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A simple screening test for the detection of metallo-β-lactamase-producing *Pseudomonas aeruginosa* and *Acinetobacter* in a tertiary care hospital

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Abstract. Clinical utilization of carbapenems remains under threat with the emergence of acquired carbapenemase-producing bacteria, particularly metallo-β-lactamases (MBL). Rapid detection of MBL-producing Gram-negative bacilli is essential to prevent their widespread dissemination. However, no standardized detection method is available for routine laboratory use. The purpose of the study was to evaluate a chelating-agent based double disk synergic test and disk potentiation test for MBL-producing strain detection and to determine the isolation rate of MBL-producing *Pseudomonas aeruginosa* and *Acinetobacter* from clinical samples in our tertiary teaching hospital. A total of 22 and 66 imipenem-resistant *P. aeruginosa* and *Acinetobacter* isolates respectively were tested with ceftazidime (CAZ) disk by modified double disk synergic test and disk potentiation test using ethylenediaminetetraacetic acid (EDTA) and 2-mercaptopropionic acid (as chelating agents) to detect MBL production. The tests were compared with EDTA-phenanthroline-imipenem (EPI) microdilution MIC test as gold standard. MBL positive strains were detected in 17 (77.3%) *P. aeruginosa* and 2 (3.5%) *Acinetobacter* isolates. The disk potentiation test with 2-mercaptopropionic acid (2-MPA) dilution of 1:12 provided the most acceptable sensitivities and specificities (88.2% sensitivity and 100% specificity in *P. aeruginosa*; 100% sensitivity and specificity in *Acinetobacter*) compared to other screening methods used in this study. This study provided useful information on the local prevalence of MBL-producing *P. aeruginosa* and *Acinetobacter* in our hospital. Disc potentiation test with CAZ/2-MPA disc appears to be reliable and convenient MBL detection method in the routine clinical laboratory.

INTRODUCTION

Carbapenems are β-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 AmpC β-lactamases. However, this scenario has changed with the emergence of metallo-β-lactamase (MBL)-producing strains (Jesudason *et al.*, 2005). Resistance to carbapenem is now of global concern and being observed more frequently among non-fermenting bacteria, such as *Pseudomonas aeruginosa* and *Acinetobacter* spp. A significant proportion of carbapenem resistance in these bacteria is due to the production of acquired MBL (Livermore & Woodford, 2000, Vitkauskiene *et al.*, 2011).

MBLs are β-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 ethylenediaminetetraacetic acid (EDTA) and thiol-based compounds (Livermore & 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. The MBLs have

been classified as Group 3 (Ambler Class B) β-lactamases according to their functional properties and molecular structure (Ambler, 1980; Bush *et al.*, 1995). These enzymes can hydrolyse all classes of β-lactam drugs and withstand neutralization by β-lactamase inhibitors.

MBL-producing Gram-negative bacilli have been increasingly reported in Asia, Europe, Latin American and the United States (Kurokawa *et al.*, 1999; Iyobe *et al.*, 2000; Chu *et al.*, 2001; Miriagou *et al.*, 2003; Toleman *et al.*, 2004). MBL-producing isolates of *P. aeruginosa* and *Serratia marcescens* probably have the highest incidence in Japan (Kurokawa *et al.*, 1999). Fritsche *et al.* (2005) reported that, the increase in resistance to carbapenems by various mechanisms has been observed in the Asia-Pacific, Europe and Latin America. 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.

In recent years, MBL genes have spread from *P. aeruginosa* to *Enterobacteriaceae*, and a clinical scenario appears to be developing that could simulate the global spread of extended-spectrum β-lactamases. Moreover, given that MBLs will hydrolyse virtually all classes of β-lactams and the implementation of a therapeutic inhibitor are several years away, their continued spread would be a clinical catastrophe (Walsh *et al.*, 2005).

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. However, the Clinical and Laboratory Standards Institute (CLSI), 2011 documents do not as yet describe methods for screening and confirmation of the suspected MBL-producing isolates of *P. areuginosa* and *Acinetobacter*. 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 or 2-mercaptopropionic acid (2-MPA), to inhibit its activity (Arakawa *et al.*, 2000; Yong *et al.*, 2002). The MBL non-molecular methods that have been developed include modified Hodge test, double disk synergic test, disk potentiation test, microdilution test, Etest and carbapenem hydrolysis. Although various MBL detection techniques have been investigated, there are currently no perfect phenotypic methods for the detection of all transferable MBLs (Walsh *et al.*, 2005). Thus, the evaluation and use of a simple, reliable and inexpensive testing method for screening of MBL-producers in routine laboratory has become necessary.

The objectives of this study were to evaluate a chelating agent based MBL detection test for routine use in laboratory and to determine the prevalence of MBL-producing *P. aeruginosa* and *Acinetobacter* in clinical samples from Hospital Universiti Sains Malaysia (HUSM), Kelantan, Malaysia.

MATERIALS AND METHODS

Bacterial isolates

A total of 88 clinical isolates consisting of 22 strains of imipenem-resistant *P. aeruginosa*, 57 strains of imipenem-resistant *Acinetobacter* spp. and 9 strains of imipenem-resistant *Acinetobacter baumannii* were used. These isolates were from selected clinical specimens received by Medical Microbiology Laboratory in HUSM, Kelantan, Malaysia in a twelve month period from January 2009 to December 2009. All of the isolated strains were screened for resistance to imipenem by the routine disc diffusion test according to the Clinical and Laboratory Standards Institute (CLSI) recommendations. *P. aeruginosa* American Type Culture

Collection (ATCC) 27853 and *A. baumannii* ATCC 19606 were included in the detection methods as negative controls.

Phenotypic detection of MBLs and AmpC β-lactamases

The phenotypic detection of MBL was performed using EDTA-phenanthroline-imipenem (EPI) microdilution test as described by Migliavacca *et al.* (2002). This test was used as gold standard for the detection of MBLs production among the isolates in this study. The EPI microdilution test was carried out as follows. Briefly, imipenem (IPM) MICs were determined with a standard microdilution assay in 96-well microtiter plates, using Mueller-Hinton (MH) broth and a bacterial inoculum of 5×10^4 CFU per well, in a final volume of 100 µl. IPM concentrations in the range of 256 to 0.25 µg/ml were tested. The MICs were determined either in the absence or in the presence of a mixture of EDTA plus 1,10-phenanthroline. The final concentration of EDTA and 1,10-phenanthroline in each well was 0.4 mM and 0.04 mM respectively. In the presence of chelator mixtures, an IPM MIC reduction of fourfold or more is considered as MBL positive.

The double disk synergic test and disk potentiation test performed in this study were modified from Arakawa *et al.* (2000); Lee *et al.* (2000); and Yagi *et al.* (2005). Double disk synergic test was employed using disks impregnated with EDTA 0.5M, EDTA 0.1M and 2-MPA 1:8 dilution to detect MBL production in these isolates. Colonies of each bacterial strain were suspended and diluted with MH broth to obtain 10^8 CFU/ml and spread on an MH agar plate with a sterile cotton swab. Four disks containing 30 µg of ceftazidime (CAZ) were placed on the plates. The distance between each CAZ disk was about 30 - 40 mm from center to center. A blank disk was placed 10 - 15 mm (center to center) from one of the CAZ disks. Subsequently, 10 µl of 0.1 M EDTA was added to the blank disk. Another two blank disks were placed near the other two CAZ disks respectively, at a distance between 15 and 20 mm (center to center). Five µl of 0.5 M EDTA was added to one of the blank disks on the agar, and 3 µl of diluted 1:8 of 2-MPA was added to another blank disk. Then, 5 µl of 300 µg 3-aminophenylboronic acid (3-APB) was added directly to another CAZ disk which did not have an adjacent blank disk (Figure 1). The agar plates were incubated at 37ºC overnight. A distinct enhancement of inhibitory zone between CAZ disk and disk containing chelators was considered as positive MBL. The difference in diameters of inhibitory zone around CAZ+APB disk and CAZ disk by $\geq$ 5 mm and a reduction of CAZ MICs by eightfold or more in the addition of APB indicate AmpC β-lactamase positive (Yagi *et al.*, 2005).

Disk potentiation test was employed with 0.1 M EDTA and 1:12 2-MPA to detect MBL production by *P. aeruginosa*, and *Acinetobacter*. The same bacterial strain suspension (10^8 CFU/ml) was spread on another MH agar plate with sterile cotton swab. Three disks containing 30 µg of CAZ were placed on the plates. The distance between each CAZ disk was kept at about 30 - 40 mm from center to center. Two blank disks were placed on the agar plate. The distance between blank disk and CAZ disk or between blank disk and blank disk was kept

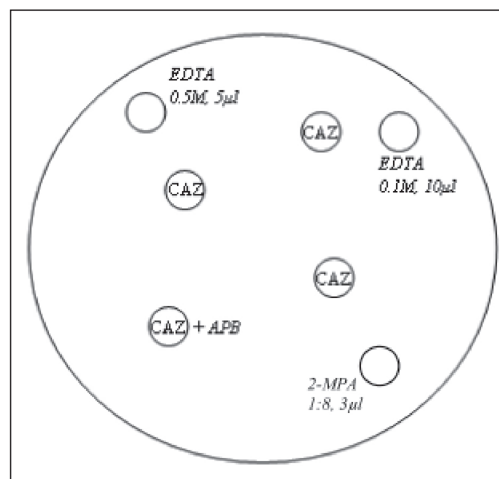

Figure 1. Double disk synergic test. Distance between CAZ disk and disk with EDTA 0.1 M: 10-15 mm; distance between CAZ disk and disk with EDTA 0.5 M: 15-20 mm; distance between CAZ disk and disk with 2-MPA 1:8: 15-20 mm; distance between every CAZ disks including CAZ+APB disk: 30-40 mm. All distances between disks were measured from center to center

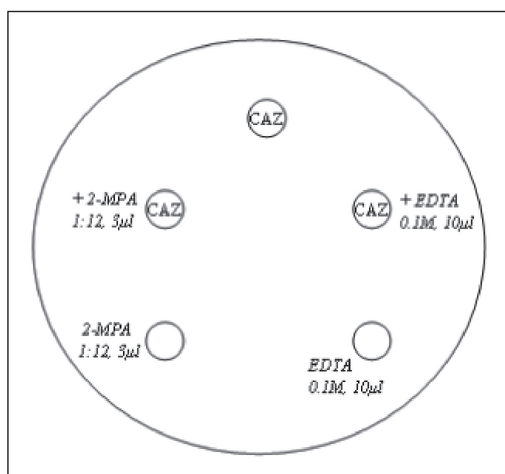

Figure 2. Disk potentiation test. Distance between every single disks (including CAZ disks and blank disks): 30-40 mm from center to center

at about 30 - 40 mm (center to center). Ten microliters of 0.1 M EDTA was added respectively to a CAZ disk and a blank disk. Three microliters of 1:12 diluted 2-MPA was added respectively to another CAZ disk and another blank disk (Figure 2). The agar plates were incubated at 37°C overnight. In disk potentiation test, there was no standardized cut-off value for a significant difference in the inhibitory zone sizes around CAZ+2-MPA disk or CAZ+EDTA disk as compared to CAZ disk without 2-MPA or EDTA. Therefore, the cut-off values were determined accordingly after obtaining the results.

RESULTS

Detection of MBLs by EPI Microdiluton MIC test

Out of 22 *P. aeruginosa* isolates, 17 (77.3%) were positive for MBLs, as they showed equal to or more than fourfold reduction of IMP MIC in the presence of chelating agents like EDTA and 1,10-phenanthroline. Thirteen (76.5%) of the *P. aeruginosa* isolates tested positive to MBLs exhibited high-level imipenem resistance (IMP MICs of >32 µg/ml). For *Acinetobacter* spp. isolates, only two strains (3.5 %) were detected as positive for MBLs by EPI microdilution MIC test. There were no MBL-producing *A. baumannii* detected from the selected samples. Majority (70%) of *Acinetobacter* isolates exhibited a high IMP MIC ($\geq$64 µg/ml) although they were negative for MBLs (Table 1).

All the *P. aeruginosa* and *Acinetobacter* isolates were also tested by disk potentiation test and Microdilution MIC Test with APB for the presence of AmpC β-lactamases as the enzymes could also produce resistance to imipenem. There were three *P. aeruginosa* isolates exhibited an enlargement of growth inhibitory zone equal to or greater than 5mm by disk potentiation test and one of the isolates was positive for MBL production. Four *Acinetobacter* spp. isolates were found to be positive for AmpC β-lactamases production by disk potentiation test and all of them were non-MBL producer. However, the microdilution MIC test result showed that all

Table 1. Imipenem MICs of MBL positive and negative isolates

Organisms	MBLs[a]	Total organisms tested (N)	Imipenem MICs (µg/ml)[b] (No. of organisms)					
			$\geq$256	128	64	32	16	$\leq$8
P. aeruginosa	Positive	17	7	3	3	2	1	1[c]
	Negative	5	3	0	1	1	0	0
Acinetobacter spp	Positive	2	0	0	2	0	0	0
	Negative	55	16	10	11	11	7	0
A. baumannii	Positive	0	–	–	–	–	–	–
	Negative	9	9	0	0	0	0	0

[a]Detection by EPI microdilution MIC test with chelator mixture (EDTA and 1,10-phenanthroline)
[b]Imipenem MICs without adding chelator mixture
[c]The imipenem MIC of the particular strain was 8 µg/ml

these isolates were negative for AmpC β-lactamases production and had ceftazidime MICs greater than 128 µg/ml.

Detection of MBL-producing *P. aeruginosa* and *Acinetobacter* by double disk synergic test

As shown in Table 2, out of the 17 MBL positive *P. aeruginosa*, 15 (68.2%) isolates were MBL positive by double disk synergic test using 0.1 M EDTA and 2-MPA disks while 12 (54.5%) isolates were positive using 0.5 M EDTA disk. An inconclusive result was considered if it did not show a typical enhancement or clear expansion of growth inhibitory zone between the CAZ disk and disk containing chelator. The use of 0.5 M EDTA disk produced more inconclusive results compared to 0.1 M EDTA and 2-MPA disks.

For *Acinetobacter* spp. only 2 isolates (3.5%) were tested as positive for MBLs by both concentrations of EDTA and 2-MPA. However, disk containing 0.1 M EDTA produced the most inconclusive results (33.3%). All the MBL negative *A. baumannii* by EPI microdilution MIC test were also negative for MBLs with EDTA and 2-MPA disks but 4 (44.4%) isolates produced inconclusive results with 0.1 M EDTA disk. All the inconclusive results obtained in all isolates by double disk synergic test were considered false positive results as they have been tested negative for MBLs by EPI microdilution MIC test.

Detection of MBL-producing *P. aeruginosa* and *Acinetobacter* by disk potentiation test

For disk potentiation test using EDTA/CAZ disk, the mean increments of inhibition zone diameter for MBL-producing *P. aeruginosa* and *Acinetobacter* spp. were 10±3 mm (range: 6-15 mm) and 7.5±0.5 mm (range: 7-8 mm) respectively while the mean increments of diameter for MBL-negative *P. aeruginosa*, *Acinetobacter* spp. and *A. baumannii* were 2±1 mm (range: 1-4 mm), 3±1 mm (range: 1-4 mm) and 3±1 mm (range 3-4 mm) respectively. For test using 2-MPA/CAZ disk, the mean increments of inhibition zone diameter for MBL-producing *P. aeruginosa* and *Acinetobacter* spp. were 14±4 mm (range: 5-19 mm) and 19±1 mm (range: 18-20 mm) respectively while the non-MBL producer showed mean increment of 2±1 mm in diameter for both *P. aeruginosa* (range: 1-4 mm) and *Acinetobacter* spp. (range: 1-2 mm). Based on the findings, we have recommended

Table 2. Detection of MBL producing isolates by double disk synergic test using 0.5 M, 0.1 M EDTA and 1:8 2-MPA disks

EPI micodilution MIC test (n=88)		MBL detection (n=88)					
Isolates	No. of strains	EDTA 0.1 M		EDTA 0.5 M		2-MPA 1:8	
		Pos	Neg	Pos	Neg	Pos	Neg
P. aeruginosa							
MBL positive	17(77.3%)	15(68.2%)	2(9.1%)	12(54.6%)	5(22.7%)	15(68.2%)	2(9.1%)
MBL negative	5(22.7%)	1(4.5%)	4(18.2%)	4(18.2%)	1(4.5%)	1(4.5%)	4(18.2%)
Acinetobacter spp							
MBL positive	2(3.5%)	2(3.5%)	0	2(3.5%)	0	2(3.5%)	0
MBL negative	55(96.5%)	19(33.3%)	36(63.2%)	3(5.3%)	52(91.2%)	0	55(96.5%)
A. baumannii							
MBL positive	0	0	0	0	0	0	0
MBL negative	9(100%)	4(44.4%)	5(55.6%)	0	9(100%)	0	9(100%)

Note 1. Each blank disk in double disk synergic test was added with the respective concentration and volume of EDTA or 2-MPA
2. The results were considered as positive when a distinct expansion of growth inhibitory zone between the CAZ disk and disk containing chelator was observed

the cut-off values of growth inhibitory zone diameter increment around EDTA/CAZ and 2-MPA/CAZ disks compared to CAZ disk alone for positive MBL as equal to or more than 6 mm and 5 mm respectively.

Out of the 17 MBL producing *P. aeruginosa*, 15 (68.2%) isolates were tested as MBL positive by both methods using 0.1 M EDTA/CAZ and 1:12 2-MPA/CAZ disks, without producing any inconclusive result. The two MBL positive isolates which became negative for MBL by disk potentiation test had large zone of inhibition (29 mm) around 30 µg of CAZ disk and there was only 1 mm increase in diameter with CAZ+EDTA or CAZ+2-MPA. Furthermore, the imipenem MICs for these two isolates were 8 µg/ml and 64 µg/ml respectively. For *Acinetobacter* spp., 2 isolates (3.5%) were positive for MBL by both EDTA and 2-MPA methods; whereas for *A. baumannii*, no isolate tested positive for MBL (Table 3).

Comparison of double disk synergic test and disk potentiation test for detection of MBLs production

Table 4 shows the comparative results of double disk synergic test and disk potentiation test with EDTA and 2-MPA in the detection of MBL producing *P. aeruginosa* and *Acinetobacter*. Double disk synergic test with EDTA and 2-MPA produced more inconclusive results but disk potentiation test with 2-MPA did not show any inconclusive results. In addition, disk potentiation test with 2-MPA exhibited higher sensitivity and specificity in detection of MBL producing *P. aeruginosa* and *Acinetobacter* compared to other methods in this study.

DISCUSSION

MBL-producing Gram-negative bacteria infections are associated with high morbidity and mortality. Therefore, rapid detection of these highly resistant bacteria is crucial for infection control management and assisting the physicians in instituting appropriate treatment options. The CLSI currently does not recommend a standard testing method for the detection of MBLs. Different studies have reported the use of phenotypic methods such as imipenem-EDTA combined disk test, double disk synergic test using imipenem and EDTA, E-test and modified Hodge

Table 3. Detection of MBL producing isolates by disk potentiation test using 0.1 M EDTA and 1:12 2-MPA

EPI micodilution MIC test (n=88)		MBL detection (n=88)			
Isolates	No. of strains	EDTA 0.1 M		2-MPA 1:12	
		Pos	Neg	Pos	Neg
P. aeruginosa					
MBL positive	17(77.3%)	15(68.2%)	2(9.1%)	15(68.2%)	2(9.1%)
MBL negative	5(22.7%)	0	5(22.7%)	0	5(22.7%)
Acinetobacter spp					
MBL positive	2(3.5%)	2(3.5%)	0	2(3.5%)	0
MBL negative	55(96.5%)	3(5.4%)	52(94.5%)	0	55(96.5%)
A. baumannii					
MBL positive	0	0	0	0	0
MBL negative	9(100%)	0	9(100%)	0	9(100%)

Note 1. Every CAZ disk was added with the respective concentration and volume of EDTA or 2-MPA. Control for the methods were also prepared by adding the respective concentration and volume of EDTA or 2-MPA onto blank disks

2. For EDTA method, the result was positive for MBL if enlargement of growth inhibitory zone diameter around the EDTA + CAZ disk was $\geq$ 6 mm compared to CAZ disk alone

3. For 2-MPA method, the result was positive for MBL if enlargement of growth inhibitory zone diameter around the EDTA + CAZ disk was $\geq$ 5 mm compared to CAZ disk alone

Table 4. Comparison of sensitivity and specificity between double disk synergic test and disk potentiation test with EDTA and 2-MPA in detection of MBL producers

Test	Method	*P. aeruginosa*		*Acinetobacter spp*		*A. baumannii*	
		Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity
Double disk synergic	EDTA 0.1 M	88.2%	80.0%	100%	65.4%	–	55.6%
	EDTA 0.5 M	70.6%	20.0%	100%	94.5%	–	100%
	2-MPA 1:8	88.2%	80.0%	100%	100%	–	100%
Disk potentiation	EDTA 0.1 M	88.2%	100%	100%	94.5%	–	100%
	2-MPA 1:12	88.2%	100%	100%	100%	–	100%

test (Arakawa *et al.*, 2000; Migliavacca *et al.*, 2002; Yong *et al.*, 2002; Lee *et al.*, 2003; Yan *et al.*, 2004; Behera *et al.*, 2008). These methods have been performed for quick and easy detection of MBL-producing strains in routine clinical laboratory but their capabilities for convenient use in local setting has to be established.

EDTA, 2-MPA and 1,10-phenanthroline have been used as chelators to block the activity of MBL for the detection of MBLs-producing strains (Goto *et al.*, 1997; Lee *et al.*, 2000; Migliavacca *et al.*, 2002). By applying the chelator, the zinc ion from the active site of the enzyme is removed and its activity is inhibited. A single chelating agent may sometimes not adequately inhibit all MBLs in certain pathogens, however in this study, we evaluated and compared the capabilities of double disk synergic test and disk potentiating test using EDTA and 2-MPA disks at different concentrations for the reliable detection of MBL-producing strains in clinical isolates. The purpose of using different concentrations of chelator was to identify the most suitable concentration to detect MBL producers among *P. aeruginosa* and *Acinetobacter* isolates as well as to optimise the detection techniques.

The detection method also included disk potentiation test using disk containing APB to determine the presence of AmpC β-lactamase in those isolates as to differentiate them from the carbapenem-resistant strains due to only MBL production. APB functions as a reversible inhibitor of AmpC β-lactamase enzyme (Beesley *et al.*, 1982). Although disk potentiation test in the presence of 0.1M EDTA has been used previously (Arakawa *et al.*, 2000; Lee *et al.*, 2000) to detect MBL production, so far there have been no reported studies on the use of 2-MPA as a chelating agent in disk potentiation technique. Moreover, there was no recommended cutoff measurement of growth inhibition in the presence of either EDTA or 2-MPA for positive MBL-producer. Therefore, cutoff values were also determined in this study.

In both double disk synergic test and disk potentiation test, 30 µg of CAZ disk was used. According to Arakawa *et al.* (2000), IMP-1 producers demonstrated high level of resistance to CAZ (MIC > 64 µg/ml), but various levels of resistance to IPM (MIC of 4 to 128 µg/ml). As a result, the inhibitory effect of 2-MPA tends to be ambiguous, especially in strains that demonstrated reduced or intermediate susceptibility to IPM (MIC of 4 to 8 mg/ml) whenever IPM disk is used. Therefore, CAZ disk seemed to be the most suitable choice over IPM disk for this test. 2-MPA (or thiol compound) used in double disk synergic test in this study was diluted to 1:8. This was different from the concentration used by Arakawa *et al.* (2000) and Lee *et al.* (2003). Both researchers used the undiluted 2-MPA in the double disk synergic test. However, at this concentration, 2-MPA itself demonstrated a large growth inhibitory zone around the disk which might be misinterpreted when reading the result. To overcome this problem, dilution of 2-MPA into 1:2, 1:4, 1:8, 1:12, and 1:16 was done. It was found that 1:8 dilution of 2- MPA was the most suitable concentration to produce optimum results in double disk synergic test. A 1:12 dilution of 2-MPA was the most suitable concentration to be used in disk potentiation method as 1:12 diluted 2-MPA itself did not produce any growth inhibitory zone around

the disk. However, there were two isolates of *P. aeruginosa* which were positive for MBL by EPI microdilution MIC test but were negative by either double disk synergic or disk potentiation test with EDTA and 2-MPA. Both isolates demonstrated imipenem MICs of 8 µg/ml and 64 µg/ml respectively. They were sensitive to CAZ disk and yielded large zones of inhibition (29mm each). The reasons for the observed findings remained unclear. We suggested that PCR screening for MBL genes such as bla_{IMP} and bla_{VIM} should be performed on all the 88 isolates so that the result could be accurately compared with double disk synergic test and disk potentiation test from our findings.

In this study, phenotypic MBL detection with various level of sensitivities (70.6% to 100%) and specificities (20% to 100%) were reported using different chelators combined with CAZ tested on different non-fermenter Gram negative isolates. Disk potentiation test using CAZ/2-MPA seemed to be the most sensitive and highly specific (100%) at detecting imipenem-resistant *P. aeruginosa* and *Acinetobacter*. The method is simple to perform, cheap and can be applied in most clinical laboratories. Overall, disk potentiation test is preferred due to its objective interpretation. Similar result was obtained by previous study (Franklin *et al.*, 2006), which reported a highly sensitive and specific disk potentiation test using IPM-EDTA disk at detecting both carbapenem-susceptible and carbapenem-resistant MBL-carrying Gram-negative isolates.

We found high prevalence of MBL-producing *P. aeruginosa* in our study and most of the isolates exhibited high level of resistance to imipenem. Previous studies conducted in a university teaching hospital in Malaysia has reported that the population of *P. aeruginosa* strains carrying MBL genes increased dramatically from year 2002 until year 2008 (Ho *et al.*, 2002; Khosravi *et al.*, 2010). In our laboratory, we did not perform routine screening test for MBL detection in all carbapenem-resistant isolates and the isolation rate of MBL carrying pathogens were unknown. Therefore, the result of this study will be useful to the clinicians and creating awareness on the presence of this highly resistant pathogen in this hospital. More attention and necessary steps are required to reduce the transmission, as the MBL genes may spread to other groups of bacteria such as *Enterobacteriaceae*. The same emerging problem has also been reported in Italy in which about 20% of all *P. aeruginosa* strains and 70% of carbapenem resistance strains produced MBLs (Lagatolla *et al.*, 2004; Luzzaro *et al.*, 2004). Most of the imipenem-resistant *Acinetobacter* spp and *A. baumannii* isolates in this study exhibited high imipenem MIC levels although they were negative for MBL. These may be due to hyperproduction of serine-β-lactamase and/ or a change in membrane permeability in the bacteria (Kohler *et al.*, 1999; Livermore & Woodford, 2000).

Our study suggested that disk potentiation test using CAZ disk impregnated with 1:12 of 2-MPA is a more reliable and convenient screening method in detecting MBL-producing *P. aeruginosa*, *Acinetobacter* spp. and *A. baumannii* compared to double disk synergic and disk potentiation test with EDTA disk. This study reports the validation of a simple and accurate MBL detection method that can be easily incorporated into the daily routine of a clinical laboratory. Early detection of MBL-producing strains is of paramount clinical importance, as it allows rapid initiation of strict infection control practices as well as therapeutic guidance for confirmed infection. Because of a modest number of isolates that produced MBLs were included in this study especially for *Acinetobacter* and determination of genotypic resistance was not performed, it is therefore necessary to further evaluate this method in future studies.

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