Carbapenem Resistance in Non-Fermenters: An Overview

Bhuvaneshwari G*, Shameembanu A S, Kalyani Mohanram

Department of Microbiology, Saveetha Medical College and Hospital, Thandalam, Chennai 602105, Tamilnadu, India

**ABSTRACT**

This study was conducted with interest in increasing carbapenem resistance in non-fermenters: an important causative agent of nosocomial infection and to standardize the methods for interpretation of their resistance. The aim of this study is to perform disk diffusion testing and minimal inhibitory concentration technique for the identification of carbapenem resistance for imipenem and meropenem. The isolates found resistant to carbapenems were confirmed with the modified Hodge test. The genes responsible for carbapenem resistance were identified by both phenotypic and genotypic methods. Out of 240 non-fermenters isolated 20% showed resistance to carbapenem by disk diffusion. Only 7% showed resistance by the micro broth dilution technique of minimum inhibitory concentration. 3% were panning drug-resistant. Out of 16 carbapenem-resistant isolates, 5 were found to have KPC (*Klebsiella pneumonia* carbapenem) genes, 9 had MBL (Metallo beta-lactamase) genes and 2 had KPC+MBL genes and none were found to have Amp C and OXA-48 genes phenotypically. Genotypically all the KPC strains had KPC genes and out of 9 MBL strains, 6 had VIM and the remaining 3 strains were negative for both IMP and VIM gene. In conclusion, the interpretation of susceptibility for carbapenems should not be made only with disk diffusion testing. Always check for Minimal inhibitory concentration methods and determination of genes responsible for carbapenem resistance, a double-disc synergy test goes in hand with genotypic detection.

*Corresponding Author
Name: Bhuvaneshwari G
Phone: 9941787147
Email: bhuvaneshwarigunasekar@gmail.com

ISSN: 0975-7538
DOI: [https://doi.org/10.26452/ijrps.v11iSPL2.2120](https://doi.org/10.26452/ijrps.v11iSPL2.2120)

© International Journal of Research in Pharmaceutical Sciences
fermenters in our setup as well as to determine the methodology to routinely do in the diagnostic laboratory for the detection of carbapenem resistance.

MATERIALS AND METHODS

A cross-sectional study during the period of April 2018 to October 2018 was conducted at Saveetha Medical College and Hospital, Thandalam, Tamil Nadu after getting approval from Human Ethical Committee and Institutional Review Board. Disk diffusion testing and micro broth dilution for minimal inhibitory concentration detection were done according to CLSI (Central laboratory standard institute) 2017 guidelines. A double-disc synergy test was done for phenotypic detection of resistant genes. Uniplex PCR was done for genotypic detection.

Double disk synergy test

Phenotypic methods for detecting carbapenemase activity and the differentiation of KPCs and MBLs was performed (Tsakris et al., 2010; Prakash, 2006). Carbapenemase activity was assessed with the modified Hodge test (MHT) using meropenem disks according to the CLSI guidelines (CLSI, 2012). The phenotypic detection of KPC- and/or MBL-possessing nonfermenter isolates was carried out by a combined disk test with meropenem as a substrate without and with phenylboronic acid (PBA), Ethylene diamine tetraacetic acid (EDTA), or both (Tsakris et al., 2009).

OXA-48 disk test

The test is based on the use of EDTA to permeabilize the bacterial cell and release _lactamases into the external environment. EDTA was also used to inhibit the production of MBL carbapenemases (Franklin et al., 2006), while a solution of PBA was used to inhibit the production of KPCs (Tsakris et al., 2009). The stock solution of EDTA was prepared by dissolving anhydrous EDTA in distilled water at a concentration of 0.1M (CLSI, 2012). From this solution, 10 μl (containing 292 μg of EDTA) was dispensed onto two blank paper disks. The stock solution of PBA was prepared as previously recommended (Tsakris et al., 2009) by dissolving PBA in dimethyl sulfoxide and water at a concentration of 60 mg/ml. From this solution, 10 μl (containing 600 μg of PBA) was dispensed onto the right of the two disks containing EDT. The disks were then dried and used within 60 min. The surface of a Mueller-Hinton agar plate was inoculated with a lawn of carbapenem-susceptible E. coli ATCC 25922 at turbidity of 0.5 McFarland standards. A 10-μg imipenem disk was placed on the inoculated surface of the Mueller-Hinton agar.

By touching the tops of well-isolated colonies, 2 to 3 colonies of the tested microorganism were applied to coat the disks containing EDTA and EDTA plus PBA. The inoculated disks were placed with the bacterial inoculum (microorganism) side down on the lawn adjacent to the imipenem disk. The plate was then incubated overnight at 35°C in ambient air. After 18 h of incubation, the plates were examined for either an indentation or a flattening of the zone of inhibition, indicating enzymatic inactivation of imipenem (positive result) for either the disk adjacent to the imipenem or the absence of a distortion of the inhibition halo, indicating no significant inactivation of imipenem (negative result). Indentation of growth toward both EDTA and EDTA/PBA disks indicated the production of OXA-48 carbapenemase. Indentation of growth toward the EDTA disk and an absence of growth toward the EDTA/PBA disks was indicative of KPC or KPC plus MBL production. An absence of growth toward both disks containing EDTA and EDTA/PBA indicated production of MBL carbapenemase or nonsusceptibility to carbapenems due to ESBL/AmpC production plus porin loss.

Genotypic detection

Pure culture of carbapenem-resistant strains was further processed for molecular detection of drug-resistant genes targeting the following sequence (Murugan et al., 2010).

**Primer sequence targeting bla** $K_{PC}$ **gene:**

KPC-F: CGGCAGCAGTTTGTGATTG
KPC-R: CGCTGTGCTTGTCATCCTTG

**Primer sequence targeting blaVim gene:**

VIM-F: TGTCGTTGATGTGATGAGT
VIM-R: GTGCTTCCGGTATGTTG

**Primer sequence targeting bla** $IMP$ **gene:**

IMP-F (5′-GAAGCCGTTTATGTTTCAC-3′)
IMP-R (5′-GTATGTTCAAGAGTGTGC-3′)

RESULTS AND DISCUSSION

In this study, out of 240 non-fermenters isolated, 49 were found to be resistant to carbapenems by disc diffusion test. Out of which only 16 were confirmed to be resistant by minimum inhibitory concentration (MIC) technique. The disk diffusion susceptibility report is given in Table 1. MIC 50 and 90 are tabulated in Table 2. The antibiotic susceptibility profile of 16 carbapenem-resistant strains was tabulated in Table 3.
Table 1: Antibiotic susceptibility test of non-fermenters

| Group of Antibiotics       | Number of isolates Sensitive | Number of isolates Resistant |
|----------------------------|------------------------------|------------------------------|
| Aminoglycoside (AK)        | 201                          | 39                           |
| Cephalosporin (CEPH)       | 117                          | 123                          |
| Quinolone (Q)              | 156                          | 84                           |
| Beta-lactam inhibitors (BLI)| 183                          | 57                           |
| Carbapenem (C)             | 191                          | 49                           |

Chi-square=91.965, P<0.001.

Table 2: Minimum inhibitory concentration of non-fermenters for carbapenems

| MIC 50 (25th no.) | MIC 90 (44th no.) |
|-------------------|-------------------|
| Imipenem          | 2                 | 8                 |
| Meropenem         | 1                 | 8                 |

Table 3: The antimicrobial susceptibility profile of 16 carbapenemase producers

| Susceptibility profile       | No. of isolates |
|------------------------------|-----------------|
| (AK, G, CAZ, CPM, CIP, OF, PIT, IMP, MR) R | (Nil) S 7 |
| (CAZ, CPM, CIP, OF, PIT, IMP, MR) R         | (AK, G) S 5   |
| (G, CAZ, CPM, CIP, OF, PIT, MR) R          | (AK, IMP) S 2  |
| (G, CAZ, CPM, CIP, OF, PIT, IMP, MR) R     | (AK) S 1       |
| (AK, G, CAZ, CPM, CIP, OF, MR) R           | (PIT, IMP) S 1 |

Figure 1: Phenotypic detection of Carbapenem-resistant gene
Table 4: Comparison of phenotypic and genotypic identification of carbapenem-resistant genes

| Organism                   | Imipenem MIC (µg/mL) | Meropenem MIC (µg/mL) | phenotypic gene detection | Genotypic detection |
|----------------------------|-----------------------|------------------------|---------------------------|---------------------|
| Pseudomonas aeruginosa     | 8                     | 4                      | KPC                       | KPC                 |
| Pseudomonas aeruginosa     | 4                     | 8                      | MBL                       | VIM                 |
| Pseudomonas aeruginosa     | 8                     | 8                      | MBL                       | VIM                 |
| Pseudomonas aeruginosa     | 4                     | 8                      | KPC+MBL                   | KPC+VIM             |
| Acinetobacter baumannii    | 4                     | 2                      | KPC                       | KPC                 |
| Acinetobacter baumannii    | 8                     | 8                      | MBL                       | VIM                 |
| Acinetobacter baumannii    | 8                     | 8                      | KPC                       | KPC                 |
| Acinetobacter lwofii       | 8                     | 8                      | MBL                       | VIM                 |
| Acinetobacter lwofii       | 8                     | 8                      | MBL                       | VIM                 |
| Acinetobacter lwofii       | 8                     | 8                      | MBL                       | KPC, VIM and KPC    |
| Acinetobacter lwofii       | 8                     | 16                     | KPC                       | KPC, VIM and KPC    |
| Stenotrophomonas maltophilia| 4                    | 2                      | MBL+KPC                   | VIM+KPC             |
| Acinetobacter lwofii       | 8                     | 4                      | MBL                       | VIM                 |
| Pseudomonas stutzeri       | 4                     | 2                      | KPC                       | KPC                 |
| Pseudomonas putida         | 8                     | 4                      | MBL                       | Negative for IMP, VIM and KPC |
| Moraxella atlantae         | 8                     | 8                      | MBL                       | VIM                 |

*KPC-Klebsiella pneumonia carbapenemase, MBL- metallo beta lactamase, VIM- Verona integronencoded metallo beta lactamase, IMP- active on imipenem.

Table 5: Comparison of phenotypic and genotypic identification of carbapenem-resistant genes

| Genotypic Identification | Phenotypic Identification |
|--------------------------|---------------------------|
|                          | KPC Positive (5 isolates) | MBL Positive (9 isolates) | KPC+MBL Positive (2 isolates) |
| KPC                      | POSITIVE                  | 5                          | NA                          | NA                          |
|                          | NEGATIVE                  | 0                          | NA                          | NA                          |
| MBL(VIM,IMP)             | POSITIVE                  | NA                         | 6                           | NA                          |
|                          | NEGATIVE                  | NA                         | 3                           | NA                          |
| KPC+MBL                  | POSITIVE                  | NA                         | NA                          | 2                           |
|                          | NEGATIVE                  | NA                         | NA                          | 0                           |
The correlation between phenotypic and genotypic detection is explained in Figures 1, 2, 3 and 4 and also in Table 4 and Table 5. This explains that 81% correlation was there between both methods.

The present study deals with the analysis of 240 non-repeatable non-fermentative gram-negative bacilli isolated from various clinical samples that came to the clinical microbiology laboratory of Saveetha Medical College and Hospital for the prevalence of carbapenem-resistant of those nosocomial infections causing organisms in our set up. In this study minimum, inhibitory concentration was proved to be the highly sensitive methodology for screening the resistance to imipenem and meropenem. Where carbapenem resistance prevalence rate is only 7%, which can be controlled by proper surveillance, disinfection and following proper regimen for patients; moreover, phenotypic methods for gene detection can be done when there is no availability of polymerase chain
reaction. Because 81% was well correlated with genotypic detection, the remaining 19% also may be correlated if the study would have identified for NDM (new Delhi Metallo carbapenemase) or GIM (German Imipenemase) or SPM (Sao Paulo Metallo beta-lactamase). As the genotyping is expensive to perform couldn’t do for other genes which were not prevalent in this locality. This is a drawback of this study.

Carbapenem resistance

The present study shows the prevalence of Carbapenem resistance among non-fermenting gram-negative bacilli is 7%, which is less when compared to the study done by (Noyal et al., 2009). which was found to be 14.3% and 15% by Shivesh et al and 10.9% by (Shashikala et al., 2006) respectively. In our study, of the total carbapenem-resistant non-fermenters isolated, 31% were KPC producers, 56% were MBL producers and 13% were both KPC and MBL producers. None were found to be positive for OXA-48. While the study done by (Datta et al., 2012), reported a 5.75% MBL-type Carbapenemase among Enterobacteriaceae strains and KPC production among Enterobacteriaceae was found to be 2.51% (13 out of 516). Most of the Indian studies reported carbapenemase production in non-fermenters like Pseudomonas aeruginosa and Acinetobacter baumannii, where the incidence ranged from 7% to 65%. (Singh et al., 2014). However, very few studies that showed carbapenemase production, including MBL and KPC in Enterobacteriaceae, have been conducted in India so far and according to those reports, the occurrence of these enzymes ranged from 1% to 18%. (Deshmukh et al., 2011). The bacteria having MBL has the potential to spread rapidly (horizontal MBL gene transfer) within the hospital environment and also across continents posing both therapeutic and control management problems.

**BlaKPC, BlaIMP and Bla IMP detection**

Molecular detection was done for KPC and MBL producing isolates where for three isolates bla\text{IMP} and bla\text{VIM} gene were not detected. This could be explained by the fact that this isolate show MBL resistance by the presence of genes other than bla\text{IMP} and bla\text{VIM}, which needs further evaluation. This is contrary to the study done by Fatemeh Fallah et al. (Fallah and Borhan, 2013), where out of 48 MBL producers, all were found to be positive for bla\text{IMP} and negative for bla\text{VIM}.

**CONCLUSION**

Minimal Inhibitory Concentration technique needs to be done for carbapenem instead of Kirby Bauer disk diffusion assay before reporting resistance to carbapenems. This will help the patients by preventing the treatment with third-line drugs like Colistin, Polymixin B and fosfomycin. Genotyping and phenotyping can go in hand for the detection of resistant genes for research and diagnostic purposes.

**ACKNOWLEDGEMENT**

I would like to thank my technicians for the immense support given by them. And would like to solicit my gratitude to my guide and co-guide for their guid-
I also thank the Saveetha Institute of Medical and Technical Sciences for the infrastructure provided for carrying out this work.

REFERENCES

CLSI 2012. Performance standards for antimicrobial susceptibility testing; 22nd informational supplement update.

Datta, P., Gupta, V., Garg, S., Chander, J. 2012. Phenotypic method for differentiation of carbapenemases in Enterobacteriaceae: Study from North India. Indian Journal of Pathology and Microbiology, 55(3).

Deshmukh, D. G., Damle, A. S., Bajaj, J. K., Bhakre, J. B., Patwardhan, N. S. 2011. Metallo-β-lactamase-producing clinical isolates from patients of a tertiary care hospital. Journal of laboratory physicians, 3(2):93–97.

Fallah, F., Borhan, R. 2013. Detection of bla (IMP) and bla (VIM) Metallo-β-lactamases genes among Pseudomonas aeruginosa strains. International Journal of Burns and Trauma, 3(2):122–124.

Franklin, C., Liolios, L., Peleg, A. Y. 2006. Phenotypic Detection of Carbapenem-Susceptible Metallo-β-Lactamase-Producing Gram-Negative Bacilli in the Clinical Laboratory. Journal of Clinical Microbiology, 44(9):3139–3144.

Lari, A., Talebi, M., Owlia, P., Alaghhehbandan, R., Asghari, B., Lari, E., Azimi, L. 2015. Inhibitory-based method for detection of Klebsiella pneumoniae carbapenemase Acinetobacter baumannii isolated from burn patients. Indian Journal of Pathology and Microbiology, 58(2):192–192.

Livermore, D. M., Woodford, N. 2006. The β-lactamase threat in Enterobacteriaceae, Pseudomonas and Acinetobacter. Trends in Microbiology, 14(9):413–420.

Murugan, S., Lakshmi, R. B., Devi, P. U., Mani, K. R. 2010. Prevalence and antimicrobial susceptibility pattern of Metallo β-lactamase producing Pseudomonas aeruginosa in diabetic foot infection. Int J Microbiol Res, 1:123–128.

Nordmann, P., Dortet, L., Poirel, L. 2012. Carbapenem resistance in Enterobacteriaceae: here is the storm! Trends in Molecular Medicine. 18:263–272.

Nordmann, P., Poirel, L. 2014. The difficult-to-control spread of carbapenemase producers among Enterobacteriaceae worldwide. Clinical Microbiology and Infection, 20(9):821–830.

Noyal, M. J. C., Menezes, G. A., Harish, B. N., Sujatha, S., Parija, S. C. 2009. Simple screening tests for the detection of carbapenemases in clinical isolates of nonfermentative Gram-negative bacteria. The Indian Journal of Medical Research, 129(6):707–712.

Prakash, S. 2006. Carbapenem sensitivity profile amongst bacterial isolates from clinical specimens in Kanpur city. Indian Journal of Critical Care Medicine, 10(4):250–253.

Shashikala, D., Kanungo, S., Srinivasan, R., S., S., S. 2006. Emerging resistance to carbapenems in hospital-acquired Pseudomonas infection: A cause for concern. Indian Journal of Pharmacology, 38(4).

Singh, R. M., Sarkar, S., Saha, P., Gupta, M., Sen 2014. Carbapenemase-producing Enterobacteriaceae among urinary isolates: scenario from a tertiary care hospital in eastern India. Journal of Evolution of Medical and Dental Sciences, 3(6):1323–1333.

Thomson, K. S. 2010. Extended-Spectrum-β-Lactamase, AmpC, and Carbapenemase Issues. Journal of Clinical Microbiology, 48(4):1019–1025.

Tsakris, A., Kristo, I., Poulou, A., Themeli-Digalaki, K., Ilkonomidis, A., Petropoulou, D., Sofianou, D. 2009. Evaluation of Boronic Acid Disk Tests for Differentiating KPC-Possessing Klebsiella pneumoniae Isolates in the Clinical Laboratory. Journal of Clinical Microbiology, 47(2):362–367.

Tsakris, A., Poulou, A., Pournaras, S., Voulgari, E., Vrioni, G., Themeli-Digalaki, K., Sofianou, D. 2010. A simple phenotypic method for the differentiation of Metallo-β-lactamases and class A KPC carbapenemases in Enterobacteriaceae clinical isolates. Journal of Antimicrobial Chemotherapy, 65(8):1664–1671.

Walsh, T. R. 2010. Emerging carbapenemases: a global perspective. International Journal of Antimicrobial Agents, 36(3):70004–70006.