

**SIMPLE SCREENING TESTS FOR THE DETECTION OF METALLO- $\beta$ -LACTAMASE (MBL) PRODUCTION IN CLINICAL ISOLATES OF *PSEUDOMONAS* AND *ACINETOBACTER***

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**Abstract**

There are no standard methods for the detection of metallo- $\beta$ -lactamase (MBL) production in gram negative organism in routine microbiology practice. The present study was undertaken to evaluate the screening tests like double disk synergy test (DDST) and disk potentiation test (DPT) using ceftazidime (CAZ) and imipenem (IPM) disks with chelating agents like EDTA, 2-mercaptopyruvic acid (2-MPA). A total of 132 *Pseudomonas* and 76 *Acinetobacter* isolates were obtained from Bangabandhu Sheikh Mujib Medical University (BSMMU) and Bangladesh Institute of Research and Rehabilitation for Diabetes, Endocrine and Metabolic Disorders (BIRDEM) hospitals of Dhaka city. A total of 53 and 29 IPM resistant *Pseudomonas* and *Acinetobacter* isolates were selected. EDTA-IPM microdilution minimum inhibitory concentration (EDTA-IPM MIC) method detected MBL in 44 (83%) IPM resistant *Pseudomonas* and 19 (65.5%) *Acinetobacter* isolates. DDST with CAZ-0.1M EDTA and CAZ-2-MPA detected MBL in 73.6% and 67.9% of IPM resistant *Pseudomonas* and 55.2% and 48.3% of *Acinetobacter* isolates respectively. The detection rate was 67.9% and 66.1% in *Pseudomonas* and 51.7% and 44.8% in *Acinetobacter* isolates by EDTA-IPM and IPM-2-MPA methods respectively. In comparison to DDST, DPT with CAZ-0.1M EDTA showed higher sensitivity (89.7%) and specificity (100%) for detection of MBL in *Pseudomonas* and *Acinetobacter*. The results showed that simple screening tests like DPT with 0.1M EDTA was able to detect MBL producing *Pseudomonas* and *Acinetobacter* from clinical samples with high sensitivity and specificity.

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**Introduction**

Carbapenem, namely imipenem is the drug of choice for the treatment of infections caused by multidrug resistant gram-negative bacilli specially *Pseudomonas* and *Acinetobacter*. Recently, metallo- $\beta$ -lactamase (MBL), a carbapenemase, has been reported to be involved in mediating resistance against imipenem.<sup>1</sup> It is a class B beta-lactamase enzyme capable of hydrolyzing all  $\beta$ -lactams except monobactam and their catalytic activities are generally not inhibited by inhibitors like clavulanic acid, salbactam and tazobactam. MBLs are sensitive to metal chelators like EDTA and thiol based compounds and these inhibitors are exploited to detect MBL activities of the organisms.<sup>2</sup> Currently, there is no recommended

method for the detection of MBL in routine laboratory practice.

E-test is presently the most widely accepted standardized screening test for the detection of MBL. But E-test does not detect all MBL producing *Enterobacteriaceae* due to low level resistance and also it is expensive to use in the routine screening procedure.<sup>3</sup> Modified Hodge test, though a simple and suitable screening procedure, is difficult to interpret and may give false positive results.<sup>4</sup> Carbapenem hydrolysis test is considered as non molecular “gold standard”. But this technique utilizes specialized spectrophotometer and is not available in the routine

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diagnostic laboratory.<sup>5</sup> DDST and DPT detect MBL by using chelating agents like EDTA, 2-MPA and other thiol compounds. But, these tests need to be standardized for each type of test organism in terms of enhancement of zone of inhibition with IPM/CAZ plus chelating agents compared to IPM/CAZ alone.<sup>6</sup>

Therefore, the aim of this study is to evaluate screening tests like DDST and DPT for the detection of MBL producing *Pseudomonas* and *Acinetobacter* isolated from clinical samples.

## Materials and Methods

### Study samples

All the 208 isolates (132 *Pseudomonas* and 76 *Acinetobacter*) from sputum, urine, tracheal aspirate, blood, wound swab were obtained from the patient admitted in ICU, ward and outpatient department of BSMMU and BIRDEM hospitals. Samples were collected from January 2009 to December 2009.

### Identification and antimicrobial susceptibility testing

All the samples were routinely cultured in MacConkey agar media and blood agar plates. All the suspected colonies of *Pseudomonas* and *Acinetobacter* isolates were identified by Gram staining, colony characteristics, pigment production, motility test and other biochemical reactions.<sup>7</sup>

All the isolates were tested for imipenem susceptibility by disk diffusion method using the Kirby-Bauer technique<sup>8</sup> and as per the recommendations of the NCCLS.<sup>9</sup> Imipenem (10 $\mu$ g) and ceftazidime (30 $\mu$ g) disks were obtained from Oxoid Ltd (Basingstoke, Hampshire, UK). Antibiotic potency of the disks were standardized against the reference *Pseudomonas* ATCC 25853 strain.

### Detection of MBL-production

#### 1. EDTA-IPM microdilution MIC test

The EDTA-IPM microdilution MIC test was a modification of EPI microdilution MIC test as described by Migliavacca *et al.*<sup>10</sup> MIC of IPM were determined with a standard microdilution assay in 96 well microtiter plates using Mueller Hinton broth (MHB) and a bacterial inoculum of 5x 10<sup>4</sup> CFU per well, in a final volume of 100 $\mu$ l. IPM concentrations in the range of 512 to 0.5  $\mu$ g/ml were tested in the study. The MICs of IPM were determined with IPM

alone and IPM plus 0.4mM EDTA. The best results of MIC of IPM were observed with a concentration of EDTA of 0.4mM. One well containing the bacterial suspension alone and another well containing 0.4mM EDTA alone were used as control. Results were recorded by visual inspection of microtiter plates after 18 hour of incubation at 37°C. A minimum fourfold reduction in the MIC of IPM in presence of EDTA in comparison to IPM alone was designated as the cutoff value for detection of MBL producers.<sup>10</sup> The test has been used as gold standard for detection of MBLs production in this study.

#### 2. Double disk synergy test (DDST)

DDST was performed according to the methods described by Kim *et al* (2007).<sup>11</sup> CAZ and IPM were used with 2-MPA and EDTA in this study. Concentrations of EDTA and 2-MPA were optimized and modified accordingly. Inoculums of test organism were prepared by emulsifying 4-5 colonies of the normal saline and turbidity adjusted to 0.5 McFarland opacity standards. After inoculating the Muller Hinton plate with test organisms, two CAZ (30 $\mu$ g) and two IPM (10 $\mu$ g) disks were placed on the plate. The distance between the disks were about 3-4 cm from center to center. A blank disk was placed near one of the CAZ/IPM disk at a center to center distance of 1-1.5 cm. 10 $\mu$ l of 0.1M EDTA and 1:8 2-MPA were added in the blank disks. The agar plate was incubated at 37°C overnight. Enhancement of growth inhibitory zone between CAZ/IPM and 0.1M EDTA/1:8 2-MPA disk was considered as positive for MBLs.

#### 3. Disk potentiation test (DPT)

DPT was performed according to the method described by Galani *et al.*<sup>12</sup> Three CAZ (30 $\mu$ g) disks and three 10 $\mu$ g IPM disks were placed on the plates. The distance between every CAZ/IPM disk was about 3-4cm from center to center. 10 $\mu$ l of 0.1M EDTA was added to one CAZ/IPM disk and 10 $\mu$ l of 1:12 2-MPA was added to another CAZ/IPM disk. The plate was incubated at 37°C overnight. Enlargement of the diameter of growth inhibitory zone around CAZ/IPM + EDTA/2-MPA disk by  $\geq$ 7mm compared to CAZ/IPM alone was considered as positive for MBL.

## Result

A total of 132 *Pseudomonas* and 76 *Acinetobacter* were studied of which 90 *Pseudomonas* was isolated from

**Table-1:** Comparison of detection of MBL positive *Pseudomonas* with DDST and DPT using CAZ/IPM with EDTA and 2-MPA

Test	Method	MBL positive <i>Pseudomonas</i> isolates (n=44)							
		With CAZ				With IPM			
		Positive for MBL	Doubtful result for MBL	Sensitivity	Specificity	Positive for MBL	Doubtful result for MBL	Sensitivity	Specificity
DDST	EDTA 0.1M	39(73.6)	02(3.8)	89.7%	81.8%	36(67.9)	03(5.6)	84.6%	75%
	2-MPA 1:8	37(69.8)	03(5.6)	86.3%	75%	35(66.1)	04(7.5)	83%	69.2%
DPT	EDTA 0.1M	39(73.6)	0(0)	89.7%	100%	37(69.8)	0(0)	86.2%	100%
	2-MPA 1:12	37(69.8)	0(0)	86.3%	100%	35(66)	0(0)	83%	100%

Note: Figures in the parenthesis indicate percentage; IMP sensitive *Pseudomonas* were tested by both DDST and DPT methods

BSMMU (53 non ICU and 37 ICU) and 42 were from BIRDEM (13 non ICU and 29 ICU). Out of 76 *Acinetobacter* 62 (36 non ICU and 26 ICU) from BSMMU and 14 were from BIRDEM (6 non ICU and 8 ICU). Amongst them, 53 (40.1%) *Pseudomonas* and 29 (38.1%) *Acinetobacter* isolates were resistant to IPM. Among 53 and 29 IPM resistant *Pseudomonas* and *Acinetobacter* isolates, 44 (83.1%) *Pseudomonas* and 19 (65.5%) *Acinetobacter* were found positive for MBL by EDTA-IPM microdilution MIC test respectively.

The details of the results of DDST and DPT are given in Table 1 and 2. In DDST, higher doubtful results were obtained by EDTA-IPM (5.6%) or IPM-2-MPA (7.5%) compared to CAZ plus EDTA/2MPA disks. Similarly, higher doubtful results were also obtained with EDTA-IPM (10.3%) or IPM-2-MPA (13.8%) for *Acinetobacter* compared to CAZ-EDTA and CAZ-2-MPA by DDST. But no doubtful results have been

observed for either *Pseudomonas* or *Acinetobacter* in detecting MBL by DPT.

#### Discussion

Among the IPM resistant *Pseudomonas* and *Acinetobacter*, MBL was found positive in 83% and 65.5% of isolates respectively by EDTA-IPM microdilution MIC method. In the present study, 30 µg CAZ and 10µg IPM disks were used in DDST and DPT. MBL producers usually demonstrate high level resistance to CAZ (MIC > 64 µg/ml), but various levels of resistance to IPM (MIC, 4 to > 128 µg/ml).<sup>1</sup> As a result, the synergistic effect of metal chelators with IPM tends to be ambiguous, especially in strains that demonstrate reduced susceptibility to IPM (MIC, 4 to 8 µg/ml). So, both CAZ and IPM disks must be used simultaneously to detect all MBL producers.

**Table-2:** Comparison of detection of MBL positive *Acinetobacter* isolates with DDST and DPT using CAZ/IPM with EDTA and 2-MPA

Test	Method	MBL positive <i>Acinetobacter</i> isolates (n=19)							
		With CAZ				With IPM			
		Positive for MBL	Doubtful result for MBL	Sensitivity	Specificity	Positive for MBL	Doubtful result for MBL	Sensitivity	Specificity
DDST	EDTA 0.1M	16(55.2)	02(6.8%)	86.3%	83.3%	15(51.7)	03(10.3)	82.6%	76.9%
	2-MPA 1:8	14(48.3)	04(13.7)	79.1%	71.4%	13(44.8)	04(13.8)	76%	71.4%
DPT	EDTA 0.1M	17(58.6)	0(0)	90.4%	100%	16(55.2)	0(0)	86.3%	100%
	2-MPA 1:12	16(55.1)	0(0)	86.3%	100%	15(51.7)	0(0)	82.6%	100%

Note: Figures in the parenthesis indicate percentage; IMP sensitive *Acinetobacter* were tested by both DDST and DPT methods

In this study, initially different concentrations of EDTA (0.1M and 0.5M) and 2-MPA (1:8, 1:12) were used in DDST and DPT. The purpose of using different concentrations was to identify the most optimum concentration of these agents to detect MBL producing organism. For detection of MBL, 25 *Pseudomonas* and 19 *Acinetobacter* were tested with 0.1M and 0.5M EDTA by DDST. All gave positive results with 0.1M EDTA without any equivocal results but with 0.5M EDTA only 76% *Pseudomonas* and 73.7% *Acinetobacter* isolates were positive for MBL while 24% and 26.3% showed equivocal results respectively.

Similarly, 2-MPA at a dilution of 1:8 and 1:12 was found most suitable to use in both DDST and DPT. These dilutions exhibited a clear and distinct growth inhibitory zone between CAZ/IPM disk and disk containing 2-MPA when the result is positive. However, 2-MPA is a volatile, odorous and expensive chelating agent. EDTA is a cheap, non toxic and easily available in all the laboratories.

Distances of 1, 1.5, 2, 2.5, 3, 4 cm (from center to center) between EDTA/2-MPA and CAZ/IPM were tested for DDST for detection of MBL production. It was found that 1-1.5 cm distance between EDTA/2-MPA and CAZ/IPM disks showed a clear and distinct synergistic zone towards EDTA/2-MPA in DDST.

Based on the enhancement of mean inhibitory zone with CAZ/IPM plus 0.1M EDTA and 1:12 2-MPA compared to CAZ/IPM alone, an enlargement of zone of inhibition by  $\geq 7$ mm around CAZ/IPM-EDTA/2-MPA disk is indicative of presence of MBL by DPT.

By comparing the sensitivity and specificity of DDST and DPT to detect MBL producing *Pseudomonas* and *Acinetobacter* isolates, DPT with 0.1M EDTA provided higher sensitivity and specificity results. DDST with 2-MPA showed the lowest specificity for detection of MBL. Franklin *et al* showed that DPT had 100% sensitivity and 98% specificity whereas DDST had a sensitivity of 79% and specificity of 98%.<sup>13</sup> DPT is preferred because of its objective interpretation compared to DDST. Interpretation of DDST depends on the technician's expertise in discriminating true synergism from intersection of the inhibitory zones.<sup>14</sup> Also, it may be noted that the synergistic zone of inhibition sometimes may be masked if the resistance to CAZ is conferred by AmpC  $\beta$ -lactamase or ESBL. MBL producing *Pseudomonas* and *Acinetobacter* are emerging in our country. Rapid detection of these MBL

is necessary to institute appropriate treatment and effective infection control measures. Simple screening tests like DPT using CAZ/IPM with 0.1M EDTA can be introduced into the routine clinical laboratories for their early detection and to prevent the consequences of this worrying resistance mechanisms.

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