

## Detection of metalloβ-lactamase producing imipenem resistant acinetobacter species in intensive care unit patient in a Tertiary Care Centre

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### Abstract

**Introduction:** Metalloβ-lactamase producing *Acinetobacter* species has been reported to be an important cause of nosocomial infection and is a critical therapeutic problem worldwide, especially in the intensive care unit.

**Objectives:** To determine the frequency of metallo-β-lactamases among imipenem-resistant *Acinetobacter* species and to compare different phenotypic methods.

**Materials and Methods:** 59 imipenem-resistant *Acinetobacter* species isolated from various clinical samples were tested for metallo-β-lactamase production using different phenotypic methods. Minimal Inhibitory Concentration (MIC) to meropenem was determined by E test.

**Results:** Of all the imipenem resistant isolates, 50.8% of *Acinetobacter* species were MBL producers. MIC of all those MBL producing isolates were  $\geq 16\mu\text{g/ml}$ . Among the *Acinetobacter* species MBL production were detected in 30 isolates (50.8%) by E test, 29 isolates (49.2%) by CDT and in 15 isolates (25.4%) by DDST and DPT. The sensitivity and specificity of CDT, DDST and DPT compared to E test was 98%, 48.1%, 48.1% and 100%, respectively.

**Conclusion:** Prevalence of MBL producing *Acinetobacter* species is being increasingly reported in ICU patients. The MIC of all the MBL producing isolates for meropenem were  $>16\mu\text{g/ml}$  (Resistant). E test and CDT were more reliable for MBL detection. CDT was cost effective, easy to perform and interpretation also straightforward. MBL producing isolates were multidrug resistant making therapeutic choices limited. Continuous antibiotic surveillance, infection control practices and an effective antibiotic policy are required to address the problem of MBL – associated infections.

**Keywords:** *Acinetobacter* species, MBL detection.

### Introduction

*Acinetobacter* species is the most common pathogen causing nosocomial infections.<sup>1,2</sup> This organism is noted for their intrinsic resistance to antibiotics and for their ability to acquire genes encoding resistance determinants.<sup>3</sup> Carbapenems are used as the last choice in treating serious infections caused by multidrug resistant gram negative bacilli. Unfortunately resistance to these antibiotics started emerging and has been reported worldwide. This resistance to carbapenem is due to decreased outer membrane permeability, increased efflux systems, alteration of penicillin binding proteins and carbapenem hydrolyzing enzymes carbapenemase.<sup>4</sup> Among carbapenemases transferable MBL are the most feared because of their ability to hydrolyze all β – lactams, including carbapenems, except monobactams. The capability of MBLs to disseminate and spread through bacterial population is facilitated by its gene being encoded on an intergron – borne mobile gene cassette.<sup>4,6</sup> The rapid detection of MBL – producing GNB is necessary to aid infection control and to prevent dissemination. Also infection with MBLs result in higher mortality rates, probably related to less frequent institution of appropriate antimicrobial therapy. PCR though highly accurate and reliable, its accessibility is often limited to reference laboratories. MBL – activity is inhibited by chelating agents. Therefore several

laboratory methods using metal chelators as EDTA have been described for the phenotypic detection of MBLs among clinical isolates.<sup>2,6</sup>

### Materials and Methods

**Source of Specimens:** This study was conducted prospectively from January 2014 to June 2015 in the Department of Microbiology in a tertiary care centre. A total of 59 consecutive, non repetitive isolates of *Acinetobacter* species were included. They were isolated from various samples (Trachial aspirate, Pus, Sputum, Urine, Ascitic fluid, Pleural fluid) from intensive care unit patients.

**Isolation and identification of organisms:** Immediately after receiving the sample, the material was subjected to microscopic examination. After Gram staining specimens were inoculated onto MacConkey agar, Blood agar and Chocolate agar. Plates were incubated at 37°C for 18-24 hours. Cultures yielding insignificant growth were excluded. The colonies were subjected to Gram's stain and other biochemical tests for identification.

**Antibiotic susceptibility testing:** Antibiotic susceptibility test was performed according to CLSI (Vol 32 No.2. M07 – A 9. Wayne PA: Jan 2012) guidelines. Susceptibility was tested against the following antibiotics:

1. Amikacin (30μg)

2. Amoxycylav(20/10µg)
3. Ampicillin (10µg)
4. Cefepime (30µg)
5. Cefuroxime(30µg)
6. Cefoperazone(75µg)
7. Ciprofloxacin(5µg)
8. Cotrimoxazole(1.25/23.75µg)
9. Gentamicin(10µg)
10. Imipenem(10µg)
11. Levofloxacin(5µg)
12. Meropenem(10µg)
13. Piperacillin-Tazobactam (100/10µg)

These discs were obtained from *HiMedia* Laboratories, Mumbai. The diameter of zone of inhibition was measured and interpreted according to CLSI (Vol 32 No.3. M100-S22.Wayne PA: Jan 2012) guidelines.

### MBL detection

**Imipenem- EDTA Combined disc test (CDT):** This test was described by Yong et al. Here the test organisms were inoculated on to plates with Mueller Hinton agar (MHA) as per CLSI (Vol 32 No.2. M07 – A 9. Wayne PA: Jan 2012) guidelines. Two 10µg Imipenem discs (Becton Dickinson) were placed on the plate, and appropriate amounts of 10µL of EDTA solution were added to one of them to obtain the desired concentration (750µg). The plates were incubated at 37°C for 24 hours. The inhibition zones of the Imipenem and Imipenem-EDTA discs were compared. The increase in inhibition zone with the Imipenem and EDTA disc  $\geq 7$ mm than the Imipenem disc alone, was considered as MBL positive.<sup>2</sup>

**Imipenem- EDTA double disc synergy test (DDST):** This test was described by Lee et al. Here the test organisms were inoculated on to MHA plates as per CLSI (Vol 32 No.2. M07 – A 9. Wayne PA: Jan 2012) guidelines. An Imipenem (10µg) disc was placed 20 mm center to center from a blank disc containing 10µL of 0.5 M EDTA (750µg). Enhancement of the zone of inhibition in the area between Imipenem 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>2</sup>

**EDTA- disc potentiation test using ceftazidime, ceftizoxime, cefepime and cefotaxime (DPT):** Here the test organisms were inoculated on to plates with MHA as described for the standard disc diffusion test. A filter paper blank disc is placed and the following discs Ceftazidime (30µg), Ceftizoxime(30µg), Cefepime(30µg), Cefotaxime (30µg) are placed 25mm center to center from the blank disc. 10µL of 0.5 M

EDTA solution is added to the blank disc and the plate was incubated overnight at 35°C for enhancement of the zone of inhibition in the area between the EDTA disc and any one of the four Cephalosporin disc in comparison with the zone of inhibition on the far side of the drug was interpreted as a positive result.<sup>2</sup>

### MBL E test

Broth microdilution methods usually detect carbapenem resistance. When performed properly, disc diffusion and agar gradient diffusion also are acceptable methods for carbapenem testing. An additional test method, such as agar gradient diffusion (i.e., E test), can be used to verify intermediate or resistant results.

Among carbapenems, imipenem degrades easily. Studies suggest meropenem may be more stable than imipenem. Hence compared to imipenem, meropenem is the best choice for calculating MIC of carbapenem *in vitro*.<sup>7</sup>

Here in this study a unique phenotypic MBL detection E test strip were used, which is capable of detecting MBLs including strains with recently discovered NDM-1 resistant gene. This strip is coated with mixture of Meropenem + EDTA and Meropenem on a single strip in a concentration gradient manner. The upper half has Meropenem+EDTA (1to 64µg/ml) with highest concentration tapering downwards, whereas lower half is similarly coated with Meropenem (4 to 256 µg/ml) in a concentration gradient in reverse direction. The E test was done according to manufacturer’s instruction. MIC ratio of MRP/MRP-EDTA of  $>8$  log<sub>2</sub> dilutions indicates MBL production and  $\leq 8$  indicates MBL negative.<sup>8,9</sup>

### Results

A total of 59 consecutive, non repetitive isolates of *Acinetobacter species* were studied in a tertiary care centre between January 2014 and June 2015.

MIC of Meropenem measured by E test (Table 1).

E Test found to be most sensitive phenotypic method for detection of MBL production among imipenem resistant isolates (Table 2/Graph 1).

Compared to E Test, CDT 98% sensitive and 100% specific (Table 3)

73% of *Acinetobacter species* from wound infection (other than post operative and diabetic foot) were MBL producers respectively (Table 4/Graph 2).

MBL producers were significantly resistant (P value  $< 0.05$ ) to beta lactam drugs with beta lactamase inhibitors, fluoroquinolones and co-trimoxazole (Table 5/Graph 3).

**Table 1: Detection of MIC of Meropenem by E test**

MIC of Meropenem	E Test
0.5 µg/ml	00
1 µg/ml	00
2 µg/ml	00
4 µg/ml	02

8 µg/ml	12
16 µg/ml	26
32 µg/ml	07
64µg/ml	12

**Table 2: Detection of MBL production by different phenotypic methods**

Test	MBL Positive	MBL Negative
CDT	29(49.2%)	30(50.8%)
DDST	15(25.4%)	44(74.6%)
DPT	15(25.4%)	44(74.6%)
E TEST	30(50.8%)	29(49.2%)

**Table 3: Comparison of different phenotypic methods with E test in detection of MBL production.**

Phenotypic method	Sensitivity	Specificity	PPV	NPV
CDT	98%	100%	100%	98%
DDST	48.1%	100%	100%	64%
DPT	48.1%	100%	100%	64%

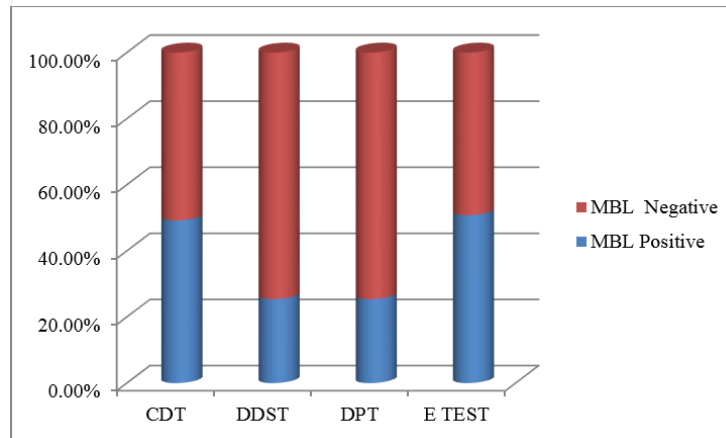
**Table 4: MBL production of *Acinetobacter* species from different clinical conditions**

Clinical condition	MBL positive	MBL negative
Wound infection (Other than postoperative and diabetic foot infection)	08 (73%)	03
Respiratory tract infections	15 (54%)	13
Post operative wound infection	04(44%)	05
Diabetic foot	02(33%)	04
Blood stream infections	01 (50%)	01
Urinary tract infections	00 (0%)	02
Peritonitis	00 (0%)	01
Total	30	29

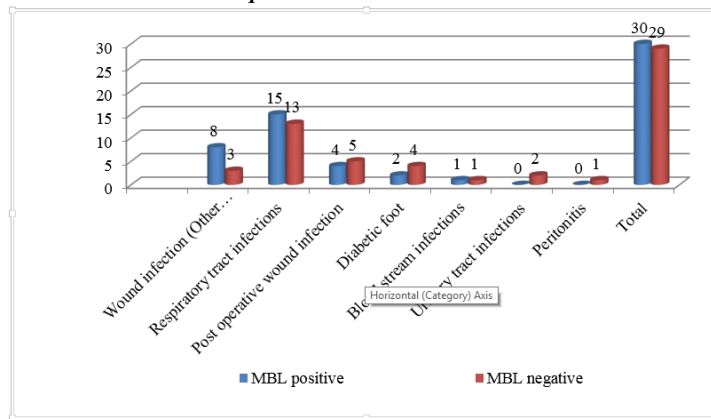
**Table 5: Antibiotic resistance pattern of MBL positive and MBL negative isolates of *Acinetobacter* species.**

Antibiotic	MBL Positive (n=30)		MBL Negative (29)		P-Value
	No	%	No	%	
AK	19	63.33	16	55.17	0.54
AMC	30	100	19	100	-
AMP	30	100	27	93.10	0.14
CPM	30	100	28	96.55	0.31
CXM	30	100	29	100	-
CPZ	30	100	26	89.66	0.07
CIP	28	93.33	17	58.62	0.002*
COT	28	93.33	19	65.51	0.008*
GEN	20	66.67	18	62.07	0.71
LE	0	00	02	6.90	0.14
PIT	12	40	01	3.45	<0.001*

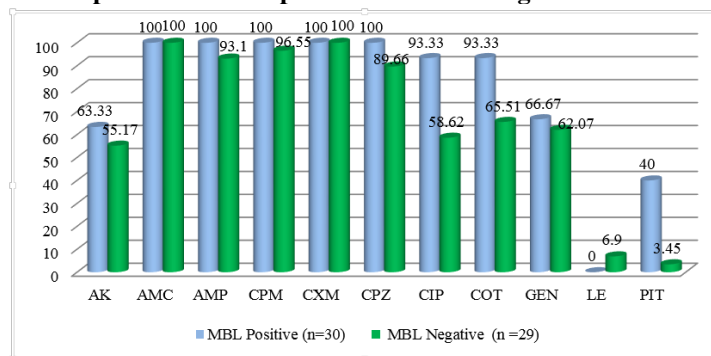
**Graph 1: Detection of MBL production by different phenotypic methods**



**Graph 2: MBL production in Acinetobacter species from different clinical conditions**



**Graph 3: Antibiotic resistance pattern of MBL positive and MBL negative isolates of Acinetobacter species**



**Statistical Analysis**

Analysis was done by Epidata analysis software version 2.22.182. Discrete variables were expressed as percentages and proportions were compared using the Chi-square test. P value <0.05 was considered significant.

**Discussion**

The incidence of nosocomial infections in critically ill patients is much higher than in general ward patients<sup>[3]</sup>. Increasing incidence of serious infections in hospital intensive care units due to *Methicillin resistant*

*Staphylococcus aureus*, *MDR Klebsiella spp*, *A. baumannii* and *P. aeruginosa* are being reported worldwide.<sup>10,11</sup> Most frequently encountered species are *Acinetobacter species* and *Pseudomonas aeruginosa* that are commonly associated with infections, such as bacteremia, urinary tract infection, meningitis, skin and soft tissue infections and pneumonia in ICU patients.<sup>12,13</sup> These organisms are noted for their intrinsic resistance to antibiotics and for their ability to acquire genes encoding resistance determinants. Because of frequent resistance to commonly used

antibiotics, carbapenems have become important for managing these infections.<sup>14</sup>

Recently resistance to carbapenems has started emerging due to carbapenamases and expression of efflux pumps. Among carbapenamases, the genes responsible for MBL production may be chromosomally or plasmid mediated and hence pose a threat of spread of resistance by gene transfer among the Gram – Negative bacteria.<sup>4</sup> Severe nosocomial infections due to MDR, MBL producing *Acinetobacter species* contribute to prolonged hospitalization, increased morbidity and mortality and of course, increased resource utilization.<sup>3</sup>

Correct identification of MBL producing organisms in due time is necessary not only for optimal patient management but also for immediate institution of appropriate infection control measures to prevent the spread of these organisms.

This study aims to identify an early, rapid and effective phenotypic method for identifying MBLs in ICU patients especially for *Acinetobacter species* which is being increasingly reported. In this study all isolates were Imipenem resistant and were subjected to E Test for measurement of their MIC to Meropenem. Imipenem degrades easily. Studies suggest that, Meropenem is slightly more active and stable than Imipenem against Gram – negative organisms.<sup>7</sup> Hence, among carbapenems, meropenem was used in this study for measurement of MIC for all the isolates by E test. MIC values were interpreted based on the current CLSI breakpoints.<sup>15</sup>

The MIC of 2 isolates were  $\leq 4\mu\text{g/ml}$  (sensitive), 12 isolates MIC were  $8\mu\text{g/ml}$  (intermediate susceptibility) and 45 isolates MIC were  $\geq 16\mu\text{g/ml}$  (Resistant) by E test.

Carbapenem resistance is usually multifactorial. Apart from carbapenem- hydrolyzing enzymes (MBLs), increased production of AmpC chromosome-encoded cephalosporinase, reduced outer membrane porin OprD expression, and associated factors are known to contribute to carbapenem resistance. Overexpression of the MexAB-OprM efflux system is known to affect meropenem efficacy but not that of imipenem. In addition, the MexCD-OprJ and MexXY-OprM efflux systems may also be involved in reduced susceptibility to meropenem. Whereas OprD inactivation alone is the source of intermediate susceptibility or resistance to imipenem. The mechanisms leading to meropenem resistance, involves overproduction of AmpC or over expression of the efflux pumps MexAB-OprM, MexXY-OprM, and MexCD-OprJ<sup>[16]</sup>. In the present study, the imipenem resistance in meropenem sensitive isolates could be due to OprDporin mutation. Hence all the isolates were tested for MBL production by different phenotypic tests. CDT, DDST and DPT were used for detecting MBL production in all the isolates and the sensitivity of CDT Test, DDST and DPT were

98%, 48.1% and 48.1% and the specificity of CDT, DDST and DPT were 100% when compared to E Test.

There are conflicting reports regarding the performance of MBL E Test in the literature. It has been reported to be insensitive to detect carbapenem sensitive MBL carrying organisms.<sup>2</sup> In our study, we screened only carbapenem resistant isolates with MBL E Test, which may have accounted for very high sensitivity of the test. We found that all isolates found to be MBL positive by CDT were also positive with the E Test and one isolate which were positive by E Test, were found to be MBL negative by CDT. These findings are consistent with the study done by Maria Omair et al.<sup>17</sup>

The CDT detected 48.2% of MBL producers among which were negative by the DDST and DPT. This may be because the sensitivity varies with the distance between the discs and the antibiotic discs chosen for the test. The major disadvantage of DDST and DPT were the subjective interpretation of result in some instances. The increase of  $\geq 7\text{mm}$  in inhibition zone with Imipenem plus EDTA disc than with Imipenem disc alone was considered as MBL positive by CDT. The CDT was found to be superior to DDST and DPT. Hence, in this study CDT was found to be one of the most sensitive techniques than DPT, DDST for detecting MBL. This findings are correlating with other studies.<sup>2,3</sup> CDT is technically much simpler and inexpensive method compared to other phenotypic methods like E Test, DDST and DPT. The interpretation is straightforward. Hence, addition of one more imipenem disc with 0.5M EDTA along with regular imipenem disc while performing the disc diffusion method would also screen for MBL production in the laboratory.

## Conclusion

MBL producing *Acinetobacter species* is being increasingly reported in ICU patients. The MIC of all the MBL producing isolates for meropenem were  $>16\mu\text{g/ml}$  (Resistant). Compared to DDST and DPT, E test and CDT were more reliable for MBL detection. Compared to E Test, CDT was cost effective, easy to perform and interpret. MBL producing isolates were multidrug resistant making therapeutic choices limited. Polymyxin B and Colistin are the next therapeutic options for carbapenem resistant isolates. Continuous antibiotic surveillance, infection control practices and an effective antibiotic policy are required to address the problem of MBL – associated infections. Molecular studies are necessary to evaluate the various MBL type.

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