CLSI.<sup>[14]</sup>

EDTA (750 $\mu$ g) disc was placed and the following discs ceftazidime (30  $\mu$ g), ceftizoxime (30  $\mu$ g), cefepime (30  $\mu$ g), and cefotaxime (30  $\mu$ g) were placed 25mm centre to centre from the EDTA (750 $\mu$ g) disc. Incubate the plates for overnight.<sup>[16]</sup> (color plate-3).

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**Interpretation:** After overnight incubation, Enhancement of the zone of inhibition in the area between EDTA disc and any one of the four cephalosporins discs in comparison with the zone of inhibition on the far side of the drug was interpreted as a positive result.<sup>[16]</sup>

**MBL E-Test:** We used the E test MBL strip containing a double sided seven-dilution range of MRP (4 to 256 mcg/ml) and MRP+EDTA (1 to 64 mcg/ml).

**METHODS:** The strains were adjusted to the McFarland 0.5 standard and inoculated on to plates with Mueller Hinton agar as recommended by the CLSI.<sup>[14]</sup>

Removed Ezy MIC™ strip container from cold and keep it at room temperature for 15 minutes before opening. Placed the strip at a desired position on inoculated agar plate with the help of applicator Incubated the plates for overnight. (Color plate-4).

**Interpretation:** MIC ratio of MRP (meropenem)/MRP+EDTA (meropenem+EDTA) of >8 interpreted as a positive result.

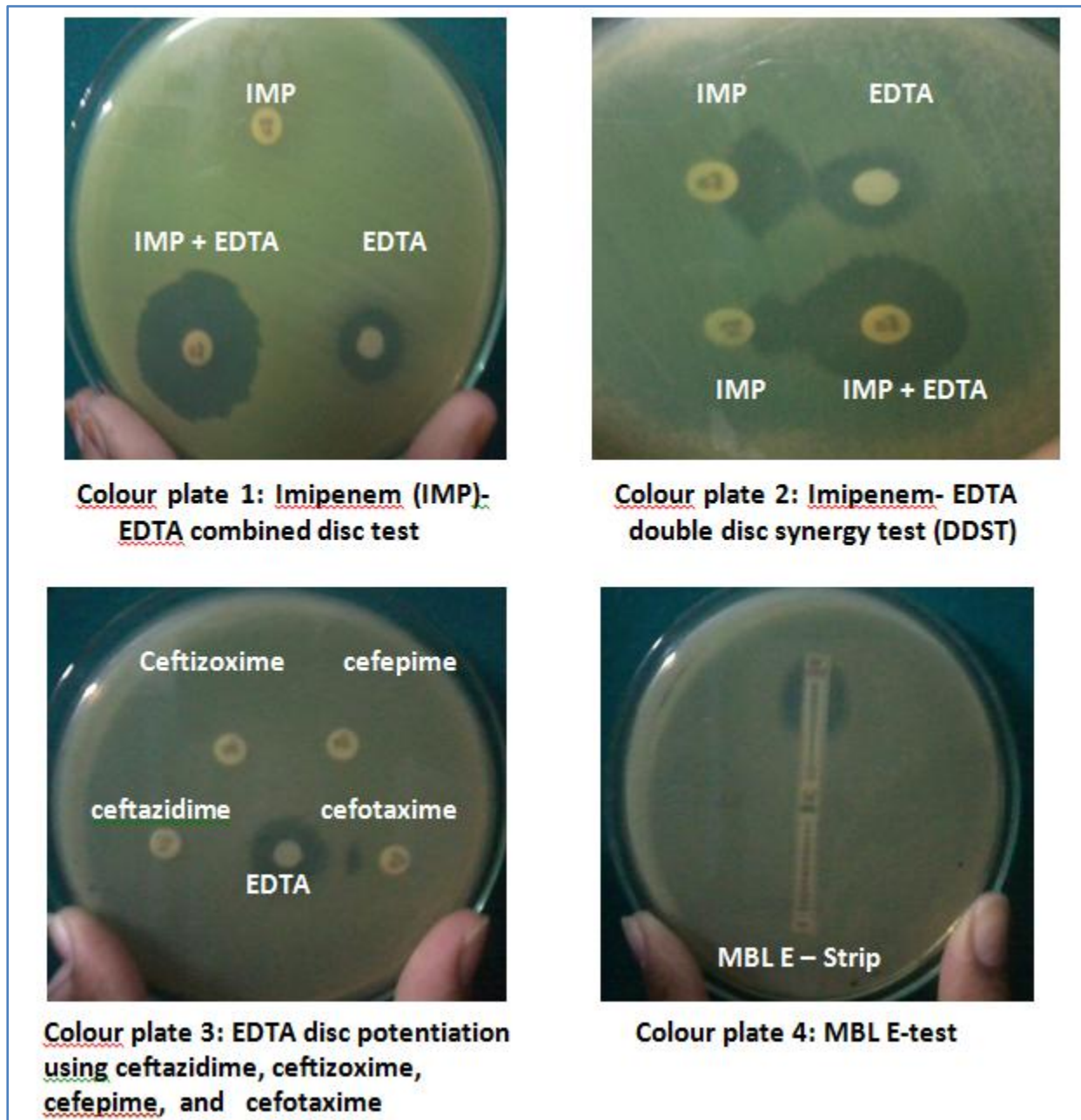
**RESULTS:** A total of 50 isolates of *Pseudomonas aeruginosa* were included in the study. Of the 50 isolates of *P. aeruginosa*, 18 were isolated from Pus, 10 from urine, 07 from Sputum, 06 from E.T., 05 from blood, and 04 from T.S.

Sr. No.	Test	Applied on No. of isolates	MBL	
			Negative	Positive
1	IMP-EDTA combined disc test	50	38	12
2	IMP-EDTA double disc synergy test	50	38	12
3	EDTA disc potentiation using ceftazidime, cefotaxime, ceftizoxime & cefepime	50	48	02
4	E-Test	30	11	19

**Table 1: Evaluation Of Various Methods**

50 isolates were tested for MBL production. 12 of these 50 isolates exhibited a  $\geq 7$  mm zone size enhancement in the combined disc test, as well as 12 isolates gave positive result by DDST. In the combined disc test, the mean  $\pm$  S.D (95% confidence interval) zone augmentation values of the MBL positive and negative isolates were  $15.16 \pm 5.54$  and  $1.78 \pm 2.39$  mm respectively.

EDTA disc potentiation with four cephalosporins. (ceftazidime, ceftizoxime, cefepime, cefotaxime) was not as good as other methods to detect MBL production only 2 isolates gave positive result by these method while MBL E- test was done in 30 isolates and it gave MBL production in 19 isolates.



**DISCUSSION:** Carbapenems have been the most successful  $\beta$ -lactam antibiotics used in the treatment of infections caused by  $\beta$ -lactam resistant Gram-negative bacteria. However, the clinical utility of these antimicrobials is under threat with the emergence of carbapenemases, particularly the Ambler class B Metallo  $\beta$ -lactamases (MBLs).

MBLs can hydrolyze most  $\beta$ -lactams except for monobactams and confer a broad-spectrum  $\beta$ -lactam resistance phenotype to the bacterial host, which is not reversible by conventional therapeutic  $\beta$ -lactamase inhibitors. The prevalence of MBLs has been increasing worldwide, notably among *Pseudomonas aeruginosa* and lately, amongst other Gram-negative bacteria as well.<sup>[17]</sup>

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Production of MBL by *Pseudomonas* spp. And other gram negative bacteria has tremendous therapeutic consequences, since these organisms also carry other multidrug resistance genes and the only viable treatment option remains the potentially toxic polymyxin B and Colistin.<sup>[18]</sup>

All the methods for detection of MBL producing bacterial isolates depend on the principle, that MBLs are affected by the removal of zinc from their active site. Still, no single screening method has been found to be perfect. Currently, there is no Clinical Laboratory Standards Institute (CLSI) recommended method available. Also, no standard method is recommended by any other international committee for the detection of MBL producers.<sup>[19]</sup>

In this study, we have used four different methods of screening for MBL production. With the Imipenem and EDTA combined disc test with a cut-off >7 mm, the positive and negative results were more clearly discriminated. DDST have a disadvantage of subjective interpretation of result in some instances. But here it also gave a similar result that of the combined disc test. hence, The combined disc test using imipenem + EDTA and DDST using imipenem - EDTA were found to be superior to EDTA disc potentiation with ceftazidime, cefotaxime, ceftizoxime, cefepime.

We also found the MBL E-test to be very sensitive for detection of MBL on *Pseudomonas aeruginosa*. In our study, we screened random isolates with MBL E -Test, We found that out of 30 isolates, 19 isolates were MBL positive by E test. The E test MBL strip, based on a combination of a  $\beta$  lactam substrate and a  $\beta$  lactam/ metallo  $\beta$  lactamase inhibitor is specifically designed to detect as many clinically relevant MBL as possible.<sup>[20]</sup>

The E- test MBL strip has the ability to detect metallo  $\beta$  lactamases, both chromosomally and plasmid mediated, in aerobic and anaerobic bacteria <sup>[20]</sup>. This novel method could be used by clinical laboratories to monitor the emergence of metallo  $\beta$  lactamase in a range of clinically significant bacteria and by surveillance network to establish the spread of the enzyme.

Though, the Imipenem-EDTA combined disc test and Imipenem-EDTA double disc synergy test (DDST) are equally effective for MBL detection but In conclusion, in our study, the best method for screening of MBL production in *Pseudomonas aeruginosa* was E-test. However, given the cost constraints of E-test, a simple screening method like combined disc test using imipenem + EDTA and DDST using imipenem - EDTA can be used.

This method provides a simple, inexpensive, and reproducible functional screen for MBL-producing *Pseudomonas aeruginosa*. It is very easy, economical and can be incorporated into the routine testing of any busy Microbiology laboratory, as has been done for ESBL screening.

### REFERENCES:

1. Hocquet D, Berthelot P, Roussel-Delvallez M, Favre R, Jeannot K, Bajolet O et al. *Pseudomonas aeruginosa* may accumulate drug resistance mechanisms without losing its ability to cause bloodstream infections. *Antimicrob Agents Chemother* 2007; 51: 3531-6.
2. Gupta, V. Metallo beta lactamases in *Pseudomonas aeruginosa* and *Acinetobacter* species. *Expert Opin Investig Drugs*. 2008; 17:131-143.
3. Lincopan, N., J. A. McCulloch, C. Reinert, V. C. Cassettari, A. C. Gales, and E. M. Mamizuka. First isolation of metallo- $\beta$ -lactamase-producing multiresistant *Klebsiella pneumoniae* from a patient in Brazil. *J Clin Microbiol*. 2005; 43:516-519.
4. Walsh TR, Toleman MA, Poirel L, Nordmann P. Metallo- $\beta$ -Lactamases: the Quiet before the Storm? *Clin Microbiol Rev* 2005; 18:306-25.

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5. Jacoby GA, Bush K. Amino acid sequences for TEM, SHV and OXA extended-spectrum and inhibitor resistant  $\beta$ -lactamases 2009.
6. Bush K, Jacoby GA. An updated functional classification of  $\beta$ -lactamases. *Antimicrob Agents Chemother.* 2010; 54: 969-976.
7. Ambler RP. The structure of  $\beta$ -lactamases. *Philos Trans R Soc Lond B Biol Sci.* 1980, 289: 321-331.
8. Huovinen P, Huovinen S, Jacoby GA. Sequence of PSE-2 beta-lactamase. *Antimicrob Agents Chemother.* 1988; 32:134-136.
9. Nordmann P, Poirel L. Emerging carbapenemases in gram-negative aerobes. *Clin Microbiol. Infect.* 2002; 8: 321-31.
10. Picao R C, S S Andrade, A G Nicoletti, E H Campana, G C Moraes, R E Mendes, A C Gales. 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.* 2008; 46: 2028-2037.
11. Franklin C, Liolios L, Peleg AY. Phenotypic detection of carbapenem- susceptible metallo- $\beta$ -lactamase producing Gram-negative bacilli in the clinical laboratory. *J Clin Microbiol* 2006; 44: 3139-44.
12. Collier JG, Diduid JP, Fraser AG. Tests for the identification of bacteria. In: Mackie and Mc Cartney - *Practical medical Microbiology*. 14<sup>th</sup> ed. (Chachill Livingstone, Edinburgh) 1996; 131-418.
13. Yong D, Lee K, Yum JH, Shin HB, Rossolini GM, Chong Y. Imipenem-EDTA disk method for differentiation of metallo- $\beta$ -lactamases producing clinical isolates of *Pseudomonas* spp. and *Acinetobacter* spp. *J Clin Microbiol* 2002; 40: 3798-801.
14. Clinical and Laboratory standards institute (CLSI). Performance standards for antimicrobial susceptibility testing, 16<sup>th</sup> informational supplements. CLSI Document M2-A9, Wayne PA: 2006.
15. Lee K, Lim YS, Yong D, Yum JH, Chong Y. Evaluation of the Hodge test and the imipenem-EDTA double disk synergy test for differentiation of metallo-  $\beta$ -lactamases producing clinical isolates of *Pseudomonas* spp. and *Acinetobacter* spp. *J Clin Microbiol.* 2003; 41: 4623-9.
16. Arakawa Y, Shibata N, Shibayama K, Kurokawa H, Yagi T, Fujiwara H, et al. Convenient test for screening metallo- $\beta$ -lactamases producing gram-negative bacteria using thiol compounds. *J Clin Microbiol.* 2000; 38: 40-3.
17. Walsh TR, Toleman MA, Poirel L, Nordman P. Mettalo  $\beta$ - lactamases. *Clin Microbiol Rev.* 2005; 18: 306-25.
18. Livermore DM, Woodford N. Carbapenemase. *Curr Opin Microbiol.* 2000; 3: 489-95.
19. Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing, 7<sup>th</sup> Informational Supplement (M100-S17). Wayne, PA: Clinical Laboratory Standards; 2007.
20. Walsh TR, Bolmstrom A, Qwarnstrom A, Gales A. Evaluation of a new E test for detecting metallo- $\beta$ -lactamases in routine clinical testing. *J Clin Microbiol* 2002; 40: 2755-9.

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