Detection of Metallo Beta Lactamase Production in Gram Negative Clinical Isolates

Vandanan B. Rajput and Jigna P. Naik

1. Arts, Science & Commerce College, Department of Microbiology (PGDMLT), Kholwad, Surat, (Gujarat) - India
2. Arts, Science & Commerce College, Department of Microbiology (PGDMLT), Kholwad, Surat, (Gujarat) - India

Abstract

Gram-negative bacteria are highly adaptive pathogens with multiple mechanisms of resistance to current therapies. Spread of Metallo-beta-lactamase (MBLs) in Gram negative bacteria (GNBs) are represents a serious threat to public health and medical community, this leaves few option to treat severe infection and leads to emergence of multi-drug resistance (MDR) and pan drug resistance organisms. This study was undertaken to detect MBLs production by GNBs and their antibiotic susceptibility pattern from variety of clinical samples in area of Surat. Total 200 non-duplicate GNBs were isolated and identified from clinical samples. Isolates were subjected to antibiotics susceptibility testing by Kirby-Bauer disk diffusion method as per Clinical and Laboratory Standard Institute (CLSI) guidelines. MDR isolates were screened for Metallo-beta-lactamase production and confirmed by phenotypic confirmatory test: Combined disk test (CDT), EDTA disk synergy (EDS) test & modified-EDS test. 200 GNBs included in this study, 100 isolates were found to be MDR. 63 isolates were found to be MBLs producers. MBLs producers showed broad-spectrum resistance profile and 100% sensitive to colistin (CL) and polymyxin B. This study indicates MBLs producing GNBs, can be prevented by detecting it from all samples by adapting MBL testing in routine laboratories and by implementation policy for proper and judicious selection of antibiotics.

Key-Words: Gram negative bacteria (GNBs), Multi drug resistance (MDR), Metallo-beta-lactamase (MBLs)

Introduction

Gram-negative organisms are generally identified as normal flora in healthy individuals, they rarely cause serious infection in healthy person, but become great concern in hospitalized, immunocompromised, and in intensive care unit patients, where these causes severe invasive infection and responsible for nosocomial outbreak due to various resistance mechanism. There are several mechanisms by which bacteria acquired resistant to β-lactam antibiotics, most clinically and recently identified as efflux reduced permeability, alteration of transpeptidase and by production of β-lactamases. Carbapenems possess broad spectrum activity against Gram-positive as well as Gram-negative organism as compare to other different β-lactams antibiotics (1). They are stable against extended-spectrum β-lactamases and AmpC β-lactamase (2), and are used as last resort of antibiotics to treat multidrug-resistant gram-negative bacilli.

However, in the recent year there is increases incidence of resistance against this last resort of antibiotics are seen in GNBs (3). Resistance may develop due to the production of carbapenem hydrolysing enzymes such as Klebsiella pneumoniae carbapenemase (KPC; Ambler class A); Verona integron-encoded metallo-β-lactamase (VIM), imipenemase (IMP), New Delhi metallo-β-lactamase (NDM) (all Ambler class B); and oxacillinase-48 (OXA-48; Ambler class D) (4-7). In addition, carbapenemase producers are usually associated with many other non-β-lactam resistance determinants, which give rise to multidrug- and pandrug-resistant isolates (8). Productions of MBLs in GNBs are associated with higher morbidity and mortality, and in present there global prevalence increases (9,10).

Metallo-beta-lactamase, characterized by its ability to hydrolyze penicillins, cephalosporins, carbapenems and as well as commercially available beta-lactamase inhibitors, but lack the ability to hydrolyzed aztreonam and remains to susceptible metal ion chelators such as EDTA, sodium mercaptoacetic acid and dipicolinic acid (11).

* Corresponding Author
E.Mail: rajputvandana11@gmail.com,
Mob.: +91-9979263433
β-lactamases can be classified according to two properties; molecular and functional. Molecular classes A, C, and D included beta-lactamases with serine at their active site while molecular class B are metallo enzyme with an active site contain Zinc ion (12). Functional classification were proposed by Bush in 1988, in which group 3 suggested as metallo enzyme (13). This study was undertaken to detect MBLs in GNBs from various clinical samples.

Material and Methods

Study period and clinical samples: The prospective study was conducted in tertiary care hospital at Surat, between December 2013 to December 2014. Various samples like urine, pus/swab, fluids, ET (Endotracheal aspirates), sputum and BAL (Bronchoalveolar lavage) collected with universal safety precautions (14), from patients admitted to hospital or attending the OPD. All samples were processed as per standard microbiological procedure to isolate the organism.

Bacterial strains: By using standard bacteriological techniques like Gram staining, colony morphology on MacConkey’s agar, motility, pigment production, oxidase reaction and other special biochemical media and test organisms were identified and speciated (14, 15).

Antimicrobial susceptibility testing: Antimicrobial sensitivity testing of GNBs wasperformed on Muller-Hinton agar plates by Kirby-Bauer disk diffusion method, according to CLSI guidelines with commercially available disk (Hi-media Laboratory Pvt. Limited; Mumbai India) (16, 17). Results were interpreted as per CLSI- M100-S21, 2011 recommendations (18).

Following antibiotic diskwere used for antibiotic susceptibility test, Ciprofloxacin 5µg/disk (CIP), Ofloxacain 5µg/disk (OF), Levofloxacin 5µg/disk (LE), Amikacin 30µg/disk (AK), Gentamicin 30µg/disk (GEN), Netilmicin 30µg/disk (NET), Tobramycin 10µg/disk (TOB), Ceftazidime 30µg/disk (CAZ), Ceftriaxone 30µg/disk (CTR), Cefotaxime 30µg/disk (CTX), Cefepime 30µg/disk (CPM), Imipenem 10µg/disk (IPM), Meropenem 10µg/disk (MRP), Ertapenem 10µg/disk (ETP), Aztreonam 30µg/disk (AT), Piperacillin 100µg/disk (PI), Piperacillin-tazobactam 100/10µg/disk (PTT), Chloramphenicol 30µg/disk (C), Polymyxin B 300U/disk (PB) and Colistin 10µg/disk (CL). For quality control Escherichia coli ATCC 25922 and Pseudomonas aeruginosa ATCC 27853 strains were used.

MBLs screening method: Isolates resistant to multi-drugs were suspected as MBLs producer. The sensitivity or resistivity pattern to imipenem was not considered for MBL detection as bacteria might harbour “hidden MBLs” and if only the carbapenem resistant phenotypes were considered, then such hidden MBL carrying isolates would be missed (19, 20).

Screening for MBLs production was done using Imipenem, Meropenem, Ertapenem, and third generation cephalosporin Ceftazidime. Screening was carried out by Kirby Bauer disk diffusion method as per CLSI guidelines (17, 18). Isolates resistant to Imipenem, Meropenem, Ertapenem and Ceftazidime were considered as screening positive.

MBLs Confirmation test: All screening positive isolates were subjected to phenotypic confirmatory test. There were various methods have been recommended for detection of MBLs (21). For confirmation of MBLs production we used combined disk test (CDT) and EDTA-Disk synergy test (EDS) test.

A 0.5 M EDTA solution was prepared by dissolving 18.61 g. EDTA (Hi-Media, India) in 100mL of distilled water and adjusting its pH 8 by using NaOH and was sterilized by autoclaving (22).

Combined Disk Test (22): In CDT method, an overnight liquid culture of test organism was adjusted to a turbidity of 0.5 McFarland standards and inoculated on the Muller-Hinton agar plates. After drying, two imipenem disk 10µg/disk, were placed on surface of agar plates. 10 µL 0.5M EDTA solution was added to one of the imipenem disk to obtained desired concentration 750 µg and commercially available IEDisk (imipenem + EDTA 10+750µg/disk) was also compared. Plates were incubated for 16-18 hours at 35°C, an increase in zone diameter of > 7mm around the IPM-EDTA disk as compared to IPM disk alone was considered positive for MBL, as shown in Figure 1.

![Combined disk test](image)

**Fig. 1:** Combined disk test (CDT) using imipenem + imipenem + EDTA

EDTA Disk Synergy Test (23): In EDS test an overnight liquid culture of the test strain was adjusted to a turbidity of 0.5 McFarland standard and spread on the surface of Mueller-Hinton agar plates. After drying, 10µg/diskimipenem disk and blank filter paper (Whatmann filter paper no. 1, 6mm in diameter) disk...
were placed 10 mm apart from edge to edge, and 10µL of 0.5 M EDTA solution was then applied to the blank disk. Carbapenem resistance have often been observed in Enterobacteriaceae producing carbapenemase of various classes, so detection is problematic, means no single carbapenem disk can be used to identify all isolates. In addition to imipenem, three different β-lactam - meropenem, ertapenem & ceftazidime were used with EDS test, for MBLs detection and named as modified-EDS test. After incubating overnight at 37°C, the strain shows a synergistic zone of inhibition between imipenem, meropenem, ertapenem, ceftazidime disks with EDTA disks were considered as MBLs positive as shown in Figure 2, while no synergistic zone of inhibition, the strain were considered as MBLs negative.

Results and Discussion

Bacterial strains: Out of 474 specimens, 200 non-duplicate GNBs were isolated. Among the GNBs, the maximum number of strains isolated were Escherichia coli (n = 94) followed by Klebsiella spp. (n = 51), Pseudomonas aeruginosa (n = 33), Acinetobacterspp (n = 15), Providenciaspp (n = 03), Burkholderiaacepecia complex (n = 01), Proteus (n = 01), Enterobacter cloaca (n = 01), and stenotrophomonasmaltophilia (n = 01).

Antibiotics susceptibility test result: Resistant to three or more antibiotics class viz. β-lactams, aminoglycosides, quinolones, third generation cephalosporins and carbapenems is considered as MDR isolates. We detected 100 MDR strains out of 200 non-duplicate GNBs.

Screening test result: Of the 100 MDR-GNBs, 63 isolates were screening positive. All 63 isolates were resistance to, meropenem, ertapenem and ceftazidime whereas 56 strains were imipenem resistance and remaining 7 strains were sensitive.

Phenotypic confirmatory test result: Out of 63 MBLs screening positive isolates, 56 (88.8%) isolates were confirmed MBLs producer by both CDT and EDS test, while remaining 7 imipenem sensitive strains and 56 strains were found MBLs producer by modified-EDS test, they showed synergistic zone of inhibition between meropenem, ertapenem, ceftazidime with EDTA disk. Results were summarised in (Table no. 1).

<table>
<thead>
<tr>
<th>Number of MDR isolates</th>
<th>Number of screening positive isolates</th>
<th>Number of confirmatory positive isolates By CDT &amp; EDS test Method</th>
<th>By Modified EDS test Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>63</td>
<td>56</td>
<td>7+56 = 63</td>
</tr>
</tbody>
</table>

The predominant source of the 63 MBLs producer isolates were from ET followed by urine, pus/swab, sputum, BAL, and fluids. Of these isolates, highest numbers of organism were from ICU 48 (76.1%) followed by 8 (12.6%) from inpatient department and 7 (11.1%) from outpatient department. Out of 63 MBLs producer isolates 35 (55.5%) were from male patients and 28 (44.4%) were from female patients, indicate no significant difference were associated with gender and age distribution. From 63 MBLs producer GNBs, the
The commonest organism was *Escherichia coli*, followed by *Pseudomonas aeruginosa >Acinetobacter* spp. >*Klebsiella* spp. >*Providencia* spp. >*Providencia* spp. >*Burkholderia cepacia* complex (Table no. 2).

### Table 2: Distribution of MBLs producing clinical isolates by Organism-wise, Sample type wise, & Ward-wise

<table>
<thead>
<tr>
<th>Source of isolates</th>
<th>Urine</th>
<th>ET*</th>
<th>BAL**</th>
<th>Sputum</th>
<th>Fluid</th>
<th>Swab/pus</th>
<th>Out of Total (n=63) No. organisms (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Acinetobacter</em> spp.</td>
<td>00</td>
<td>07</td>
<td>02</td>
<td>00</td>
<td>02</td>
<td>02</td>
<td>13 (20.6%)</td>
</tr>
<tr>
<td><em>Pseudomonas</em> spp.</td>
<td>02</td>
<td>04</td>
<td>00</td>
<td>01</td>
<td>00</td>
<td>01</td>
<td>08 (12.6%)</td>
</tr>
<tr>
<td></td>
<td>02</td>
<td>03</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>05 (7.9%)</td>
</tr>
<tr>
<td></td>
<td>01</td>
<td>01</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>02 (3.1%)</td>
</tr>
<tr>
<td><em>Klebsiella</em> spp.</td>
<td>02</td>
<td>05</td>
<td>00</td>
<td>02</td>
<td>00</td>
<td>02</td>
<td>11 (17.4%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>08</td>
<td>04</td>
<td>00</td>
<td>01</td>
<td>00</td>
<td>01</td>
<td>14 (22.2%)</td>
</tr>
<tr>
<td></td>
<td>04</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>03 (4.7%)</td>
</tr>
<tr>
<td><em>Providencia</em> spp.</td>
<td>01</td>
<td>00</td>
<td>00</td>
<td>00</td>
<td>00</td>
<td>00</td>
<td>01 (1.5%)</td>
</tr>
<tr>
<td><em>Burkholderia cepacia Complex</em></td>
<td>00</td>
<td>01</td>
<td>00</td>
<td>00</td>
<td>00</td>
<td>00</td>
<td>01 (1.5%)</td>
</tr>
<tr>
<td>Total no. of MBLs GNBs sample-wise</td>
<td>24 (38%)</td>
<td>25 (39%)</td>
<td>02 (3.1%)</td>
<td>04 (6.3%)</td>
<td>02 (3.1%)</td>
<td>06 (9.5%)</td>
<td>Total no. of MBLs-GNBs ward Wise</td>
</tr>
</tbody>
</table>

ICU: Intensive-Care-Unit, IPD: Indoor Patients Department, OPD: Outdoor Patients Department. *ET*: Endotracheal aspirates, **BAL**: Bronchoalveolar lavage.

Antibiotics susceptibility of MBLs producers showed 100% sensitive to polymyxin B & colistin, and 100% resistance to aztreonam. Antibiotics resistance profile of MBLs producing isolates were as shown in (Graph no.1). The increasing incidences of MBLs in GNBs indicate, higher morbidity and mortality. The resistance may spread rapidly to various species of GNBs, as the MBLs genes reside in mobile gene cassettes inserted in integrons. The occurrence of MBLs producing isolates poses not only therapeutic problem but also a serious concern for infection control management; their continued spread would be a clinical disaster. Since there are no standard guidelines are available for detection of MBLs. Different studies have reported, the use of different phenotypic methods viz. Hodge test, E-test, DDST, CDT and EDS test. Genotypic methods are also available like PCR, which known as gold standard test but is cost effective, infrastructure and technical expertise not possible in routine microbiology laboratory. Accurate result may not be obtained by single test; hence we undertook these two techniques for phenotypic confirmatory test. Use of combination test would increase the sensitivity to detect the presence of MBLs producer, therefore we used two phenotypic confirmatory tests. Out of these two methods we found modified-EDS test showed better and more reliable than EDS test, although its subjective interpretation. The modified-EDS test was able to detect, all 7 imipenem sensitive strains were MBLs producer, which were not detected by CDT & EDS test. Introduction of ertapenem and EDTA disk in modified-EDS test were seem to be most appropriate for detection with low level resistance to carbapenems, while introduction of meropenem & ceftazidime with EDTA disk were useful in detection of meropenem & ceftazidime resistance isolates.
Among the all 63 MBLs in GNBs, predominant source of MBLs producer was endotracheal secretion 39.6%, followed by urine 38%, swab/pus, 9.5%, sputum 6.3%, fluid & BAL 3.1% respectively. Sangeetha et al., 2014 also observe similar observation as maximum numbers of MBLs-GNBs were isolated from endotracheal secretion (28). It may correlates, indwelling device are major risk factor for the development of MBLs (29, 30).

Isolation of MBLs producer in urine had significant role, in our study second predominant MBLs producer were isolated from urine 38%. Jain et al., 2012 also reported second predominant source of MBLs was urine 35% (31). According to Sendet et al., 1996b MBLs were isolated from the ICU (32).

In our study, Escherichia coli was the most predominant organism isolated 33.3%, followed by Pseudomonas spp. 23.8%, Acinetobacter spp. 20.6%, Klebsiella spp. 19%, Providencia spp., & Burkholderiacepecia complex were 1.5% respectively, while in study of Zahooret al., 2014, the predominant organism was Pseudomonas 40%, followed by Escherichia coli 30%, Acinetobacterspp. 6.7% & Klebsiella spp. 16.7% (33).

Out of 63 MBLs producing strains, maximum number 76.1% were isolated from ICU patients. A similar observation was noted by Prashanth et al., 2001, where the maximum numbers of MBLs producer were isolated from the ICU (34). Since, MBLs producer are largely associated with hospitalized patients, especially in ICU where they share numerous risk factors, excessive use of broad spectrums antibiotics, invasive procedures and associated septicaemia (35, 36).

Identification of Escherichia coli, Pseudomonas spp., Acinetobacter spp., Klebsiella spp., Providencia, & Burkholderiacepecia complex, such MBLs producing nosocomial isolates were essential, because they are associated with high level of mortality especially patients in ICU setting.

MBLs producers were generally associated with broad spectrum resistance profile. In addition such organisms also carry gene encoding other antibiotics resistance determinants such as aminoglycosides, as a result multidrug resistance and pan-drug resistance organisms were arises. These were left limited therapeutic option, however they usually remain susceptible to toxic peptide antibiotics such as polymyxin B and colistin. In our study we found all 63 MBLs producing isolates were 100% susceptible to polymyxin B & colistin. These antibiotics are associated with high incidence of nephrotoxicity and neurotoxicity, which limits their use (27, 37, 38).

Aztreonam susceptibility was common feature of MBLs producing organisms, but resistance showed other co-existing resistance mechanism such as ESBL, AmpC β-lactamase, permeability defects and efflux mechanism (39, 40), in our study all 63 MBLs producing isolates were 100% resistance to aztreonam.

In present study, Providencia sp. and Burkholderiacepecia complex isolates were found to be positive for MBLs by both methods, although, there numbers are too small but attribute significant role in infection. As a result of being difficult to identified & detect, such organisms poses significant risks particularly due to their role in unnoticed spread within institution and their ability to participate in horizontal MBLs gene transfer, with other pathogens in the hospital (41). Emergence of MBL producing fungi in alarming in India, where already facing problem of higher level of antibiotics resistance. Therefore detection of metallo-β-lactamase was important tool for control of the spread of resistance.

Conclusion
In conclusion our study shows that, there is need of routine detection of MBLs from all clinical samples, as infection control measures. There should be judicious use of carbapenems to prevent the spread of resistance and use of effective antibiotics as per the antibiotic-sensitivity report, which contributes towards the optimal treatment of patients.

Acknowledgement
We are thankful to Management staff, Pathologist, Infection control Manager, and all laboratory staff of Pathology and Microbiology department of tertiary-care hospital for helping and providing the suitable environment to carry out this study. We also thankful to all the patients whose samples were used in this study.
Research Article

[Graph 1: Resistance profile of MBLs producing isolates (n=63)]

References


