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Original Article

Molecular Detection of Metallo-β-Lactamase Genes in Carbapenem-Resistant Non-Fermenting Gram-Negative Bacilli from a Tertiary Care Hospital in Lahore, Pakistan

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Abstract

Objective: This study aims to identify the most common metallo-beta-lactamase (MBL) genes in non-fermenting Gram-negative bacilli (NFGNB) through both phenotypic and genotypic analysis, with the goal of informing new strategies to mitigate hospital-acquired infections.

Study type, settings & duration: This cross-sectional study was conducted at General Hospital, Lahore and Institute of Public Health, Lahore from January to December 2015.

Methodology: A total of 170 NFGNB isolates were identified and tested in this study. Antimicrobial susceptibility testing was performed using the disc diffusion technique. Isolates were analyzed for MBL production through a phenotypic method (DDST) and for the presence of MBL genes (*blaIMP* and *blaVIM*) via PCR.

Results: A total 170 out of 253 samples (67.19%) were identified as NFGNB (blood: 11.3%, pus: 16.4%, urine: 26.4%, genital: 5.6%, respiratory: 39.6%). Among the isolates, 53 (53/170: 31.17%) exhibited resistance to imipenem whereas highest resistance was found against Amikacin (96.2%). The current study revealed that 54.05% isolates of *P. aeruginosa*, 84.61% of *A. baumannii*, and 50% of *P. luteola* were MBL positive on DDST. On PCR 51.35% *P. aeruginosa*, and 38.46% *A. baumannii* were found positive for MBL genes (n=24) as *blaIMP* (14/24: 58.33%) and *blaVIM* (8/24: 33.33%).

Conclusion: DDST is effective for ascertaining antimicrobial susceptibility followed by PCR for the detection of MBL producers among NFGNB showing a sensitivity and specificity of 100% and 72% respectively.

Key words: Non-fermenter gram negative bacilli, metallo β-lactamase, imipenem resistant, DDST.

Introduction

The rapid global emergence of MBL-producing bacteria, particularly in *Pseudomonas aeruginosa* and *Acinetobacter* species, threatens the effectiveness of antibiotics that save millions of lives.[1] Bacterial multidrug resistance is widely recognized as a major global health threat in the 21st century.[2] The discovery of new drugs is particularly challenging due to MBLs, given their diversity, unique structure, and function.[3] Nearly 75% of deaths in patients with burn injuries have been documented to be caused by *Acinetobacter baumannii* infections, which are among the most frequent nosocomial infections and cause high mortality and morbidity among hospitalized patients, especially in burn and critical care units.[4]

Carbapenems group of beta lactam antibiotics was introduced first in 1980.[5] Carbapenem resistance arises from modifications in penicillin-binding proteins, loss of the OprD porin, increased efflux mechanisms, and reduced outer membrane permeability. This resistance is further

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AS & MM conceptualized the project. MA & MM* did the data collection. AS & IJ performed the statistical analysis. MM* & IJ did the literature search. Drafting, revision & writing of manuscript were done by SZ, OQ & SZHN.

driven by carbapenemases, enzymes that hydrolyze carbapenems.[6] Genes encoding carbapenem-hydrolyzing metallo-beta-lactamases are arranged as cassettes within integrons, enabling their propagation and expression.[7] To detect MBLs on plasmids capable of horizontal gene transfer and reorganization, as well as to confirm the presence of metallo-beta-lactamases, the use of both phenotypic and genotypic techniques is urgently required.

In clinical laboratories, phenotypic techniques are essential for differentiating the horizontally acquired mechanisms of carbapenem resistance in Gram-negative bacilli. Various screening methods have been developed to inhibit MBL activity, including the use of metal-chelating agents like 2-mercaptopropionic acid for detecting MBL-producing enzymes.[8]

Phenotypic tests such as the DDST (Double Disc Synergy Test) can detect the synergistic effects between antibiotics which is relatively simple, sensitive, and easy to perform for detecting metallo-beta-lactamases. Integrons carried by large, transferable plasmids facilitate the presence of *blaIMP* genes, which are responsible for producing IMP-1. Given the role of *P. aeruginosa* in the development of nosocomial infections, it is essential to analyze the genetic background of MBL genes to identify potential links with other antibiotic resistance genes and determine possible routes of dissemination.

This study aims to identify the most common metallo-beta-lactamase (MBL) genes in non-fermenting Gram-negative bacilli (NFGNB) through both phenotypic and genotypic analysis, with the goal of informing new strategies to mitigate hospital-acquired infections.

Methodology

The study was cross-sectional and conducted at General Hospital, Lahore and Institute of Public Health, Lahore from January 2015 to December 2015. Culture positive samples taken from adult population of both genders having no medico-legal issues were included while the samples from patients on, long term antimicrobial therapy, having multiple co-morbid conditions and drug addicts were excluded from study.

A sample size of 53 imipenem resistant cases was statistically calculated. To achieve this target, using convenient sampling technique 170 out of 253 specimens were non fermenter Gram negative bacilli identified through standard characteristics and API 20E. These 170 NFGNB were further screened for resistance to imipenem. Out of 170 non fermenter Gram negative bacilli 53 were isolated as imipenem resistant by Modified Kirby-Bauer method recommended by CLSI 2013 guidelines. Based on the pattern of antibiotic sensitivity, 53 isolates resistant to imipenem were then subjected to metallo-beta-lactamase analysis utilizing phenotypic and molecular methods.

The Kirby-Bauer method was used to evaluate the clinical isolates of NFGNR for antibiotic susceptibility. In compliance with the guidelines provided by CLSI 2013, this was carried out using Mueller-Hinton agar plates [Oxoid]. Imipenem-resistant isolates that had a zone diameter of less than 13 mm were thought to be MBL producers. The double disc synergy method was used to further analyse isolates that were resistant to imipenem for the development of metallo beta lactamase. The test isolate was incubated overnight on Mueller Hinton agar (0.5 McFarland opacity standard). All metallo beta lactamase producing isolates that were phenotypically detected by DDST were further confirmed by PCR [Gold standard].

Phenotypically MBL positive were further confirmed by PCR. By using a DNA extraction kit [QIAGEN, USA], bacterial DNA was extracted. After DNA extraction the tubes having purified DNA were labeled and stored at -20^{o}C in freezer that were further used in PCR. Amplification was done in thermal cycler after DNA extraction. PCR was done to screen for the genes blaIMP and blaVIM. PCR is comprised of forward and reverse primers [Table 2]. PCR amplification was done in thermal cycler after DNA extraction. The cycling conditions for PCR were optimized comprising of initial denaturation, annealing and elongation. Following one last extension in the last step, the amplification result was verified using agarose gel electrophoresis. P. aeruginosa strains that generated the MBL genes (blaIMP and blaVIM) served as the positive controls for MBL detection, whereas ATCC 27853 served as the negative control. Polyacrylamide gel was used to test the final PCR result. The PCR results were then examined under a UV lamp in a Gel documentation system (Bio Rad) and captured on camera. 1µl of 50 bp and 100 bp DNA ladders were utilized as DNA size markers.

Statistical analysis of the demographic data was carried out. Categorical descriptive variables like gender, age, imipenem resistance, isolates with metallo-beta-lactamases were presented as frequencies and percentages. Statistics showed that (P-value < 0.05) was significant.

The ethical approval was obtained from the Ethical Committee of Postgraduate Medical Institute, Lahore vide reference no. UHS/Education/126-15/2289.

Results

Out of total 53 cases, 31 isolates were collected from females and 22 from men (Table-1). Of all the age groups, the age group under 20 years old had the lowest isolation rate and the age group over 60 years old the highest (Figure-1).

Table 1: The gender-specific distribution of non fermenter, imipenem-resistant Gram-negative bacilli isolates. (n=53)

Gender	*Percentage (%)*
Female	(31) 58.49%
Male	(22) 41.50%

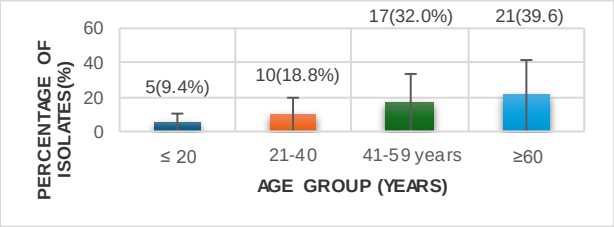


Figure 1: Percentage of isolates in relation to age groups.

Sample-wise isolation of NFGNB was respiratory samples n=21 [39.6%], urine n=14 [26.4%], pus n=9 [16.9%], blood n=6 [11.3%] respectively (Figure-2).

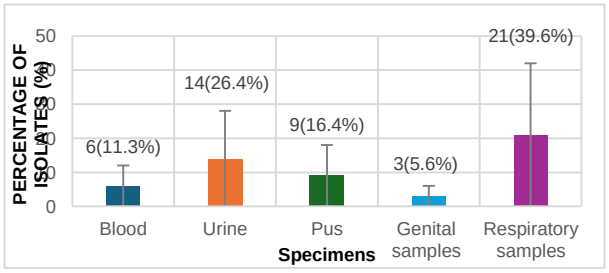


Figure 2: Percentage of isolates with relation to the type of specimen.

Figure-1 shows that out of 53 imipenem resistant isolates there was a maximum number 21 (39.6%) with age group 4. It was lowest with group 1, 5 (9.4%). With age group 2 and 3 the frequency was 10 (18.8%) and 17(32 .0%) respectively.

Figure-2 shows distribution of clinical specimens based on frequency of non-fermenter Gram negative bacilli isolates.

The samples were handled in accordance with standard operating procedures. The highest susceptibility was demonstrated by ciprofloxacin 8 [15%]. Most of the isolates showed resistance to multiple antibiotics. Among them *Acinetobacter junii* was the organism which showed 100% resistance to all 14 drugs.

The isolates underwent a double disc synergy test with 2-MPA in order to detect metallo-beta-lactamase phenotypically. Of the 53 imipenem resistant NFGNB, *Pseudomonas aeruginosa* accounted for 54.05%, 84.61% were *Acinetobacter baumannii spp* (Table-3). For confirmation polymerase chain reaction was done on all the phenotypically identified isolates for MBL gene confirmation.

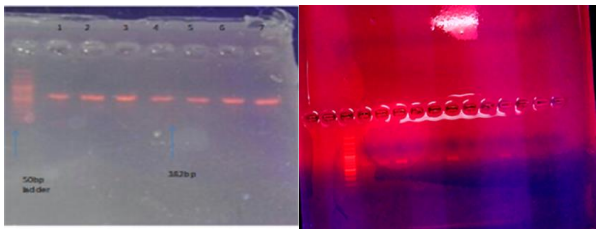
Table 3: MBL producers' frequency as determined by a DDST with a 2-MPA disk.

NFGNB isolated	*MBL positive (%) on DDST*
Pseudomonas aeruginosa	54.05%
Acinetobacter baumannii	84.61%
Pseudomonas luteola	50%
Acinetobacter junii	0%

The MBLs genes were confirmed by polymerase chain reaction (PCR) processing of many imipenem-resistant NFGNB isolates with phenotypically characterized MBL producers. Out of 37 *Pseudomonas aeruginosa,* 19 (51.35%) were positive for MBL genes and out of 13 *Acinetobacter baumannii,* 05 (38.46%) were found positive for MBL genes. *Pseudomonas luteola* and *Acinetobacter junii* showed no production for MBL genes. Out of 24 MBL gene producers on PCR blaIMP gene was observed in 14 (58.33%) and blaVIM gene in 8 (33.33%) isolates and 2(8.33%) of them carried both blaIMP and blaVIM genes.Sensitivity and specificity patterns between

Table 2: Names and Corresponding sequences of the primers used in PCR [Macrogen].

Primers	*Oligonucleotide Sequence [5'-3']*	*Specificity*	*Amplification Size*
Type 1-F	GTTTATGTTCATACWTCG	blaIMP	587 bp
Type 1-R	GGTTTAAYAAAACAACCAC	blaIMP	587 bp
Type 11–F	TTTGGTCGCATATCGCAACG	blaVIM	382 bp
Type 11–R	CCATTCAGCCAGATCGGCAT	blaVIM	382 bp



(a) (b)

(a) Agar Gel Electrophoresis Photograph showing bands for blaVIM gene (382bp) for different non fermenter Gram negative bacilli isolates after PCR Amplification. (b) Agar Gel Electrophoresis Photograph showing bands for blaIMP gene (587bp) for different non fermenter Gram negative bacilli isolates after PCR Amplification.

Figure 3: Agar Gel Electrophoresis Photograph showing bands for blaVIM gene and blaIMP gene.

DDST using 2-MPA and PCR for the MBL genes. The study's findings indicated 100% SN, 72% SP, 75% PPV and 100% NPV. The overall diagnostic accuracy of the tests was 84.9%.

Discussion

The rising incidence of antimicrobial-resistant bacteria has rapidly become a major global health concern.[9] The absence of advanced antimicrobials and the emergence of pan-resistant bacterial strains pose a serious threat to the management of nosocomial infections.

This study was conducted in response to the alarming increase in metallo-beta-lactamase production. The identification of these potentially deadly enzymes underscores the need for strict infection control measures to prevent the spread of carbapenemase-encoding genes.

This study reported maximum imipenem resistance 39.6% in 60 years and above followed by 32% in 41 to 59 years of age, 18.8% in 21-40 years and 9.4% in age group ≤ 20 years. Other studies conducted in 2011-2015 also reported imipenem resistance in 60 years and above in accordance with our study.[10] The current study shows the distribution of non-fermenter Gram negative bacilli detected in several clinical samples. A 2009 study revealed that the highest incidence of non-fermenter Gram negative bacilli was seen in respiratory samples, followed by pus samples.[11]

Present study reports that out of total 53 imipenem resistant NFGNB, 19 (51.35%) *Pseudomonas aeruginosa* and 05 (38.46%) *Acinetobacter baumannii* were found positive for MBL genes. Similar studies conducted from 2015-2016 reported high prevalence of *Pseudomonas aeruginosa* showing MBL production by PCR.[12] The prevalence of MBL gene is rapidly spreading among non-fermenter Gram negative bacilli and is getting worse all over the world day by day. For therapeutic reasons, our laboratories must regularly perform the rapid detection of MBL genes, since this will aid in containing the spread of illnesses acquired in hospitals and the community. The frequency of several MBL genes in MBL positive isolates found by PCR is displayed in the current study. MBL-producing isolates showed a high frequency of the blaIMP gene, followed by the blaVIM gene. In this study the sensitivity and specificity of DDST was compared with the PCR for MBL detection among non-fermenter Gram negative bacilli. According to our study there is 100% sensitivity and 72% specificity, 75% PPV and 100% NPV. Early pathogen detection is necessary to stop the morbidities and deaths brought on by these MBL-producing organisms. A good MBL test should be chosen for a certain pathogen under test in standard microbiological labs by consulting research that offer sensitivity and specificity data.

In conclusion, the synthesis of different enzymes, multidrug-resistant organisms have become more common and a global danger in recent years. It is advised to use quick diagnostic tests to identify MBL producers in order to create plans to stop the spread of these resistant bacteria. The gold standard for identifying MBL producers among NFGNB is PCR, but the double disc synergy test also works well in this regard. In our standard clinical microbiology laboratory, DDST can be configured for MBL detection, especially in isolates resistant to imipenem due to its affordability.

Conflict of interest: None declared.

References

1. Ventola CL. The antibiotic resistance crisis: part 1: causes and threats. Pharm Therapeu2015; 40(4): 277.
2. Eggleston K, Zhang R, Zeckhauser RJ. The Global Challenge of Antimicrobial Resistance: Insights from Economic Analysis. Int J Environ Res Public Health 2010; 7(8): 3141-9.
3. Boyd SE, Livermore DM, Hooper DC, Hope WW. Metallo-β-Lactamases: Structure, Function, Epidemiology, Treatment Options, and the Development Pipeline. Antimicrob Agents Chemother 2020; 64: e00397-20.
4. Gupta V, Chhina D, Kaur A. Incidence of metallo-beta-lactamase (MBL) producing non-fermenters isolated from respiratory samples in ICU patients. Int J Pharm Bio Sci 2013; 4(2): 580-5.
5. Tsakris A, Poulou A, Pournaras S, Voulgari E, Vrioni G, Diagalaki K T, et al. A simple phenotypic method for the differentiation of metallo-β-lactamases and class A KPC carbapenemases in Enterobacteriaceae

clinical isolates. J Antimicrob Chemother 2010; 65(8): 1664-71.
6. Queenan AM, Bush K. Carbapenemases: the versatile β-lactamases. Clin Microbiol Reviews 2007; 20(3): 440-58.
7. Tsakris A, Poulou A, Pournaras S, Voulgari E, Vrioni G, Diagalaki K T, et al. A simple phenotypic method for the differentiation of metallo-β-lactamases and class A KPC carbapenemases in Enterobacteriaceae clinical isolates. J Antimicrob Chemother 2010; 65(8): 1664-71.
8. Li X, Zhao J, Zhang B, Duan X, Jiao J, Wu W, et al. Drug development concerning metallo-β-lactamases in gram-negative bacteria. Front Microbiol 2022; 13: 959107.
9. Khalid S, Migliaccio A, Zarrilli R, Khan AU. Efficacy of Novel Combinations of Antibiotics against Multidrug-Resistant New Delhi Metallo-Beta-Lactamase-Producing Strains of Enterobacterales. Antibiotics 2023; 12(7): 1134.
10. Kabic J, Fortunato G, Moreira I.V, Kekic D, Jovicevic M, Pesovic J, et al. Dissemination of Metallo-β-Lactamase-Producing Pseudomonas aeruginosa in Serbian Hospital Settings: Expansion of ST235 and ST654 Clones. Int J Mol Sci 2023; 24(2): 1519.
11. Khan S, Beattie TK, Knapp CW. The use of minimum selectable concentrations (MSCs) for determining the selection of antimicrobial resistant bacteria. Ecotoxicology 2017; 26(2): 283-92.
12. Tarashi, S., Goudarzi, H., Erfanimanesh, S., Pormohammad, A., Hashemi,A. Phenotypic and Molecular Detection of Metallo-Beta-Lactamase Genes among Imipenem Resistant Pseudomonas aeruginosa and Acinetobacter baumannii Strains Isolated from Patients with Burn Injuries. Arch Clin Infect Dis 2016; 11(4): e39036.