Metallo-β-Lactamase Detection in Imipenem-Resistant *Acinetobacter* spp. and *Pseudomonas aeruginosa*: A Combined Phenotypic and Molecular Study

Running title: MBL Detection in Imipenem-Resistant *Acinetobacter* and *Pseudomonas aeruginosa*

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Abstract

Background: The incidence of healthcare-associated infections (HAIs) is increasing rapidly, primarily driven by multidrug-resistant gram-negative pathogens, notably *Pseudomonas aeruginosa* and *Acinetobacter* spp. Metallo-beta-lactamases (MBLs), including IMP and VIM types, are frequently produced by these organisms, contributing to therapeutic challenges through gene dissemination and prolonged colonization in clinical settings. This study, conducted in Rasht, Iran, aimed to evaluate phenotypic resistance and MBL production in imipenem-resistant clinical isolates of *P. aeruginosa* and *Acinetobacter*.

Methods: In this descriptive cross-sectional study, 52 *Acinetobacter* and 25 *P. aeruginosa* isolates were obtained from healthcare facilities in Rasht. Species identification was confirmed using standard biochemical assays. Antimicrobial susceptibility was assessed via the disk diffusion method in accordance with CLSI recommendations. MBL-producing isolates were screened using the Imipenem-EDTA combined disk test. Genomic DNA was extracted with a commercial kit, followed by PCR amplification to detect *bla*$_{IMP}$ and *bla*$_{VIM}$ genes.

Results: According to the findings of this study, the highest percentage of resistance in *Acinetobacter* was observed with cefotaxime antibiotics at 94.23%, and in *P. aeruginosa* with ceftazidime at 84%. Among the isolates not susceptible to Imipenem, 18 (40%) were *Acinetobacter* and 2 (15.38%) were *P. aeruginosa*, as determined by the combined disc method. Both *P. aeruginosa* and *Acinetobacter* isolates demonstrated high resistance to ceftazidime. The *bla*$_{IMP}$ and *bla*$_{VIM}$ genes were detected in 7 (13.46%) and 11 (21.15%) of the *Acinetobacter* samples, respectively, and in 8 (32%) and 5 (20%) of the *P. aeruginosa* samples, respectively.

Conclusion: Early detection of *Acinetobacter* and *P. aeruginosa* strains producing metallo-β-lactamases is key to guiding effective clinical treatment and improving patient outcomes. These results suggest that the *bla*$_{IMP}$ and *bla*$_{VIM}$ genes play important roles in the antibiotic resistance and potentially influence the virulence of *Acinetobacter* species and *P. aeruginosa* in the region.

Keywords: *Acinetobacter*; *Pseudomonas aeruginosa*; blaIMP; blaVIM; Metallo beta lactamases

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Introduction

The growing prevalence of antimicrobial resistance (AMR) poses a major challenge to public health in the 21st century (1-3). The incidence of multidrug-resistant Gram-negative bacterial infections has escalated significantly in every region of the world. (4). Environmental pathogens, particularly nonfermentative Gram-negative species including *Pseudomonas aeruginosa* (5) and *Acinetobacter* spp. (6, 7), are now frequently implicated in healthcare-associated multidrug-resistant infections (8-10). The transfer of bacteria and genes between humans, animals, and the environment renders antibiotics increasingly ineffective, leading to more difficult-to-treat infections and higher rates of death (11).

Resistance to carbapenems, which serve as a critical final option in antibiotic therapy, has become a significant global issue. Carbapenems, along with penicillins and cephalosporins, are central to this problem as they are hydrolyzed by metallo-beta-lactamases (MBLs) (12, 13). Metallo-beta-lactamases (MBLs) are zinc-dependent hydrolases that inactivate almost all beta-lactam antibiotics. Gram-negative bacteria expressing MBLs face a severe reduction in available therapeutic options. MBLs function by binding critical metal ions in the periplasm, but their activity diminishes when zinc is limited due to the innate immune defenses. (11-13). The activity of this enzyme group is susceptible to inhibition by substances including Ethylenediaminetetraacetic acid (EDTA) and sodium mercaptoacetic acid. (10, 14).

Metallo-beta-lactamases are classified into different types based on their molecular structure, which include IMP, VIM, NDM, SPM, GIM, SIM, DIM, KHM, TMB, FIM, and AIM. IMPs and VIMs represent the most frequently encountered MBLs, with production largely seen in Enterobacteriaceae and Gram-negative nonfermentative bacteria such as *P. aeruginosa* and *Acinetobacter* spp. Beta-lactamase-producing strains pose a significant risk in medical centers due to their ability to transfer genes to other bacteria and their tendency for long-term colonization in hospitals (15-17).

In the MENA region, carbapenem-resistant *Acinetobacter* isolates, often producing carbapenemases, are prevalent in hospitals and contribute to multi-drug resistance, posing a significant threat in healthcare-associated infections (18). Similarly, *P. aeruginosa* strains carrying metallo-beta-lactamases (MBLs) such as VIM-2, VIM-5, and NDM-1 are widespread across countries like Saudi Arabia, Iran, Tunisia, and Algeria. These resistance mechanisms, particularly VIM-2 and NDM-1, are commonly found in MBL-positive strains, underscoring the escalating difficulty of managing infections due to these resistant bacteria in the region (19).

The antibiotic resistance of *P. aeruginosa* and *Acinetobacter*, which are significant causes of clinical cases in hospitals, to effective antibiotics such as Imipenem, is a critical concern (20, 21). Prior research has examined MBL gene distribution in these pathogens across various Iranian provinces. In this research, we simultaneously investigated the frequency of Imipenem-resistant *P. aeruginosa* and *Acinetobacter* isolates from Rasht, along with the presence of metallo-beta-lactamase-producing genes in them. Finally, we compared the results obtained from phenotypic and genotypic studies.

Methods

This descriptive cross-sectional study involved the collection of 52 *Acinetobacter* and 25 *P. aeruginosa* isolates from clinical specimens previously identified in the microbiology laboratories of Razi University Hospital, Poursina Medical and Educational Center, Al-Zahra Maternity, and Rasool Akram Hospital in Rasht over a one-year period (June 2021 – June 2022). All *Acinetobacter* and *P. aeruginosa* isolates were sent to the laboratory at the Institute of Microbiology, Department of Biology, Islamic Azad University in Rasht for further investigation. The isolates were then examined at the Microbiology Laboratory of Azad

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University, Rasht. Identification and confirmation of the isolates were performed according to the Clinical & Laboratory Standards Institute (CLSI) guidelines.

Samples were cultured on MacConkey Agar and incubated at 37°C for 24 hours. Identification of bacterial isolates were accomplished by conventional biochemical tests including catalase, oxidase, triple-sugar-iron (TSI) agar, oxidation/fermentation of glucose using OF media and growth at 42°C**.**

Metallo-β-lactamase-producing strains were screened using the Combination Disk Diffusion Test (CDDT) to determine their prevalence among the isolates (22). Imipenem (IMP) and a combination of IMP with Ethylenediaminetetraacetic acid (EDTA), an MBL inhibitor, were applied to identify MBL-producing strains among Imipenem-non-susceptible isolates.

A solution of 0.5 M EDTA was freshly prepared. Using a 0.5 McFarland standard, a lawn culture of the isolate was prepared on Mueller-Hinton agar. The Imipenem disk and Imipenem plus EDTA (0.5 M and 5 µl) were placed at least 4 cm apart. The test was considered positive when the Imipenem + EDTA disc produced an inhibition zone more than 5 mm larger than that of Imipenem alone after overnight incubation.

Total DNA of the bacterial isolates was isolated with the aid of the SinaPure EX6011 DNA extraction kit.

All Imipenem-non-susceptible strains were screened by PCR for the *bla*$_{VIM}$ and *bla*$_{IMP-1}$ genes using gene-specific primers. PCR amplification was carried out using VIM-F (5′-TTTGGTCGCATATCGCAACG-3′) and VIM-R (5′-CCATTCAGCCAGATCGGCAT-3′) for blaVIM, and IMP-F (5′-CTACCGCAGCAGAGTCTTTG-3′) and IMP-R (5′-AACCAGTTTTGCCTTACCAT-3′) for blaIMP. The PCR conditions were as follows: initial denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, annealing at 50°C for 1 min for blaVIM and 40°C for 1 min for blaIMP, and extension at 72°C for 1 min, with a final extension at 72°C for 10 min.

Following electrophoresis of PCR products on a 1% agarose gel in 1× TBE containing ethidium bromide at 120 V for 45 minutes, the resulting bands were examined under UV light with a transilluminator.

Results

In this current research, we observed that *Acinetobacter* was highly sensitive to Imipenem (13.46%), whereas 94.2% were resistant to Cefotaxime. In contrast, *P. aeruginosa* exhibited the highest resistance to Ceftazidime (84%) and the greatest susceptibility to Cefotaxime (52%).

Both *P. aeruginosa* and *Acinetobacter* spp. isolates displayed substantial resistance to Ceftazidime, with resistance rates exceeding 80% in most cases.

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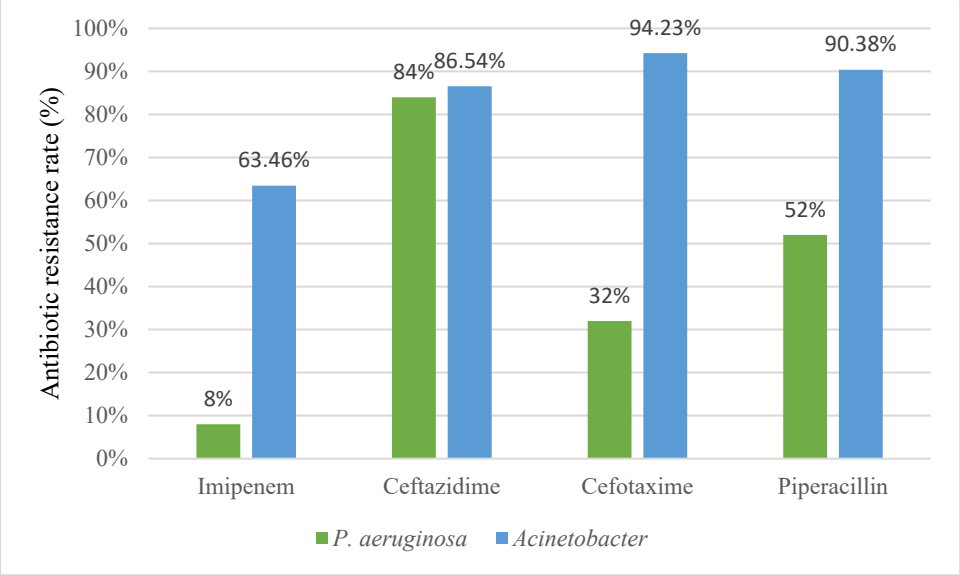

Figure 1. Antibiotic resistance rates in *Pseudomonas aeruginosa* vs. *Acinetobacter* spp. isolates

The resistance pattern to Imipenem and Cefotaxime and Piperacillin showed a significant difference between *P. aeruginosa* and *Acinetobacter* isolates ($p < 0.05$). Antibiotic resistance patterns of *P. aeruginosa* and *Acinetobacter* spp. against the other groups of antibiotics is shown in Table 1.

Table 1. The Antimicrobial susceptibility results of *Acinetobacter* spp. and *Pseudomonas aeruginosa* isolates

Antibiotic	**Antimicrobial Agent**	**MIC interpretation**			**Asymptotic Significance (p-value)**
		Susceptible (S)	**Intermediate (I)**	**Resistant (R)**	
Imipenem	*Acinetobacter* spp. (n=52)	7(13.36%)	12(23.08%)	33(63.46%)	< 0.05
	P. aeruginosa (n=25)	12(48%)	11(44%)	2(8%)	
Ceftazidime	*Acinetobacter* spp. (n=52)	6(11.56%)	1(1.92%)	45(86.54%)	> 0.05
	P. aeruginosa (n=25)	3(12%)	1(4%)	21(84%)	
Cefotaxime	*Acinetobacter* spp. (n=52)	1(1.92%)	2(3.85%)	49(94.23%)	< 0.05
	P. aeruginosa (n=25)	13(52%)	4(16%)	8(32%)	
Piperacillin	*Acinetobacter* spp. (n=52)	4(7.70%)	1(1.92%)	47(90.38%)	< 0.05
	P. aeruginosa (n=25)	6(24%)	6(24%)	13(52%)	

Phenotypic Screening of MBLs via the Combination Disk Diffusion Test (CDDT)

In our analysis, within the 25 clinical isolates of *P. aeruginosa*, 13 strains were Imipenem-non-susceptible, and 2 (15.38%) were determined to be MBL producers by the CDDT test. Additionally, of the 52 *Acinetobacter* strains, 45 isolates were Imipenem resistant, and 18 (40%) were MBL producers.

In Figure 1, the combined disc test (CDT) shows an enhanced inhibition zone of >5 mm around IPM + EDTA disc, indicating MBL positivity in clinical samples of *Acinetobacter* spp. and *P. aeruginosa.*

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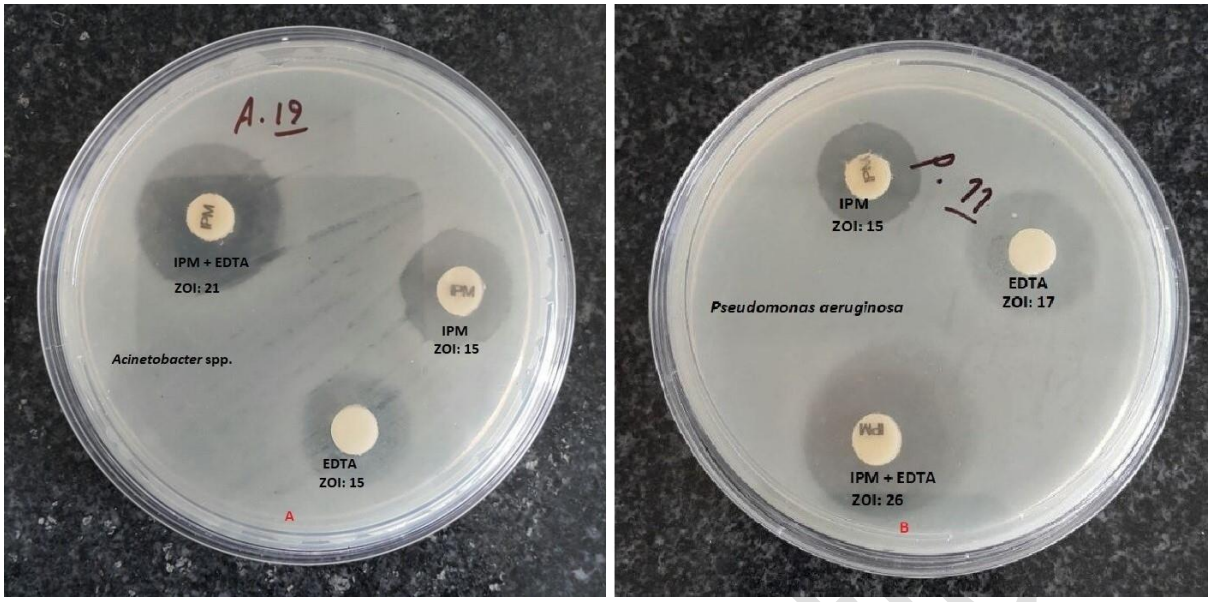

Figure 2. Representive images of the phenotypic detection of the MBL production in A: *Acinetobacter spp.* (positive control) And B: *P. aeruginosa* (positive control). Imipenem: 10 µg/disk; EDTA: 0.5 µl/disk; ZOI: Zone of inhibition.

Identification of Metallo-β-Lactamase Genes

PCR analysis showed that *bla*$_{IMP}$ and *bla*$_{VIM}$ genes were found in 7 (13.46%) and 11 (21.15%) isolated strains of *Acinetobacter*, respectively. The *bla*$_{IMP}$ and *bla*$_{VIM}$ genes were identified in 8 (32%) and 5 (20%) out of 25 *P. aeruginosa* isolates, respectively.

Discussion

The clinical utility of Carbapenems has been compromised by the emergence of Carbapenemase-producing bacterial strains, primarily metallo-β-lactamases, thus undermining their role as reserve drugs (23). Carbapenem resistance is primarily observed in Gram-negative bacteria, such as *P. aeruginosa* and *Acinetobacter* species (24), and may arise either intrinsically or through transferable Carbapenemase-encoding genes. (25). Patients infected with Carbapenem-resistant pathogens experience higher mortality than those infected with Carbapenem-sensitive organisms (26).

In this study, *P. aeruginosa* showed high resistance to Cefotaxime and piperacillin (84% and 52%, respectively), while *Acinetobacter* spp. displayed elevated resistance to all tested antibiotics except Imipenem. These results align with previous reports by Fallah et al. regarding the resistance of *Acinetobacter* (27). Similar rates of resistance in *Acinetobacter*, as observed in our study, were also reported by Namaei et al. (28). Furthermore, Moosavian et al. reported that clinical *P. aeruginosa* isolates exhibited high resistance to Ceftazidime, consistent with the results of the present study (29).

The trend observed in these studies indicates fluctuations in antibiotic resistance rates. The causes of these variations may include differences in resistance across regions, the infection type, sample size, and the antibiotics employed in disk assays. High antibiotic pressure, resulting from increased empirical or excessive use of antibiotics, seems to play a role in the emergence of Carbapenem resistance among hospital isolates.

Higher rates of antimicrobial resistance were observed in *Acinetobacter* than in *P. aeruginosa* in the present study. Additionally, a significant percentage of the clinical isolates of *P. aeruginosa* exhibited intermediate susceptibility to antibiotics, indicating a potential need for further research into the resistance patterns of these isolates in the future.

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The rise and global distribution of Metallo-β-Lactamases (MBLs), a leading mechanism of Carbapenem resistance, represent a significant threat to healthcare systems. This threat stems not only from their capacity to confer high-level resistance but also from the presence of highly mobile genetic elements in genes such as *bla*$_{IMP}$ and *bla*$_{VIM}$ (30). Consequently, another aim of this research was to identify MBL-producing samples and *bla*$_{IMP}$ and *bla*$_{VIM}$ genes in clinical *P. aeruginosa* and *Acinetobacter* spp. isolates.

In 2018, Subramaniyan et al. utilized the E-strip test method to identify MBL producers and the PCR method to detect the bla gene. They stated that 26.1% of MBL producers were *P. aeruginosa,* while 25% were *Acinetobacter*. The *bla*$_{VIM}$ gene, which encodes Metallo-β-Lactamase, was identified in 26% of *P. aeruginosa*, whereas the *bla*$_{IMP}$ gene was absent in *P. aeruginosa.* In *Acinetobacter*, the metallo-β-lactamase-producing gene, including the *bla*$_{VIM}$ gene, was reported in 4.4% of the strains, and the *bla*$_{IMP}$ gene in 5.2% (31). These results differ from those of the present study, showing a much higher abundance of metallo-β-lactamase-producing genes in native strains, possibly due to differences in the horizontal transfer of resistance genes.

In the study by Namai et al. in 2021, MBL production was observed in 40% of *P. aeruginosa* and 93.3% of *Acinetobacter*. It was found that 33.3% and 46.7% of *P. aeruginosa* isolates were resistant to Carbapenems, and 13.3% and 28.9% of Carbapenem-resistant *Acinetobacter* harbored *bla*$_{IMP-1}$ and *bla*$_{VIM-1}$ genes, respectively(28). The results of this research show a higher frequency of MBL compared to the current research, but the abundance of *bla*$_{IMP}$ and *bla*$_{VIM}$ genes is somewhat similar to the current study.

In an earlier study by Tarashi et al., the presence of *bla*$_{IMP-1}$ and *bla*$_{VIM-1}$ genes was observed in 30 (16.8%) and 52 (29.2%) strains of *P. aeruginosa*, respectively. *bla*$_{IMP-1}$ and *bla*$_{VIM}$-1 genes were detected in 10 (5.3%) and 34 (18.18%) of *Acinetobacter*, respectively (32). The results of this study are close to the present study, with the difference being the lower reported frequency of the *bla*$_{VIM}$ gene in *Acinetobacter*.

Conclusion

The results of this research carry significant consequences for both clinical practice and public health. Rapid identification and tracking of clinical isolates of *P. aeruginosa* and *Acinetobacter* producing metallo-beta-lactamase enzymes is crucial for tailoring effective treatment strategies and inhibiting the transmission of infections that are resistant to multiple drugs in healthcare environments. Resistance to Carbapenems is primarily caused by the presence of metallo-beta-lactamase-producing genes, although non-enzymatic factors may also play a role. The increasing prevalence of Carbapenem-resistant strains underscores the need for continuous monitoring and surveillance, as well as the implementation of rapid diagnostic tests to guide therapy. From a public health perspective, the results highlight the urgent need for comprehensive infection control programs and antimicrobial stewardship policies to combat the growing threat of antimicrobial resistance. Furthermore, global efforts are needed to reduce the misuse of antibiotics and promote responsible prescribing practices to slow the spread of resistance in healthcare settings.

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Author contribution

All authors were involved in: study design, data collection, article approval and statistical analysis.

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Ethics approval and consent to participate

Not applicable since the study did not involve human or animal subjects.

Conflict of interest

The authors declare no conflict of interest.

Data availability statement

The data supporting this study are available from the corresponding author upon reasonable request.

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