

**A COMPARATIVE STUDY ON DIFFERENT PHENOTYPIC METHODS FOR DETECTION OF METALLO BETA LACTAMASE PRODUCING BACTERIA IN A TERTIARY HOSPITAL OF EASTERN INDIA**

Anirban Bhaduri<sup>1</sup>, Rajdeep Saha<sup>2</sup>, Papiya Biswas<sup>3</sup>, Suranjana Mitra<sup>4</sup>, Sharmistha Datta (Basu)<sup>5</sup>, Paramita Das<sup>6</sup>, Sougata Ghosh<sup>7</sup>, Manas Sarkar<sup>8</sup>

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**ABSTRACT: BACKGROUND:** Metallo beta lactamase (MBL) producing bacteria are popularly known as superbugs due to their increased development of drug resistance to carbapenems. MBL producing bacteria mainly cause healthcare associated infections (HAI) and are the causes of increased mortality among patients infected with MBL producers. The aim of this study was to compare the different phenotypic methods available for detecting MBL producing bacteria. **MATERIALS AND METHODS:** 500 samples from different clinical specimens were processed and samples from which gram negative bacilli was isolated were screened for imipenem resistance by Kirby –bauer disc diffusion methods and further tested for MBL detection by Imipenem+EDTA combined disk diffusion method, Imipenem+EDTA double disk synergy test and MBL E-test. **RESULTS AND ANALYSIS:** Out of the 125 strains of GNB, 32 strains were found to be Imipenem resistant by Kirby Bauer disk diffusion, 18 (56.25%) were *Pseudomonas aeruginosa*, 7 (21.87%) were *K.pneumoniae*, 4 (12.5%) were *Acinetobacter* spp, 2 (6.25%) were *Citrobacter* and 1 (3.12%) was *E.coli*. When these 32 isolates were tested for MBL production by IMP+EDTA Combined disk diffusion test, 29 (90.62%) of the isolates were found to be MBL producers, by Imipenem+EDTA double disk synergy test, 26(81.25%) of the isolates showed MBL production and by MBL E-test, 30 (93.75%) of the isolates were MBL producers. **CONCLUSION:** From our study it appears that metallo-  $\beta$ - lactamase production in gram negative bacilli has become a significant problem in health care associated infection control in this part of India and is producing many carbapenemase resistant strains in India.

**KEYWORDS:** Metallo beta lactamase, Health care associated infections, E-test.

**INTRODUCTION:** The introduction of carbapenems into clinical practice represented a great advance for the treatment of serious bacterial infections caused by  $\beta$ -lactam resistant bacteria. Due to their broad spectrum of activity and stability to hydrolysis by most  $\beta$ -lactamases, the carbapenems have been the drug of choice for the treatment of infections caused by penicillin or cephalosporin resistant gram negative bacteria.<sup>1</sup>

Metallobetalactamases (MBL) was first detected in 1960 in *Bacillus cereus* which was chromosomal in location. Then, first plasmid mediated MBL isolates was found in *Pseudomonas aeruginosa* in 1991 in Japan. Since early 1990's metallo- $\beta$ -lactamase encoding genes have been reported all over the world in clinically important pathogens such as *Pseudomonas* spp, *acinetobacter* spp and members of the Enterobacteriaceae family.<sup>2</sup> MBL in gram negative bacilli is becoming a therapeutic challenge as these enzymes usually possess a broad hydrolysis profile that includes all  $\beta$ -lactam antibiotics including carbapenems.<sup>3</sup>

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Carbapenem resistance has been observed more commonly in nonfermenters such as *Pseudomonas* spp and *Acinetobacter* spp. The common form of resistance is through lack of drug penetration i.e. porin mutations and efflux pumps and for carbapenem –hydrolysing  $\beta$ -lactamases. Based on molecular studies two classes of carbapenem hydrolyzing enzyme have been described: serine enzymes possessing a serine moiety at the active site, and metallo- $\beta$ -lactamase (Class B) requiring divalent cations as co-factors for enzyme activity.<sup>4</sup>

Acquired MBL have recently emerged as one of the most worrisome resistance mechanisms owing to their capacity to hydrolyse all  $\beta$ -lactams including carbapenems. Such strains are not susceptible to therapeutic serine  $\beta$  lactamase inhibitors such as clavulanate or sulfones. Moreover these genes are carried on highly mobile elements allowing early dissemination <sup>5</sup>.The occurrence of MBL positive isolates in a hospital environment possess not only therapeutic problem but is also a cause of serious concern for infection control.

As a result of being difficult to detect such organisms pose significant risk particularly due to their role in unnoticed spread within institutions and their ability to participate in horizontal MBL gene transfer with other pathogens in hospital. In recent years, MBL genes have spread from non-fermenters to members of Enterobacteriaceae.<sup>6,7</sup> Till now seven main types of MBL have been described throughout the world – IMP, VIM, SPM, GIM, SIM, AIM-1<sup>8</sup> and NDM-1.<sup>9</sup> Among them bla<sub>IMP</sub> and bla<sub>VIM</sub> are the most common types of MBL's with worldwide distribution.<sup>10</sup>

From India only bla<sub>VIM</sub><sup>11,12</sup> and NDM-1<sup>13</sup> have been reported from *P. aeruginosa* in the past. Veronese Imipenemase (VIM) enzymes have been grouped into 3 main clusters designated VIM-1, VIM-2 and VIM-7. To date, VIM-2 is more widely spread among *P. aeruginosa* isolates whereas VIM-1 is normally confined to Enterobacteriaceae.<sup>13</sup> MBL determinants are mostly found in *P. aeruginosa*, *Acinetobacter baumannii* and Enterobacteriaceae isolates like *Klebsiella pneumonia*, *Citrobacter freundii*, *Serratia* spp, *Alcaligenes xylosoxidans* and *Escherichia coli*.<sup>14</sup>

Overall prevalence of nosocomial isolates of *Pseudomonas aeruginosa* possessing MBLs have been reported to be 20%<sup>15</sup>. Reports regarding the prevalence of MBLs are available from other countries like Canada, Brazil, Italy, Australia<sup>16</sup> and also from Asian countries like Korea, Pakistan, Taiwan,<sup>17</sup> etc. Few reports regarding the prevalence of MBLs are available from Delhi and South India also.<sup>18</sup>

Most of the studies from different parts of the world compared some of the available tests but studies comparing different phenotypic methods for MBL detection are very few in India and more so in this eastern part. This study is aimed to investigate the impact of this highly virulent group of bacteria among patients attending a tertiary care Government Hospital in Eastern India.

**MATERIALS AND METHODS:** This study was carried out from April 2012 to March 2013 in the department of Microbiology. 500 clinical samples were taken from ICU and wards of the tertiary hospital and processed as per standard microbiological procedures<sup>19</sup>.No specific exclusion criteria was envisaged.

All specimens were inoculated in 10% sheep blood agar and in Mac Conkey's Agar media and incubated at 37°C for 18-24 hrs and those showing typical colony was further identified by gram staining and standard biochemical reactions. Antimicrobial susceptibility was done on these strains by Kirby Bauer disk diffusion method as per CLSI 2011 guidelines.

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Those strains found to be resistant to Imipenem were further evaluated by three phenotypic tests viz Imipenem +EDTA combined disk diffusion method, Imipenem +EDTA double disk synergy test and by E –TEST:

1. **Imipenem +EDTA combined disk diffusion method.<sup>20</sup> (CDDT):** Lawn culture of the test organism were done on Mueller Hinton Agar plates and Imipenem 10µg discs were placed on the surface of the agar plate and 0.5 M EDTA solution was added to one of the Imipenem disc to obtain a desired concentration of 750µg. Then the inhibition zones of Imipenem and Imipenem+EDTA discs were compared after 16-18 h of incubation at 37°C. Zone difference of  $\geq 7$ mm between Imipenem alone and with Imipenem+ EDTA was interpreted as positive MBL producing strain.
2. **Imipenem-EDTA Double disk Synergy test<sup>21</sup>(DDST):** Test organisms were inoculated on Mueller Hinton agar plates as recommended by CLSI2011. Two 10 µg imipenem discs are placed on the surface of the agar plate. 0.5 M EDTA solution is added to one of the disks to obtain a desired concentration of 750µg. The inhibition zones of Imipenem and Imipenem EDTA discs are compared after 16-18 hrs of incubation at 35°C. Zones of enlarged inhibition around Imipenem EDTA discs of  $\geq 5$ mm was taken as positive indicator for MBL production.
3. **MBL E-test (Biomerieux):** A 0.5 Mc Farland standard well emulsified solution of the test strain was plated on a Mueller Hinton Agar plate by swabbing it by a sterile swab rotating thrice 60 degrees each to get an uniform distribution of inoculum. A MBL E-test strip is then put carefully on the agar surface and the plates were incubated at 35°C in a incubator for 16-18 hrs. The result was interpreted as MIC break points by the zone of intersection around the strip:
  - IMIPENEM/IMIPENEM +EDTA (IP/IPI) 128/12 = 10.7 MBL positive strain.
  - IP/IPI  $>256/1 = >256$  MBL positive strain.
  - IP/IPI 64/ $<1 = >64$  MBL positive strain.
  - IP/IPI 64/ $>64 = <1$  MBL negative strain.

*Pseudomonas aeruginosa* ATCC 27853 was used as a negative control for performing MBL E-test.

**Statistical analysis:** Statistical analysis was done with Epi info software and  $P < 0.05$  was considered significant.

**DISCUSSION:** The present study was done in a tertiary care hospital in Kolkata between April 2012 to March 2013, 500 consecutive, non- duplicate samples were collected from patients suffering from different types of infections. Different types of clinical samples like urine, pus/wound swab/body fluid, blood, sputum, ET tube suction etc were collected from those patients after obtaining informed written consent from them. Table 1 shows that out of total 500 clinical samples processed, 125 were found to be gram negative bacilli and of which 28(22.4%) were isolated from urine, 72 (57.6%) from pus/wound swab/body fluids, 18 (14.4%) from blood and 7(5.6%) from sputum/ET suction/iv cannula tip. Our study (Table 2) indicates 55 isolates were *Pseudomonas aeruginosa* followed by 42 isolates of *Klebsiella pneumoniae*, 17 isolates of *Escherichia coli*, 6 isolates of *Acinetobacter* spp and 5 isolates of *Citrobacter*.

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The present study (Table 3) gave an insight into the strains of GNB found to be Imipenem, resistant by Kirby Bauer disk diffusion method as per CLSI guidelines and 32 strains were found to be Imipenem resistant, 18 (56.25%) were *Pseudomonas aeruginosa*, 7 (21.87%) were *K. pneumoniae*, 4 (12.5%) were *Acinetobacter* spp, 2 (6.25%) were *Citrobacter* and 1 (3.12%) was *E.coli*. Table 4 in this study shows that when the 32 isolates were tested for MBL production by IMP+EDTA Combined disk diffusion test, 29 (90.62%) of the isolates showed positive result. In a study done by Galani et al<sup>3</sup> showed 94.7% positivity by IMP+EDTA CDDT, Picao et al<sup>2</sup> showed 80% positive result by the same phenotypic method and Franklin et al<sup>23</sup> showed 100% positive result by the same method. An Indian study conducted by Behera et al<sup>5</sup> at AIIMS, New Delhi showed 85.71% positive result by CDDT.

Table 5 shows that when out of the 32 GNB isolates found to be Imipenem resistant by KBDD was tested for MBL production by Double disk synergy test, 26(81.25%) isolates showed positive result. Picao et al<sup>2</sup> study showed 82.6% positivity by DDST, Galani et al<sup>3</sup> showed 100% positive MBL production by DDST and Franklin et al<sup>23</sup> showed 79 % positive result by DDST. Behera et al<sup>5</sup> showed 75% positivity by DDST. Table 6 shows that MBL E-test for MIC break points was done on the 32 Imipenem resistant isolates and 30 (93.75%) of the isolates showed MIC breakpoints within the range (10 -256µg/ml) to be labeled as MBL POSITIVE isolates.

The study by Behera et al<sup>5</sup> found 100% MBL positive isolates by MBL E-test MIC method. Walsh et al<sup>22</sup> found 94% MBL positive strains by MBL E test method. Table 7 & 8 shows the comparative efficacy of the three different phenotypic tests done for MBL detection and MBL E-test method was found to be most sensitive (93.75%) for detection of MBL production followed by Imipenem+EDTA Combined disk method (90.62%) and DDST(81.25%) in sensitivity respectively.

*Pseudomonas aeruginosa* was the main organism producing MBL (32.7%) followed by *Klebsiella pneumoniae*, (16.6%) *Acinetobacter* spp and *Citrobacter freundii* by all the three phenotypic methods.

In this study the prevalence of MBL producers among GNB was 24% by MBL E-test method, 23.2% by IMP+EDTA combined disk method and 20.8% by DDST method. By MBL E test. 56.6% isolates were of *P. aeruginosa*, 23.3%isolates of *K. pneumoniae*, 13.3%isolates of *Acinetobacter* spp and 6.6% isolates of *Citrobacter* was found to be MBL producers by MIC break points. A study done by Pandya et al<sup>24</sup> in Gujarat, West India, showed a 6% prevalence of MBL production among gram negative bacilli, and *Pseudomonas aeruginosa* showed highest prevalence (9.92%) followed by *Klebsiella pneumoniae* (7.26%), *Acinetobacter* (7.26%) and *E.coli* (2.87%).

Using Imipenem as substrate 26(96.3%) isolates were MBL producer by Imipenem +EDTA combined disk synergy test and 22 (81.48%) isolates were MBL producers by DDST. So at a nutshell this present study shows that Metallo  $\beta$  lactamase producing gram negative bacilli were indeed a matter of concern due to their carbapenem resistance property and these bugs are sure to stay in long run and by applying different phenotypic methods they can be detected in routine laboratory antimicrobial susceptibility reports.

Though MBL E –test was been the most sensitive method to detect MBL production but it is costly and not affordable to all laboratories in a developing country like India for routine testing of MBL detection.

**RESULTS AND ANALYSIS:** Out of the 125 strains of GNB, 32 strains were found to be Imipenem resistant by Kirby Bauer disk diffusion, 18 (56.25%) were *Pseudomonas aeruginosa*, 7 (21.87%)

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were *K. pneumoniae*, 4 (12.5%) were *Acinetobacter* spp, 2 (6.25%) were *Citrobacter* and 1 (3.12%) was *E.coli*. When these 32 isolates were tested for MBL production by IMP +EDTA Combined disk diffusion test, 29 (90.62%) of the isolates were found to be MBL producers, by Imipenem+EDTA double disk synergy test, 26 (81.25%) of the isolates showed MBL production and by MBL E-test, 30 (93.75%) of the isolates were MBL producers.

**CONCLUSION:** The sensitivity of MBL E-test was the highest (93.75%) followed by Combined disk test (90.62%) and lastly by DDST (81%). The IMP+EDTA Combined disk method for detection of MBL can be adopted as a routine test in laboratories of a developing country like India.

From our study it appears that metallo-  $\beta$ - lactamase production in gram negative bacilli has become a significant problem in health care associated infection control in this part of India as well and further study with more sample size and using molecular methods can justify the findings of this study. The sensitivity of IMP+EDTA combined disk test has showed to be very close to MBL E test (90.62% vs. 93.75%) in this study and this test can be an effective and economical test for MBL detection in laboratories both in the government and the private sector hospitals.

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Type of sample	Total no. of samples	Gram negative Bacteria isolated
Urine	315(63%)	28(22.4%)
Wound swab/pus/body fluids	128(25.6%)	72(57.6%)
Blood	40(8%)	18(14.4%)
Sputum/ETsuction iv cannula tip	17(3.4%)	7(5.6%)
<b>TOTAL</b>	<b>500(100%)</b>	<b>125(100%)</b>

Table 1: Distribution of gram negative bacteria isolated from different Clinical samples

ORGANISMS	Number
Klebsiella pneumoniae	42(33.6%)
Pseudomonas aeruginosa	55(44%)
Escherichia coli	17(13.6%)
Acinetobacter spp	6(4.8%)
Citrobacter freundii	5(4%)
<b>TOTAL</b>	<b>125(100%)</b>

Table 2: Distribution of the Gram negative bacteria according to type of organisms

Organisms	Urine	Pus/wounds swab/body fluids	Blood	Sputum/ET suction/iv cannula tip	TOTAL
Klebsiella pneumoniae	1(14.28%)	4(57.14%)	1(14.28%)	1(14.28%)	7(100%)
Pseudomas aeruginosa	Nil (0%)	15(83.33%)	2(11.11%)	1(5.55%)	18(100%)
Escherichia coli	1(100%)	nil	nil	nil	1(100%)
Acinetobacter spp	nil	3(75%)	nil	1(25%)	4(100%)
Citrobacter freundii	2(100%)	nil	nil	nil	2(100%)
<b>TOTAL</b>	<b>4</b>	<b>22</b>	<b>3</b>	<b>3</b>	<b>32</b>

Table 3: Distribution of Imipenem resistant GNB isolates among the different types of clinical samples

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Organisms	Isolates resistant to carbapenems by KBDD	Isolates positive by IMP+EDTA Combined disk diffusion test
Pseudomonas aeruginosa n=55	18(56.25%)	17(58.63%)
Klebsiella pneumoniae(N=42)	7(21.88%)	6 (20.69%)
Acinetobacter sppN= 6	4(12.5%)	4 (13.80%)
Citrobacter freundii N=5	2(6.25%)	2 (6.90%)
Escherichia coli N=17	1(3.12%)	NIL
<b>TOTAL</b>	<b>32(100%)</b>	<b>29 (100%)</b>

**TABLE 4: COMPARISON OF IMPENEM+EDTA COMBINED DISK DIFFUSION TEST WITH KBDD SCREENING FOR CARBAPENEM RESISTANCE**

Organisms	Isolates resistant to Imipenem by KBDD	Isolates positive by IMP+EDTA Double disk SYNERGY test
Pseudomonas aeruginosa(N=55)	18 (56.25%)	15 (57.70%)
Klebsiella pneumoniae(N=42)	7 (21.88%)	5 (19.23%)
Acinetobacter spp N= 6	4 (12.5%)	4 (15.38%)
Citrobacter freundii N=5	2 (6.25%)	2 (7.70%)
Escherichia coli N=17	1 (3.12%)	nil
<b>TOTAL</b>	<b>32(100%)</b>	<b>26(100%)</b>

**TABLE 5: COMPARISON OF DOUBLE DISK SYNERGY TEST WITH KBDD SCREENING FOR CARBAPENEM RESISTANCE**

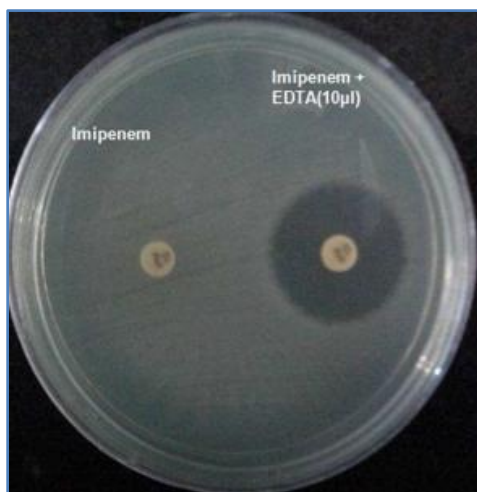
Organisms	Isolates resistant to Imipenem by KBDD	Isolates positive by MBL E-test MIC breakpoint
Pseudomonas aeruginosa(N=55)	18 (56.25%)	17 (56.66%)
Klebsiella pneumonia(N=42)	7 (21.88%)	7 (23.33%)
Acinetobacter spp N= 6	4 (12.5%)	4 (13.33%)
Citrobacter freundii N=5	2 (6.25%)	2 (6.67%)
Escherichia coli N=17	1 (3.12%)	NIL
<b>TOTAL</b>	<b>32 (100%)</b>	<b>30(100%)</b>

**TABLE 6: COMPARISON OF MBL E-TEST MIC BREAKPOINT WITH KBDD METHOD FOR CARBAPENEM RESISTANCE**



Organisms	Isolates positive by IMP+EDTA Combined disk diffusion test	Isolates positive by MBL E-test MIC breakpoint	Isolates positive by DDST
<i>Pseudomonas aeruginosa</i> (N=55)	17	17	15
<i>Klebsiella pneumoniae</i> (N=42)	6	7	5
<i>Acinetobacter</i> spp N= 6	4	4	4
<i>Citrobacter freundii</i> N=5	2	2	2
<i>Escherichia coli</i> N=17	NIL	NIL	NIL
<b>TOTAL</b>	<b>29(90.62%)</b>	<b>30(93.75%)</b>	<b>26(81.25%)</b>

**TABLE 7: COMPARISON OF COMBINED DISK DIFFUSION METHOD, DDST AND MBL E-TEST**



**Figure 1: Combined disc diffusion test (CDDT)**



**Figure 2: Double disk synergy test (DDST)**



**Figure 3: MBL E-test**

**AUTHORS:**

1. Anirban Bhaduri
2. Rajdeep Saha
3. Papiya Biswas
4. Suranjana Mitra
5. Sharmistha Datta (Basu)
6. Paramita Das
7. Sougata Ghosh
8. Manas Sarkar

**PARTICULARS OF CONTRIBUTORS:**

1. Demonstrator, Department of Microbiology, Calcutta National Medical College, Kolkata.
2. Assistant Professor, Department of Microbiology, Calcutta National Medical College, Kolkata.
3. Post Graduate Trainee, Department of Microbiology, Calcutta National Medical College, Kolkata.
4. Post Graduate Trainee, Department of Microbiology, Calcutta National Medical College, Kolkata.

5. Post Graduate Trainee, Department of Microbiology, Calcutta National Medical College, Kolkata.
6. Post Graduate Trainee, Department of Microbiology, Calcutta National Medical College, Kolkata.
7. Professor, Department of Microbiology, Medical College, Kolkata.
8. Professor, Department of Microbiology, Calcutta National Medical College, Kolkata.

**NAME ADDRESS EMAIL ID OF THE CORRESPONDING AUTHOR:**

Dr. Anirban Bhaduri,  
22/1 RAK Road,  
Dum Dum,  
Kolkata-55.  
Email: anir8365@gmail.com

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