Original Article:

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Abstract
Background: Carbapenem resistance due to the production of metallo-β-lactamase (MBL) in Gram-negative organism is an increasing public health problem. Aim: The purpose of this study is to detect MBL in Gram Negative bacterial isolates among Ventilator Associated Pneumonia patients. Materials and Methods: Phenotypic detection of MBL was done by three methods: 1. Modified Hodge Test (MHT) 2. Combined disc test (CDT) 3. Double Disc Synergy Test (DDST). Results: Out of 126 gram negative bacterial isolates, 80 (63.49%) showed resistance to carbapenem group of drugs. Among them maximum resistance was shown by Acinetobacter baumanii (90.32%), followed by Klebsiella pneumoniae (45.7%), Pseudomonas aeruginosa (26%). Out of 80 isolates, 66 were positive for MBL by MHT, 64 by CDT and 61 were detected positive for MBL by DDST. Conclusion: MHT and CDT were found equally efficient method to detect MBL. Maximum MBL production was detected in Acinetobacter baumanii. Simple and accurate screening test is required to prevent the spread of nosocomial strain in hospitals.

Key Words: Metallo-β-lactamases, VAP, Screening methods, Modified Hodge test, Combined Disc test, Double disc synergy test.

Introduction:
With the emergence of carbapenemases, particularly Ambler class B metallo-β-lactamases (MBLs) the utility of carbapenem is under threat. Such enzymes have emerged in many geographical locations and often confer high-level resistance to all β-lactams except aztreonam. Carbapenemases have been the drugs of choice for treatment of infections caused by MDR because of its broad spectrum activity. Metallo-β-lactamase was first detected in Bacillus cereus in 1960s and was chromosomal in location. Since then, MBL encoding genes have been reported all over the world among fermenters and non fermenters gram negative bacilli. The MBL producing strains are frequently resistant to aminoglycosides and fluoroquinolones but remain susceptible to polymyxins. Unlike carbapenem resistance due to several other mechanisms, the resistance due to MBL and other carbapenemase production has a potential for rapid dissemination, as it is often plasmid mediated. MBLs spread easily and cause nosocomial infections and outbreaks. Such infections mainly concern patients admitted to Intensive Care Units with several co-morbidities and a history of prolonged administration of antibiotics. There was no standard guideline for screening of Carbapenemases though lot of authors have described various phenotypic methods for its detection. PCR gives reliable specific and sensitive result for gene coding for carbapenem resistance, but due to cost and labour constrain this method is of limited practical use for daily application. A simple and inexpensive testing method for screening of carbapenemase producers is the need of hour. Therefore this study was undertaken to detect MBLs in clinical isolates by a low cost, convenient and sensitive procedure.

Materials and Methods:
This was a cross sectional study conducted in the Department of Microbiology, for a period of 24 months. Total 989 patients received mechanical ventilator support during the study who were admitted in the ICUs; Respiratory Intensive Care Unit (RICU), Surgical Intensive Care Unit (SICU), Medical Intensive Care Unit (MICU)and Intensive Cardiac Care Unit (ICCU). Among them 669 patients were on ventilator for more than 48 hours. Out of 669 patients, 100 Ventilator Associated Pneumonia (VAP) patients were included in the present study who satisfied the Clinical Pulmonary Infection Score (CPIS) >6.6 Samples of these patients were sent to the microbiology laboratory for routine culture identification and sensitivity testing. The isolates were identified based on standard
bacteriological techniques\textsuperscript{7,8} and were screened for meropenem resistance by Kirby-Bauer disk diffusion method according to CLSI guidelines.\textsuperscript{8}

Phenotypic detection of Metallo-beta-lactamase was done by performing three methods:
1. Modified Hodge Test (MHT)
2. Combined disc test (CDT: Meropenem and Meropenem+EDTA)
3. Double Disc Synergy Test (DDST: Meropenem and EDTA)

Modified Hodge Test\textsuperscript{10-14}

A 0.5 McFarland matched suspension of E.coli ATCC 25922 was diluted 1:10. From this diluted suspension lawn culture was done on Muller Hinton agar (MHA) plate with sterile cotton swabs. The plate was allowed to dry for 3-4 minutes at room temperature. A 10 mcg meropenem disc (BD Company) was placed at the centre and test organism was streaked in a straight line from edge of the disc. Plate was incubated at 37\textdegree c for 24 hours in ambient air. The presence of clover leaf type of indentation at the intersection of the test organism and ATCC E.coli 25922, within the zone of inhibition of meropenem susceptibility disc was interpreted as positive result as per CLSI guideline 2013. (Figure 1)

![Figure 1: Modified Hodge Test – Positive](image1)

Combined Disc Test (CDT)\textsuperscript{11,13}

0.5 M EDTA solution was prepared by dissolving 18.61 gram of disodium EDTA.2H2O in 100 ml of distil water and its pH was adjusted to 8.0 by using NaOH. The mixture was then sterilized by autoclaving. 10µl EDTA solution was poured on meropenem disk to obtain a desired concentration of 1900 µg per disc. Two to three identical colonies of test organism were inoculated in to nutrient broth and incubated at 37\textdegree c for 4-6 hours then turbidity was adjusted to 0.5 Mcfarland. Lawn culture of this suspension of test organism was done on Muller Hinton Agar (MHA) plate with a sterile cotton swab. One 10µg meropenem disk and one sterile blank disc (6mm size) was placed on Muller Hinton Agar plate (MHA) plate at a distance of 20 mm from centre to centre. 10µl of 0.5 M EDTA solution was put on to sterile blank disc to obtain 1900µg/disc concentration. Plate was kept for incubation at 35\textdegree c at ambient air for 24 hours. Enhancement of zone of inhibition in the area between Meropenem and EDTA disc in comparison with the zone of inhibition on the far side of the drug (meropenem) was interpreted as a positive result. (Figure 2)

![Figure 2: Positive CDT Test](image2)

Double Disc Synergy Test (DDST)\textsuperscript{12,13}

A Lawn culture was done on to Muller Hinton Agar (MHA) plate from 0.5 McFarland matched suspension of Test organism by using sterile cotton swab. A meropenem disc (10µg) and one sterile blank disc (6mm size) was placed on Muller Hinton Agar plate (MHA) plate at a distance of 20 mm from centre to centre. 10µl of 0.5 M EDTA solution was put on to sterile blank disc to obtain 1900µg/disc concentration. Plate was kept for incubation at 35\textdegree c at ambient air for 24 hours. Enhancement of zone of inhibition in the area between Meropenem and EDTA disc in comparison with the zone of inhibition on the far side of the drug (meropenem) was interpreted as a positive result. (Figure 3)

![Figure 3: Positive DDST Test](image3)

Figure 3: Positive DDST Test

Statistics:
No statistical method was required for screening method

Results:
Out of 126 gram negative bacterial isolates, 80 isolates (63.49\%) showed resistance to carbapenem group of drugs (meropenem & imipenem). Among them maximum resistance was shown by Acinetobacter baumanii (90.32\%), followed by Klebsiella pneumoniae (45.7\%), Pseudomonas aeruginosa (26\%). (Table 1)
Combined Disc Test (CDT) are better than Double Disk Synergy Test (DDST) and Modified Hodge Test (MHT) & Combined Disk Test (CDT) are better method of choice for MBL detection as compared to DDST.

In the present study, most of the carbapenem resistant A. baumannii isolates were MBL producer. Among A. baumannii 49 isolates were MBL positive by MHT test, 47 isolates by CDT test and 45 isolates by DDST. In case of K. pneumoniae, 10 isolates were MBL positive by MHT test and by CDT test where as 9 were positive by DDST (Table 2). All the carbapenem resistant isolates of P. aeruginosa and K. oxytoca were detected MBL positive by all three methods.

### Table 1: Susceptibility pattern of gram negative bacilli to carbapenem

<table>
<thead>
<tr>
<th>Organisms</th>
<th>Isolates number</th>
<th>Carbapenem resistance (%)</th>
<th>Carbapenem sensitive (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acinetobacter baumannii</td>
<td>62</td>
<td>56 (90.32%)</td>
<td>06 (9.68%)</td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>35</td>
<td>16 (45.7%)</td>
<td>19 (54.3%)</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>23</td>
<td>06 (26%)</td>
<td>17 (74%)</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>01</td>
<td>00 (00%)</td>
<td>01 (100%)</td>
</tr>
<tr>
<td>Klebsiella oxytoca</td>
<td>02</td>
<td>01 (50%)</td>
<td>01 (50%)</td>
</tr>
<tr>
<td>Serratia marcescens</td>
<td>01</td>
<td>00 (00%)</td>
<td>01 (100%)</td>
</tr>
<tr>
<td>Citrobacter species</td>
<td>02</td>
<td>01 (50%)</td>
<td>01 (50%)</td>
</tr>
<tr>
<td>Total no</td>
<td>126</td>
<td>80 (63.49%)</td>
<td>46 (36.51%)</td>
</tr>
</tbody>
</table>

Out of 80 isolates, 66 were positive for MBL by MHT, 64 by CDT and 61 were detected positive for MBL by DDST (As in Graph 1). These three methods were done for detection of metallo-beta-lactamases (MBL) & it was found that Modified Hodge Test (MHT) & Combined Disc Test (CDT) are better method of choice for MBL detection as compared to DDST. In the present study, most of the carbapenem resistant A. baumannii isolates were MBL producer. Among A. baumannii 49 isolates were MBL positive by MHT test, 47 isolates by CDT test and 45 isolates by DDST. While in case of K. pneumoniae, 10 isolates were MBL positive by MHT test and by CDT test where as 9 were positive by DDST (Table 2). All the carbapenem resistant isolates of P. aeruginosa and K. oxytoca were detected MBL positive by all three methods.

### Table 2: Results of modified Hodge test, combined disk test and EDTA disc synergy test

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Number of meropenem resistant isolates</th>
<th>Modified Hodge test</th>
<th>Combined disk test</th>
<th>EDTA disc synergy test</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. baumannii</td>
<td>56</td>
<td>49 (87.5%)</td>
<td>47 (84%)</td>
<td>45 (80.3%)</td>
</tr>
<tr>
<td>K. pneumoniae</td>
<td>16</td>
<td>10 (62.5%)</td>
<td>10 (62.5%)</td>
<td>9 (56.25%)</td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>06</td>
<td>6 (100%)</td>
<td>6 (100%)</td>
<td>6 (100%)</td>
</tr>
<tr>
<td>K. oxytoca</td>
<td>01</td>
<td>1 (100%)</td>
<td>1 (100%)</td>
<td>1 (100%)</td>
</tr>
<tr>
<td>Citrobacter spp.</td>
<td>01</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

### References