Journal of Institute of Medicine Nepal
Institute of Medicine, Kathmandu, Nepal

OPEN ACCESS

Original Article

JIOM Nepal. 2025 Aug;47(1):108-113.

Phenotypic and Genotypic Detection of Metallo-β Lactamase in Non-Fermenting Gram Negative Bacilli Obtained from Clinical Samples in a Tertiary Care Hospital in Nepal

Jyotshna Sapkota, Divya Shree GC, Ram Prasad Adhikari, Shusila Khadka, Ritu Pandey, Anil Kumar Sah, Laxmi Kant Khanal

Author(s) affiliation

Department of Microbiology, Nepal Medical College Teaching Hospital, Kathmandu, Nepal

Corresponding author

Jyotshna Sapkota, MBBS, MD
21jyots@gmail.com

ABSTRACT

Introduction

Non-fermenting Gram-negative bacilli (NFGNB) are significant nosocomial pathogens with limited treatment options. Among these, metallo-β-lactamases (MBL) are of major concern which hydrolyze carbapenems and contribute to antimicrobial resistance. This study aimed to determine the prevalence of MBL-producing non-fermenting gram negative bacilli in clinical specimens using both phenotypic and genotypic methods.

Methods

A descriptive cross-sectional study was conducted at Nepal Medical College Teaching Hospital from January to December 2024. A total of 16,954 clinical specimens were processed for culture and sensitivity testing. MBL production was detected phenotypically using the Imipenem-EDTA combined disc method. The presence of IMP and VIM genes was confirmed by conventional PCR.

Results

Among 16,954 specimens, 163 (0.96%) NFGNB isolates were identified, with *Pseudomonas aeruginosa* (52.1%) being the most prevalent, followed by *Acinetobacter* species (39.3%) and *Burkholderia* species (8.6%). MBL production was detected in 22 (13.5%) isolates: *Pseudomonas aeruginosa* (59.1%), and *Acinetobacter* species (40.9%). Genotypically IMP and VIM genes were found in 36.4% and 31.8% of MBL-positive isolates, respectively, while one isolate harbored both genes. Notably, 27.3% of phenotypic MBL producers tested negative for both IMP and VIM, suggesting the potential involvement of other MBL genes.

Conclusion

The significant prevalence of MBL-producing NFGNB, particularly among *Pseudomonas aeruginosa* and *Acinetobacter* species, highlights a serious challenge for antimicrobial therapy and underscores an urgent need for robust infection control and antimicrobial stewardship strategies.

Keywords

Metallo-β-lactamase; Non-fermenting Gram-negative bacilli; Pseudomonas aeruginosa; Acinetobacter species; Carbapenem resistance; IMP gene; VIM gene

DOI

10.59779/jiomnepal.1383

Submitted

Jan 28, 2025

Accepted

Mar 20, 2025

INTRODUCTION

Non-fermenting Gram-negative bacilli (NFGNB), including *Pseudomonas aeruginosa* and *Acinetobacter baumannii* are major nosocomial pathogens known for their resistance to treatment due to both intrinsic and acquired resistance mechanisms. β-lactams antibiotics, particularly carbapenems, have traditionally been the mainstay for managing infections caused by these organisms.[1-3] Carbapenems are often used for the treatment of infections caused by extended-spectrum-β-lactamase (ESBL)-producing non-fermenters, particularly Pseudomonas spp. and *Acinetobacter* spp.[4]

Metallo-β-lactamases (MBLs) are a long-recognized class of β-lactamases that confer resistance to most β-lactam antibiotics, with the exception of monobactams such as aztreonam, and have gained increasing clinical relevance due to their global spread in recent decades.[5,6] The genes responsible for MBL are often located on mobile genetic elements prompting their widespread dissemination.[7] Recent surveillance studies have reported a continued rise in MBL producing *Pseudomonas aeruginosa* and Acinetobacter spp., with prevalence rates ranging from 20% to over 60% in certain hospital settings, particularly in low- and middle-income countries..[8,9]

Several types of MBLs genes have been identified including clinically significant variants such as IMP, VIM, SPM, NDM, GIM and SIM, with IMP and VIM being the most predominantly encountered globally. Detecting MBL production in NFGNB is crucial for guiding appropriate treatment and reduce spread of resistance. Adding genotypic detection of MBL gene enhances diagnostic accuracy, supports infection control and epidemiological surveillance, and guides appropriate antimicrobial therapy, specially in settings with high rates of multidrug resistance.[10-11]

This study aims to determine the prevalence of MBL producing NFGNB in clinical samples using both phenotypic and genotypic approach.

METHODS

Study design and Settings

A descriptive cross-sectional, laboratory-based study was conducted in the Microbiology Laboratory of Nepal Medical College Teaching Hospital (NMCTH). Over a one-year period (January 2024 to December 2024), a total of 16,954 clinical specimens submitted for culture and sensitivity testing from both outpatient and inpatient departments were processed and included in the study. Ethical approval was obtained from the Institutional Review Committee of NMCTH (Ref No: 079-078.079). Data on isolated NFGNB were recorded in Microsoft Excel 2013, and and analysis was focused on the detection and characterization of MBL production among the isolates.

Sample Collection, Bacterial Identification and Antibiotic Sensitivity Testing

All the clinical samples received in microbiology laboratory during study period were inoculated onto blood agar and MacConkey agar for detection of NLF, following standard routine practices in microbiology laboratory for the isolation of clinically significant bacteria, while urine specimens were cultured on cysteine lactose electrolyte-deficient (CLED) medium. The inoculated media were incubated aerobically at 37°C for 24 hours. Blood samples were first inoculated into brain heart infusion (BHI) broth and incubated aerobically for 24 hours before subculturing onto blood agar and MacConkey agar. [12,36]

Colonies obtained on solid media were identified based on colony morphology, Gram staining, and biochemical tests according to standard microbiological guidelines. [12,36]

Antimicrobial susceptibility testing (AST) was performed on Mueller-Hinton agar (MHA) using the Kirby-Bauer disk diffusion method, according to Clinical and Laboratory Standards Institute (CLSI) guidelines, mainly for NLFGNB. The antibiotics were chosen based on their relevance to *Pseudomonas aeruginosa* and *Acinetobacter* spp, while *Burkholderia* spp were included in the study but not analysed separately due to their small number The organisms were tested against the following antibiotic discs: amikacin (30 µg), gentamicin (10 µg), tobramycin (10 µg), ciprofloxacin (5 µg), imipenem (10 µg), aztreonam (30 µg), piperacillin (100 µg), piperacillin-tazobactam (100/10 µg), ceftazidime (30 µg), and carbenicillin (100 µg) [Hi-Media, India]. The zone of inhibition was interpreted according to CLSI guidelines.[14]

All culture media (blood agar, MacConkey agar and CLED agar) and antibiotic discs used for susceptibility testing were procured from HiMedia Laboratories Pvt. Ltd., Mumbai, India through institutional supplier.

Phenotypic Detection of MBL

All imipenem resistant NFGNB on AST were further tested for MBL production using the Imipenem-EDTA combined disc method. A 10-µg imipenem disc was placed on Mueller-Hinton agar inoculated with the test organism, both with and without 10 µL of 0.5 M EDTA. After 18 hours of aerobic incubation at 35°C, the inhibition zones around the imipenem and Imipenem-EDTA discs were measured and compared. An increase of ≥7 mm in the inhibition zone diameter with the Imipenem-EDTA disc compared to the imipenem disc alone was considered indicative of MBL production.[15] (Figure 1) For *Burkholderia* spp., MBL screening was

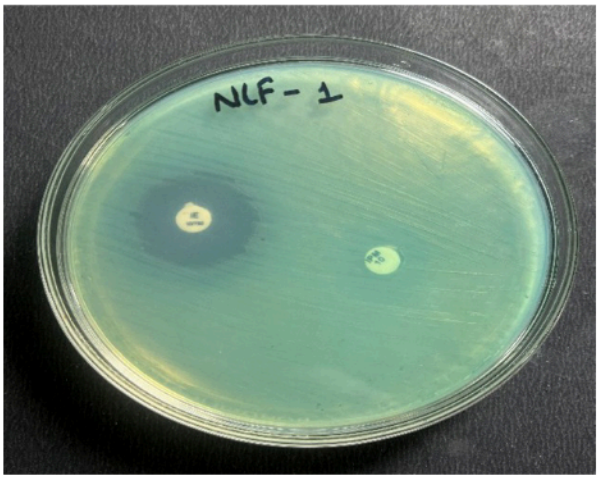

Figure 1. *Phenotypic detection of MBL using Imipenem-EDTA disc method*

carried out using the imipenem–EDTA combined disc method. Due to the absence of specific CLSI breakpoints for imipenem susceptibility in *Burkholderia*, interpretation was extrapolated from the CLSI 2021 breakpoints for *Pseudomonas aeruginosa*.[16]

Quality control of the phenotypic MBL detection was ensured using *Pseudomonas aeruginosa* ATCC 27853 as a negative control and a PCR-confirmed MBL-producing clinical isolate as a positive control.

Genotypic Detection of MBL Genes

All MBLNLF by phenotypic method was tested for MBL gene IMP and VIM by conventional PCR. PCR amplification targeted the IMP and VIM MBL genes due to their predominant local prevalence and available resources. DNA extraction was performed using a heat lysis method.[17] In brief, DNA was extracted. Then, it was run in PCR after mixing DNA with forward and reverse primers, master-mix, and distilled water according to mentioned standard guideline. Positive control for IMP and VIM genes and negative control (Distilled water) were also run in each series of PCR reaction. The PCR product was subjected to electrophoresis on 1.5% agarose gel stained with 0.5ug/ml ethidium bromide at a concentration of 0.5 µg/mL. The bands were visualized under UV illumination using a gel documentation system (GeNei™, UVI TECMumbai, India). Amplicon sizes were 179 bp for the IMP gene and 206 bp for the VIM gene[18]

Primers used were as follows:

VIM-F:TGGTTGTATACGTCCCGTCA
VIM-R: TGTGTGCTGGAGCAAGTCTA

IMP-F:TAACGGGTGGGGCGTTGTTCCT
IMP-R: CGCCCGTGCTGTCGCTATGAAA

Data was aanalyzed using Microsoft excel.

RESULTS

Among the 16,954 specimens, 163 non-fermenting Gram-negative bacilli (NFGNB) were isolated. The distribution of isolates is summarized in Table 1.

Table 1. *Distribution of Non-Fermenting Gram-Negative Bacilli (NFGNB) isolates*

Bacterial Isolate	Number (%)
Pseudomonas aeruginosa	85 (52.1)
Acinetobacter species	64 (39.3)
Burkholderia species	14 (8.6)

Phenotypic MBL Detection

Using the Imipenem-EDTA combined disc method, 22 (13.5%) of the 163 NFGNB isolates tested positive for MBL production (Figure 2). Among the NLF, P. aeruginosa was the most prevalent MBL producer, accounting for 59.1%. The details of phenotypic MBL producers are provided in Table 2.

Table 2. *Distribution of phenotypic MBL producers*

Bacterial isolate	MBL oositive (%)
Pseudomonas aeruginosa	13 (59.1)
Acinetobacter species	9 (40.9)
Burkholderia species	0 (0)

Genotypic MBL Detection

Among the 22 MBL-positive isolates, genotypic analysis revealed the presence of vim and imp genes, as detailed in Table 3.

Table 3. *Distribution of MBL genes among phenotypic MBL producers*

Gene detected	Number of isolates (%)
IMP	8 (36.4)
VIM	7 (31.8)
VIM and IMP	1 (4.5)
None	6 (27.3)

DISCUSSION

The results of this study reveal a notable prevalence of MBL-producing NFGNB in clinical samples, which presents significant challenges for antimicrobial treatment. With an overall MBL production rate of 13.5% among NFGNB isolates, our findings are in line with earlier reports from similar healthcare

environments, where carbapenem resistance is becoming more common.[19-21] However, studies conducted in Nepal by Shrestha et al. and in India by Kaur et al. found a higher prevalence of MBL producers among NFGNB isolates.[22,23] The differences in MBL production rates could be due to variations in healthcare infrastructure, infection control practices, patient demographics, and prior antimicrobial treatment histories.

In our study, *P. aeruginosa* was the most frequently isolated NFGNB, accounting for 59.1% of MBL-positive cases. This is consistent with other studies conducted in Nepal, Shrestha et al. (2015) and Adhikari et al. (2021), which also reported Pseudomonas species as the predominant NFGNB associated with carbapenem resistance in clinical isolates from tertiary care centers in Nepal.[24,25] Conversely, *Acinetobacter* spp. accounted for 40.9% of MBL-positive isolates in our study, a proportion somewhat lower than reported in other studies from Nepal where *Acinetobacter* has been identified as the leading NFGNB with higher rates of MBL production.[26,27] Globally, while P. aeruginosa is recognized as a major contributor to carbapenem resistance and MBL production in many regions, several studies from South Asia and the Middle East have documented *Acinetobacter* spp. as the predominant MBL producer, highlighting regional variations in the epidemiology of these pathogens [18,21] On the other hand, several studies have pointed to *Acinetobacter* spp. as the leading NFGNB, showing a greater prevalence of MBL production compared to our findings, which accounted for 40.9% of cases.[22,28,29]

The existence of MBL-producing NFGNB emphasizes the pressing need for enhanced infection control strategies, as these bacteria are notorious for causing severe hospital-acquired infections, particularly in immunocompromised individuals.[21]

The dominance of the IMP gene (36.4%) over VIM (31.8%) in MBL-positive isolates is consistent with previous observations in Southeast Asia, where IMP genes are frequently reported in *Pseudomonas* and *Acinetobacter* species.[30]

One important finding in our study was the detection of one isolate containing both VIM and IMP suggesting at the possibility of horizontal gene transfer, which could worsen the spread of resistance in hospital environments. This co-occurrence of MBL genes has been noted in various studies, including one that documented the coexistence of IMP and VIM genes in clinical isolates, underscoring the role of horizontal gene transfer (HGT) in the dissemination of these resistance factors. Similarly, another study found that *A. baumannii* can acquire multiple resistance genes through horizontal gene transfer mechanisms, contributing to its multidrug-resistant phenotype.[31,32] The dissemination of MBL genes among NFGNG is largely facilitated by HGT mechanisms, such as plasmids, integrons, and transposons, which contribute to the spread of multiple MBL types beyond those detected in this study.[5,33] Although our study lacked detailed epidemiological linkage data such as ward distribution or temporal clustering of isolates, previous studies have demonstrated that outbreaks involving MBL-producing strains often occur in specific hospital wards or during defined time periods, supporting the role of patient-to-patient transmission and clonal spread.[36] Future studies with integrated epidemiological and molecular data would further elucidate these transmission dynamics.

Notably, 27.3% of phenotypic MBL producers tested negative for both VIM and IMP genes, indicating the possible involvement of other MBL genes such as NDM and SPM, which were not screened in this study. This observation aligns with findings from other studies that have reported the presence of various MBL genes contributing to carbapenem resistance. For instance, Kumari et al. identified NDM-1 and VIM as predominant genes in Gram-negative bacilli associated with ventilator-associated pneumonia. Similarly, a study by Hamid et al. revealed a high prevalence of MBL genes, including VIM, IMP and NDM among Gram-negative isolates.[34,35]

As CLSI does not provide imipenem susceptibility breakpoints for *Burkholderia* spp., the interpretation of MBL screening results was based on extrapolated criteria from *Pseudomonas aeruginosa*. This may affect the accuracy of detection and represents a methodological limitation of our study. Another limitation of this study is the exclusive focus on VIM and IMP genes due to funding issues, potentially underestimating the true burden of MBL-mediated resistance. Future studies should incorporate a broader range of MBL genes and employ whole genome sequencing to better understand the molecular epidemiology of resistant strains. Moreover, surveillance of antibiotic use in the hospital setting is necessary to develop targeted interventions for reducing selection pressure and limiting the emergence of resistance.

CONCLUSION

This study highlights the concerning prevalence of MBL-producing NFGNB in clinical specimens, particularly among *P. aeruginosa* and *Acinetobacter* species. The presence of VIM and IMP genes underscores the genetic diversity of MBL-mediated resistance. Strengthening infection control measures and implementing routine MBL screening are critical steps in mitigating the threat posed by these resistant pathogens.

ACKNOWLEDGEMENT

The authors would like to express their gratitude to all the technical staff of the Microbiology Laboratory at NMCTH for their assistance during the data collection for this study.

FINANCIAL SUPPORT

The author(s) did not receive any financial support for the research and/or publication of this article.

CONFLICT OF INTEREST

The author(s) declare that they do not have any conflicts of interest with respect to the research, authorship, and/or publication of this article.

AUTHOR CONTRIBUTIONS

Study concept, design, data collection, data analysis and manuscript writing: Dr. Jyotshna Sapkota; Data collection, laboratory work and manuscript review: Dr. Divya Shree G.C, Dr. Anil Kumar Sah; Data analysis and manuscript review: Mr. Ram Prasad Adhikari, Dr. Sushila Khadka, Dr. Laxmi Kant Khanal. All authors read and approved of the final manuscript.

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