Utility of Phenotypic Methods in Detection of Metallo-Beta-Lactamases in Gram-Negative Bacteria

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Abstract

Background: Metallo-beta-lactamases (MBLs)-producing Gram-negative bacilli (GNB) are one of the significant multidrug-resistant pathogens causing healthcare-associated infection (HAI) worldwide. The present study aimed to compare the phenotypic and molecular methods to detect MBLs-producing GNB causing HAI, namely Acinetobacter baumannii, Escherichia coli, Klebsiella pneumoniae, and Pseudomonas aeruginosa in a tertiary care hospital. Materials and Methods: Antimicrobial susceptibilities were determined for 232 isolates identified during 8 months period, and the Modified Hodge Test confirmed carbapenemases production in carbapenem-resistant isolates as per the Clinical and Laboratory Standards Guidelines 2013. MBLs production was detected by the disc enhancement test (DET), combined disk test (CDT), and polymerase chain reaction (PCR) and statistically analyzed. Results: One hundred (43.1%) isolates were found to be carbapenem-resistant, of which 85% were positive on the Modified Hodge Test. The DET was 96.67%, whereas the CDT was 93.33% sensitive as compared to the PCR. Kappa coefficient ranged from 0.918 to 0.959 for different methods indicating the excellent agreement between the three methods. Conclusion: The DET and CDT showed excellent agreement with molecular methods to detect MBLs-producing isolates. They are easy to perform, inexpensive, and can be routinely used in clinical laboratories.

Keywords: Carbapenem, metallo-beta-lactamases, MBL phenotypic comparison

Introduction

The development and the spread of multidrug-resistant Gram-negative pathogens are a noteworthy public health concern.[1] The most prevalent Gram-negative pathogens, Escherichia coli (E. coli), Klebsiella pneumoniae (K. pneumoniae), Pseudomonas aeruginosa (P. aeruginosa), and Acinetobacter baumannii (A. baumannii), cause a variety of diseases in humans. These pathogens can acquire multidrug resistance in health-care settings due to various factors such as direct contacts and can spread swiftly. They are a threat of immense epidemiological concern being major pathogens related to healthcare-associated infection. They are also known to acquire resistance against carbapenems which are usually used as last resort drugs against multidrug-resistant organisms.[2,3]

Resistance to third-generation cephalosporin in the eighties and nineties through extended-spectrum beta-lactamase production by pathogens led to the increased usage of carbapenems. Due to heavy reliance on carbapenem agents, resistance to these antimicrobials started emerging, especially in the last decade.[4] Various mechanisms of carbapenem resistance have been attributed to such as reduced outer membrane permeability, augmented efflux systems, modification of penicillin-binding proteins, and the most frequent carbapenem-hydrolyzing enzymes, i.e., production of carbapenemases.[5] A variety of carbapenemases have been reported in these pathogens which belong to Ambler class A,
B, and D such as *K. pneumoniae* carbapenemase (KPC), Verona integron-encoded metallo-beta-lactamase (VIM), and New Delhi metallo-beta-lactamase (NDM). Broadly, two different families of these carbapenemases are seen in these Gram-negative bacteria: serine-beta-lactamasess and metallo-beta-lactamasess (MBLs). MBL enzymes exhibit a broad spectrum of hydrolytic activity against many antimicrobials, including all penicillins, cephalosporins, and carbapenems. Their activity is also not inhibited by beta-lactamase inhibitors clavulanic acid, tazobactam, and sulbactam. The genes encoding MBL are carried on mobile genetic elements, and they spread laterally among Gram-negative pathogens. This spread of enzymes has lead to the widespread dissemination of carbapenem-resistant pathogens. Since there are fewer antibiotics in the pipeline to combat them, the detection of MBL-producing Gram-negative bacilli (GNB) is of paramount significance for the optimal treatment of patients and to curb the spread of resistance.

Thus, it is necessary to detect and characterize MBL by dependable phenotypic and molecular detection techniques to ensure early treatment and check their further propagation.

**Materials and Methods**

The present study was carried out in the department of microbiology of an urban tertiary care center for 8 months. Two hundred and thirty-two consecutive nonrepeat isolates of four species of GNB (*A. baumannii, E. coli, K. pneumoniae*, and *P. aeruginosa*) were collected from various clinical samples such as blood, body fluids, pus, sputum, tracheal aspirate, urine, and wound swabs from inpatients of all age groups and both sexes. Assuming a 95% confidence interval, α = 5% and ROL value of 16.2% detection of carbapenemases and precision value of 5%, the total sample size required was 209 isolates. To lessen uncertainty due to frequent contamination of samples, the team collected more isolates.

All samples were collected using strict aseptic precautions and processed immediately as per the standard microbiological protocols. Patients’ details were collected, and consents were taken. Antimicrobial susceptibility testing was performed on all isolates using Kirby–Bauer disc diffusion method as per the Clinical and Laboratory Standards Institute (CLSI) guidelines M100-S23, including screening for carbapenem resistance, by using 10 μg imipenem (IPM) and meropenem (MRP) discs (HiMedia).

Carbapenemases production was detected by performing the Modified Hodge Test (MHT) in carbapenem-resistant isolates as recommended by the CLSI guidelines to see the clover-leaf indentation of the *E. coli* ATCC 25922 growing along with the test organism growth streak within the disc-diffusion zone. Further, all carbapenem-resistant isolates were subjected to two different phenotypic tests for the detection of MBL.

First, the disc enhancement test (DET) using IPM, IPM + Ethylenediaminetetraacetic acid (EDTA), and IPM + Zinc sulfate heptahydrate (ZnSO₄ 7H₂O) discs were carried out for MBL detection. In the DET on a Mueller Hinton Agar plate, 10 μ of 0.5M EDTA chelates the metal ion present in MBL which increases the zone of inhibition around IPM + EDTA disc and an increase of ≥7 mm as compared to IPM disc alone is taken as positive for MBL production. One IPM disc with 10 μl of 50 mM ZnSO₄ 7H₂O (140 μg) was also put to check if zone decreases by ≤3 mm compared to IPM disc alone to rule out the false-positive results.

Second, the combined disc test (CDT) with KPC + MBL Confirm ID Kit (ROSCO Diagnostica, Denmark) was used to detect AmpC with/without porin loss, KPC and MBL. The CDT kit consists of 9-mm diameter NeoSensitabs of MRP (10 μg), MRP + Boronic acid (10 + 250 μg), MRP + Cloxacinilin (10 + 500 μg), and MRP + Dipicolin acid (250 μg). Boronic acid inhibits KPC and AmpC production, cloxacinilin inhibits AmpC, and dipicolinic acid inhibits MBL production. The interpretation of the CDT with KPC + MBL kit is depicted in Table 1.

Finally, the molecular characterization was done with polymerase chain reaction (PCR) by the setting of simultaneous detection of common genes responsible for carbapenemases production in all IPM-resistant isolates. The presence of MBL-producing genes VIM, NDM, and IMP was evaluated. Furthermore, KPC was assessed, as together, these are the most frequently encountered carbapenemases. Control strains were used as positive and negative controls. DNA was extracted (HiPura™ kit, HiMedia) and amplified in a thermocycler (Touchgene Gradient, TECHNE). Multiplex and uniplex PCR were performed on all carbapenem-resistant isolates. The separated DNA fragments on agarose gel electrophoresis with specific bands at particular gene amplicon size were considered as positive PCR reactions. The primers (Eurofins Genomics) used and DNA fragments of a specific size are depicted in Table 2.

Taking PCR as the gold standard, sensitivity and specificity of the DET and CDT were calculated. To rule out high sensitivity

| Table 1: The interpretation of combined disc test (*Klebsiella pneumoniae* carbapenemase + metallo-beta-lactamases) kit |
| :------------------------------------------------------------------------------------------------------------------|
| **Enzyme** | **Neo-sensitab concentration** | **Meropenem + dipicolinic acid (mm)** | **Meropenem + boronic acid (mm)** | **Meropenem + cloxacinilin (mm)** |
|------------|---------------------------------|-------------------------------------|---------------------------------|---------------------------------|
| MBL        | Meropenem 10 μg                 | ≥5                                  | ≤3                             | ≤3                             |
| KPC        | Meropenem 10 μg                 | ≤3                                  | ≥5                             | ≤3                             |
| AmpC + porin loss | Meropenem 10 μg | ≤5                                      | ≥5 AND                          | ≥5                             |

MBL: Metallo-beta-lactamases, KPC: *Klebsiella pneumoniae* carbapenemase
and specificity due to the chance factor and find a level of agreement between three methods (pairwise), the kappa coefficient was used. Kappa coefficient can range from −1 to +1, where 0 represents the amount of agreement that can be anticipated from random chance, and 1 signifies a perfect agreement between the raters or methods.  

**Results**

A total of 232 consecutive, nonrepeat samples of *A. baumannii*, *E. coli*, *K. pneumoniae*, and *P. aeruginosa* were isolated from various clinical samples of inpatient. About 25% were from females and 75% were from males. The mean age of the patient was 48.9 years, with the minimum age being 1 month and a maximum of 91 years making the sample heterogeneous. The distribution of various sample types is depicted in Figure 1.

Of 232 (43.1%) isolates, 100 were found to be carbapenem resistant, as described in Table 3. A total of 48 of 73 (66%) *A. baumannii* isolates were carbapenem resistant. In case of *E. coli*, only 9 of 49 (18%), while 24 of 54 (44%) *K. pneumoniae*, and 19 of 56 (34%) *P. aeruginosa* were carbapenem resistant. Many of these isolates were resistant to multiple antimicrobial drugs with the highest resistance shown by carbapenem-resistant isolates of *A. baumannii*. The majority of the strains were sensitive to colistin and polymyxin B.

Eighty-five of 100 (85%) carbapenem-resistant isolates gave positive MHT. Forty-two of 48 (87.5%) carbapenem-resistant *A. baumannii*, 6 out of 9 (66.7%) *E. coli*, 21 out of 24 (87.5%) *K. pneumoniae*, and 16 out of 19 (84.2%) isolates of *P. aeruginosa* were MHT positive.

Fifty-eight isolates of 100 (58%) were positive for MBL production by the DET. Sixteen (27.6%) isolates of *A. baumannii*, 6 (10.3%) *E. coli*, 22 (37.9%) *K. pneumoniae*, and 14 (24.1%) *P. aeruginosa* were positive for MBL using the DET. The DET result for one isolate is depicted in Figure 2.

In the CDT, overall, 56 samples of 100 (56%) were tested positive for MBL-producing isolates. All isolates which were positive by the CDT were also positive by the DET. No KPC and AmpC with/without porin loss-producing strains were found. Fifteen (26.8%) *A. baumannii*, 6 (1.7%) *E. coli*, 22 (39.3%) *K. pneumoniae*, and 13 (23.2%) *P. aeruginosa* were positive for MBL using the CDT. The CDT result for one isolate is depicted in Figure 3.

Of the four MBL genes studied, NDM gene was detected in a total 60 of 100 carbapenem-resistant isolates, of which seven isolates had both VIM and NDM genes, and the PCR results are depicted in Table 4. There were

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**Table 2: Primers used in the polymerase chain reaction and DNA fragments of the specific size**

| Gene   | Primer sequence (bp) | Amplicon size (bp) | Cycling condition |
|--------|----------------------|--------------------|-------------------|
|        |                      |                    | Denaturation | Annealing | Extension |
| KPC    | (F):TGCAGAGCCCCGTTCAGTGT | 138                | 94°C        | 55°C      | 72°C      |
|        | (R):CGCTCTATGCGGCGATACCA |                    |             |           |           |
| VIM    | (F):GAGATTCACGCAYTCTCTAGA | 94                 | 94°C        | 55°C      | 72°C      |
|        | (R):AATGCGCAGACACCAGGATAG |                    |             |           |           |
| NDM    | (F):CATAGCCGTGCATTGATG | 83                 | 94°C        | 55°C      | 72°C      |
|        | (R):GTGCGCAGTTCACATTGCC |                    |             |           |           |
| IMP    | (F):AAGTTAGTCA(CC/T)TTTGTTTGAGGAC | 169              | 94°C        | 55°C      | 72°C      |
|        | (R):CAGACCTACCTGTATGTCGGATG |                    |             |           |           |

KPC: *Klebsiella pneumoniae* carbapenemase, NDM: New Delhi metallo-beta-lactamase, VIM: Verona integron-encoded metallo-beta-lactamase, IMP: Active-on-imipenem
18 A. baumannii (including 2 VIM + NDM), 6 E. coli, 22 K. pneumoniae (including 3 VIM + NDM), and 14 P. aeruginosa (including 2 VIM + NDM) isolates which had the presence of MBL genes. DNA bands on agarose gel electrophoresis for uniplex NDM gene are depicted in Figure 4. The strain-wise distribution of MBL/non-MBL isolates is depicted in Table 5.

The sensitivity, specificity, and kappa coefficients were calculated to find the concordance in results of tests to detect MBL production by all isolates by comparing the DET and CDT with the PCR and the DET with the CDT separately. The pairwise agreement of the DET, CDT, and PCR is depicted in Table 6.

The sensitivity of the DET was 96.7% (95% confidence interval [CI]: 88.5%–99.6%), whereas the CDT was 93.3% (95% CI: 83.8%–98.2%) sensitive as compared to the PCR. The specificity was 100% in both phenotypic tests (95% CI: 91.2%–100.0%). Kappa coefficient, which is a measure of agreement, was 0.959 for the DET and PCR (95% CI, 0.901–1), 0.918 for the CDT and PCR (95% CI, 0.838–0.998), and 0.959 for the DET and CDT (95% CI, 0.903–1). All the kappa coefficients were significant with \( P < 0.0001 \). There is a STARD flowchart provided in Figure 5.

**Discussion**

A total of 43.1% isolates were carbapenem resistant by screening test which is in accordance with other studies done in Gram-negative bacteria as reported by Adhikari et al.[20] In the study carried out by Hodiwala et al., which specifically included these four genera, 16.2% isolates were carbapenem resistant, but this study had samples from both outpatient and inpatient departments.[21] Our study had samples from inpatients with the predominance of the sample from acute wards. The carbapenem resistance has varied in GNB between different studies from 1.8% to 90% depending upon the region, location, and patient profile of the study.[22,23]

A high level of carbapenem resistance was exhibited by A. baumannii, i.e., 66% (\( n = 48/73 \)) isolates followed by 44% (\( n = 24/54 \)) of K. pneumoniae, 34% (\( n = 19/56 \)) of P. aeruginosa, and 18% (\( n = 9/49 \)) of E. coli. A. baumannii and P. aeruginosa are shown to be the most common carbapenem-resistant isolates in the other studies also.[24] The second highest resistant organism was K. pneumoniae with 44% carbapenem-resistant isolates, followed by 34% P. aeruginosa. Eighteen percent of E. coli showed resistance against carbapenems which is in concordance with the earlier studies.[25,26]
A total of 85% of carbapenem-resistant isolates were MHT positive which varies from 60% to 97.5% in various studies. Fifty-eight percent of total carbapenem-resistant isolates were MBL positive by the DET, whereas 56% were MBL positive by the CDT. In multiplex PCR, 60 isolates showed the presence of NDM gene, of which seven isolates were with both NDM and VIM genes. No isolate was found to have IMP and KPC genes. Other methods such as loss of porin channel or increased efflux mechanism may be responsible for IPM resistance in the rest of MHT-negative isolates. Eight MHT-negative isolates, which were DET and CDT positive, showed the presence of NDM genes. MHT has less sensitivity to detect...
NDM producers, despite the correct detection of class A and class D carbapenemase producers. In a study by Girlich et al., MHT’s sensitivity to detect NDM producers was found to be only 50%, which was justified by possible secretion of colicin, a bacteriocin peptide by some strains, which may suppress the growth of the indicator strain and therefore interfere with the results.[23]

High sensitivities of the DET (96.67%) and CDT (93.33%) in the detection of MBL and specificity of 100% make them a suitable replacement for the PCR in peripheral hospitals. Kappa values of 0.959 (or 91.96% agreement) for the DET and 0.918 (or 84.27% agreement) for the CDT which are significant at \( P < 0.0001 \) emphasize the excellent agreement between the DET and CDT which further supports the proposition. These are cost-effective, need less expertise, and can be relied upon for the detection of MBL in the routine clinical laboratories.

Infections due to Gram-negative MBL-producing pathogens are on the rise, especially in the intensive care unit patients who have conditions compromised by invasive procedures and multiple comorbidities. Several studies have shown that an effective initial empirical antibiotic therapy guided by local antibiotic policy improves outcome and survival, but this principle can only be implemented through ongoing surveillance of susceptibility data of clinical pathogens. Both the phenotypic tests, namely the DET and CDT showed excellent agreement with the PCR for the detection of MBL-producing pathogens. Hence, the DET and CDT can be used to confirm the detection of these isolates routinely in the clinical laboratories.

**Conclusion**

The DET and CDT phenotypic tests showed excellent agreements with the PCR to detect MBL-producing isolates. They are easy to perform, inexpensive, and can be routinely used in clinical laboratories. There are limited treatment options available against MBL-producing pathogens,[27] therefore, rapid detection of these organisms by simple confirmatory methods such as the DET and CDT is vital to improving patient outcomes, limit their spread, and enhance infection control.

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**Conflicts of interest**

There are no conflicts of interest.

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