Cerrahpaşa Med J 2024; 48(3): 207-211 ORIGINAL ARTICLE

Detection of Metallo-β-lactamase Carbapenemase Genes in Carbapenem-Resistant Pseudomonas Strains from Bloodstream Infections

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This study was presented as a poster at the 17th International Congress on Infectious Diseases.

Cite this article as: Yılmaz Yücel F, Köksal Çakırlar F. Detection of metallo-β-lactamase carbapenemase genes in carbapenem-resistant pseudomonas strains from bloodstream infections. *Cerrahpaşa Med J.* 2024;48(3):207-211.

Abstract

Objective: Metallo-β-lactamase (MBL) production is one of the most important mechanisms causing the development of resistance to carbapenems. In this study, we intended to investigate the presence of Verona integron mediated (VIM)-, imipenemase (IPM)-, and imipenemase (NDM)-type MBL in *Pseudomonas* strains isolated from blood cultures in Cerrahpaşa Medical School Hospital.

Methods: In our study, a total of 48 'carbapenem-resistant/intermediate *Pseudomonas* strains were isolated from blood samples of patients with bacteremia over 4 years. For all strains, screening for the presence of "MBL genes" was performed by "multiplex PCR," and "phenotypic MBL E-test" was used.

Results: The VIM-type MBL gene was detected in 3 isolates (1 *Pseudomonas putida* and 2 *Pseudomonas aeruginosa*). Imipenemase- and NDM-type MBL genes were not detected in any of the strains. Specificity and sensitivity of MBL E-test were determined to be 100% according to multiplex PCR results.

Conclusion: In our study, VIM-type MBL gene was first shown in *Pseudomonas* strains at our hospital. Detection of carbapenemase genes will contribute to the implementation of infection control measures that will reduce their spread and the rational use of antibiotics.

Keywords: Carbapenem-resistant *Pseudomonas aeruginosa*, *Pseudomonas putida*, metallo-betalactamase genes, VIM

Introduction

Carbapenems are antibiotics that have a strong and broad spectrum effect. They are the most important agents preferred for the treatment of *Pseudomonas* infections.[1] *Pseudomonas* is widely found in nature and it can grow in cleaning solutions, disinfectants, antiseptics, and invasive respiratory support units because of its resistance to adverse environmental conditions. It is an opportunistic pathogen and is responsible for approximately 10%-15% of nosocomial infections worldwide. The rates of carbapenem resistance continue to increase due to their extensive usage. Multidrug-resistant *Pseudomonas* infections increase morbidity and mortality. Carbapenem-resistant *Pseudomonas* strains are a significant problem in worldwide.[2]

Carbapenemase enzymes are one of the most common mechanisms of carbapenem resistance detected in *Pseudomonas* strains. Carbapenemases are grouped according to the Ambler classification (A, B, D), and class B metallo-β-lactamases (MBLs) have an important place in carbapenemase development.[3] Many MBL group carbapenemases have been defined in *Pseudomonas* species, such as imipenemase (IMP), German imipenemase (GIM), New Delhi MBL (NDM), Seul imipenemase (SIM), Sao Paulo MBL (SPM), and Verona integron-mediated (VIM) MBL. Metallo-β-lactamases, which can easily spread by plasmids and transposons among gram-negative pathogens such as *Acinetobacter, Pseudomonas,* and *Enterobacterales* spp., pose a great problem in the world and our country. Verona integron-mediated, IMP, and NDM are the most common MBL enzymes in gram-negative pathogens.[4] In this study, we aimed the detection of VIM-, NDM-, and IMP-type MBL genes in *Pseudomonas* strains isolated from blood cultures.

Received: January 11, 2024 **Revision Requested:** April 29, 2024
Last Revision Received: March 27, 2024 **Accepted:** April 29, 2024
Publication Date: July 31, 2024
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DOI: 10.5152/cjm.2024.23118

Methods

In our study, a total of 48 carbapenem-resistant/intermediate *Pseudomonas* strains were isolated from blood cultures of patients hospitalized in various departments and intensive care units at Cerrahpaşa Medical School Hospital over 4 years (2011-2015). The blood culture samples were incubated in the BD BACTEC system (Becton Dickinson, USA). Bacterial identification and antimicrobial resistance of the isolates were performed using the BD Phoenix automated system (BD Diagnostics, Sparks, MD). Antibiogram results were evaluated in accordance with The European Committee on Antimicrobial Susceptibility Testing (EUCAST) criteria.[5]

All isolates were studied for the presence of MBL enzymes using the gradient method "MBL E-test" (BIOMERIEUX, France). They were screened for the presence of MBL genes by "multiplex real-time PCR" (Bio-Rad, USA). Bacterial DNA was extracted with extraction reagents (Seegene, Seoul, South Korea) in accordance

Table 1. Specific Primer Sequences for VIM-, IMP-, and NDM-Type MBL Genes[30]

Target	Primer Name	Sequence (5′-3′)	Amplicon Size (bp)	Primer Concentration (mM)[a]	Tm[b]
*bla*VIM type	VIM-F VIM-R	GTTTGGTCGCATATCGCAAC AATGCGCAGCACCAGGATAG	382	0.2	90.3
*bla*IMP type	IMP-F IMP-R	GAGTGGCTTAATTCTCRATC AACTAYCCAATAYRTAAC	120	1.2	80.1
*bla*NDM-1 type	NDM-F NDM-R	TTGGCCTTGCTGTCCTTG ACACCAGTGACAATATCACCG	82	0.2	84

[a]Final concentration in the multiplex real-time PCR.
[b]Melting point.

with the manufacturer's proposals. Primer sequences for the genes (Sentromer, Türkiye) of IMP-, VIM-, and NDM-type enzymes were used (Table 1).

All strains were analyzed by multiplex PCR using primer sequences for blaVIM, blaIMP, and blaNDM. Polymerase chain reaction conditions were performed as follows: 5 minutes at 95°C, 36 cycles at 95°C for 20 seconds, at 55°C for 45 seconds, at 72°C for 30 seconds, and then the melt curve step. The PCR products were run on a 2% agarose gel at 100 V for 1 hour (Figure 1). The images of the resulting curves are shown in Figure 2.

Statistical Analysis

Statistical analysis was performed through the NCSS 2007 program (Number Cruncher Statistical System, LLC Kaysville, Utah, USA). The results were evaluated at a 95% CI and significant $P < .05$.

Our study was executed by courtesy of İstanbul-Cerrahpaşa Faculty of Medicine Clinical Investigations Ethics Committee (Approval no: A-21, Date: March 3, 2015). All series of steps were executed in accordance with the ethical rules and the principles of the Declaration of Helsinki. Due to the nature of the study, because it is a retrospective study using patient samples coming to routine laboratory, informed consent is not required.

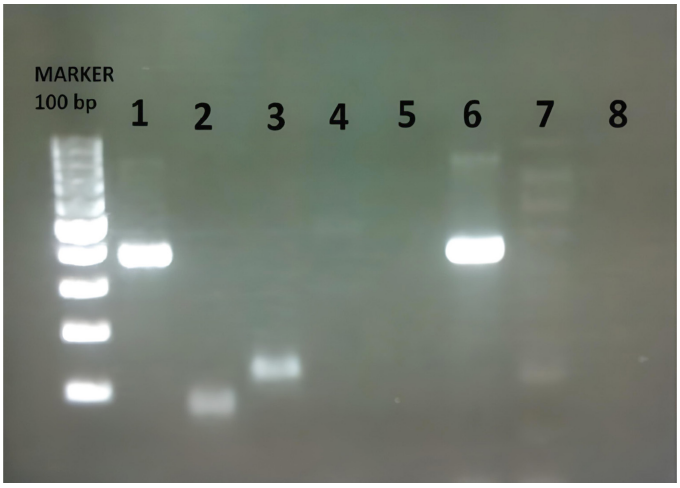

Figure 1. 2% agarose gel image of Multiplex PCR products. 100 bp Marker 1: positive control VIM 382 bp, 2: Positive control NDM 82 bp, 3: Positive control IMP 120 bp, 4: Negative control (*E. coli* ATCC 25922), 5: Negative control (*P. aeruginosa* ATCC 27853), 6: VIM positive patient that numbered 18, 7: VIM, IMP, and NDM negative patients that numbered 14, 8: non-template control-NTC.

Results

Pseudomonas strains were most frequently isolated from the intensive care unit and emergency department (emergency internal medicine and emergency surgery). The strains were as follows: 33.3% from the intensive care unit, 33.3% from the emergency department, 25% from internal medicine, 4.2% from pediatric services, 2.1% from infectious diseases services, and 2.1% from general surgery services.

Species distribution of the isolates was as follows: *Pseudomonas fluorescens* 1 (2.1%), *P. putida* 5 (10.4%), and *P. aeruginosa* 42 (87.5%). Twelve (25%) of isolates were intermediate resistant (MIC 6 µg/mL), and 36 (76%) carbapenem-resistant (MIC 8-≥32 µg/mL). The MIC90 and MIC50 for both meropenem and imipenem were 32 µg/mL each.

Resistance rates of the isolates to the antimicrobial agents, respectively, were as follows: amikacin 29%, gentamicin and aztreonam 37.5%, tobramycin and netilmicin 44%, piperacillin/tazobactam 46%, ceftazidime 48%, ciprofloxacin 50%, levofloxacin 52%, cefepime, and ticarcillin 56%. None of the isolates were resistant to colistin (Figure 3).

In the MBL screening performed with the MBL E-test, 6.3% of the isolates (n = 3) were positive. In these 3 isolates with MBL positive, in 1 *P. putida* and 2 *P. aeruginosa*, VIM-type MBL gene was detected. However, IMP and NDM-type MBL genes were not detected in any of the isolates. When compared with PCR results, the specificity and sensitivity of MBL E-test were detected as 100%.

Discussion

The most important mechanisms causing the development of carbapenem resistance in *P. aeruginosa* include β-lactamase production, deterioration of outer membrane permeability, changes in outer membrane proteins (Oprs), and the efflux pump system.[6] In recent years, the most common carbapenem resistance mechanism that has been discussed is the increasingly prevalent MBLs.[3]

Pseudomonas putida is an opportunistic pathogen with low virulence and susceptibility to various antibiotics. *Pseudomonas putida* leads to nosocomial infections including bacteremia, urinary tract infections, pneumonia, and neonatal infections. In the recent past, however, the isolation frequency of imipenem-resistant *P. putida* strains is increasing steadily. *Pseudomonas putida* infections that produce MBLs are difficult to treat due to the lack of effective antibiotics. More importantly, it has been reported that the MBL gene of *P. putida* can be horizontally transferred to *P. aeruginosa*.[7] Reasons such as the absence of an MBL inhibitor, the presence of resistance genes belonging to different classes of antibiotics in MBL-carrying integrons, the ability of MBL to be transferred to other bacteria by mobile genetic elements, and to spread to different geographical regions increase the importance of MBL.[8,9]

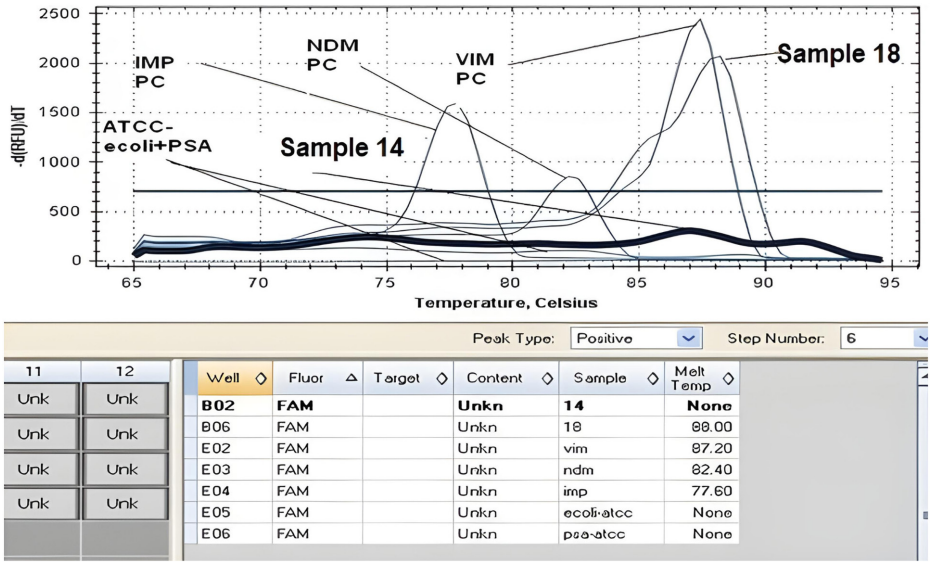

Figure 2. The results of high-resolution melting (HRM) analysis of the MBL genes that investigated in our study. ATCC *E. coli*+PSA: Negative control (*E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853), Sample 14: VIM-, IMP-, and NDM-negative patients numbered 14, IMP PC: Positive control IMP 120 bp, NDM PC: Positive control NDM 82 bp, VIM PC: Positive control VIM 382 bp, Sample 18: VIM-positive patient numbered 18.

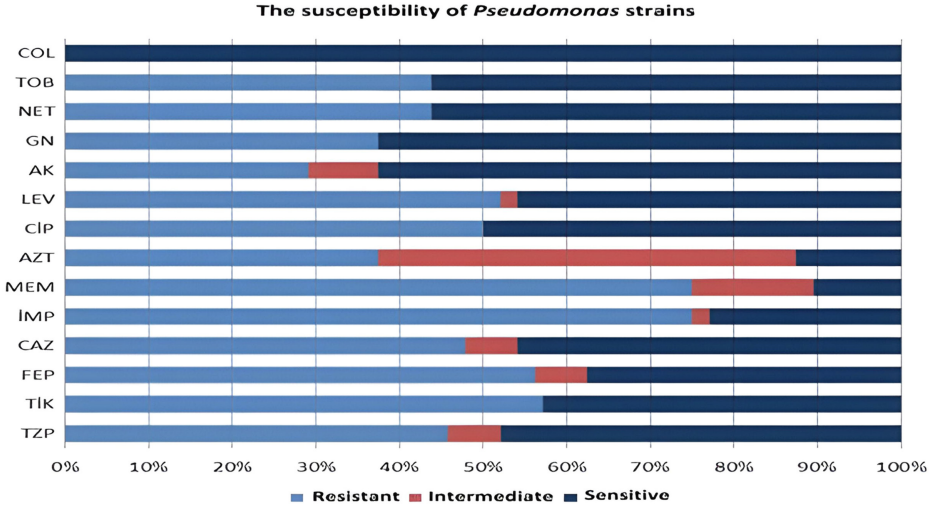

Figure 3. The susceptibility of *Pseudomonas* strains. TZP, piperacillin–tazobactam; TIK, ticarcillin; FEP, cefepime; CAZ, ceftazidime; IMP, imipenem; MEM, meropenem; AZT, aztreonam; CIP: ciprofloxacin; LEV, levofloxacin; AK, amikacin; GN, gentamicin; NET, netilmicin; TOB, tobramycin; COL, colistin.

Phenotypic methods are often used in screening genes encoding carbapenem resistance because PCR, a more reliable method, cannot always be used routinely in hospitals.[10] The MBL E-test method, a phenotypic test, is easy to use and evaluate to detect the presence of MBL, and it has high specificity and sensitivity.[11] However, both "the false-negative and false-positive results" have also been reported to occur with MBL E-test.[12,13] Therefore, MBL E-test results should be interpreted with caution in bacteria that may contain oxacillinase such as *Acinetobacter baumannii* and *P. aeruginosa*.[13]

Solanki et al also investigated the carbapenemase genes of KPC, VIM, IMP, and NDM in 100 gram-negative bacteria. They found MBL positive by phenotypic tests in 3 out of 18 *P. aeruginosa* strains and in the same strains, they found VIM by multiplex PCR.[14] Similarly, PCR and phenotypic test (E-test) were compared in our study; the specificity and sensitivity of the E-test were 100% according to PCR results. Phenotypic tests need to be confirmed by molecular methods. But when PCR cannot be performed, the E-test can be recommended as an appropriate phenotypic method.

In our study, we used the multiplex PCR method in which different IMP-, NDM-, and VIM-type MBL can be detected at one time. Multiplex PCR method is more sensitive than phenotypic tests, and molecular methods such as multiplex PCR are the most appropriate one for carbapenemase detection.[14,15] This method is fast and easy to use. VIM-, IMP-, and NDM-type MBL genes can be detected in a single reaction. The multiplex PCR method used in this study is a method that can be easily used to investigate the spread of VIM-, IMP-, and NDM-type MBL.

The MBL gene was first described in *P. aeruginosa* in Japan in 1988, and then several MBLs were identified in various countries, especially in *Pseudomonas* isolates. VIM-1 was first reported in *P. aeruginosa* in Italy. The rapidly increasing VIM enzyme is the most

common MBL enzyme in the world.[16,17] In a study involving 14 European countries, performed by Castanheira et al, 106 of the 529 carbapenem-resistant *P. aeruginosa* strains isolated in 2009-2011 were found to be positive for MBL: 4 IMP and 102 VIM. In 2011, the prevalence of MBL (30.6%) increased when compared to other years (13.4% in 2009 and 12.3% in 2010). The increase in MBL is worrying for European countries.[3]

In neighboring countries and our country, MBL enzymes have been reported to be positive in various proportions: In Greece, in *P. aeruginosa* and *Enterobacterales*: VIM-1, VIM-2, VIM-4, VIM-12, VIM-17, VIM-19[18]; in Bulgaria, in *P. aeruginosa*: VIM-15, VIM-16[19]; and in Iran, in *P. aeruginosa*: VIM-type MBL.[20] In conducted studies including *Pseudomonas*, usually IMP and VIM have been identified that are the most common MBL enzymes all over the world.[12] The first reported type of MBL in our country is VIM-5, which was detected in a *Klebsiella pneumoniae* strain at Cerrahpasa Medical School Hospital.[21] Because MBL genes are transferable to elements like plasmid and transposon and have been previously detected in *Enterobacterales* members in our hospital, MBL has been detected in *P. aeruginosa* strains in our study as we would expect. In our hospital, Kucukbasmaci et al[22] investigated the MBL gene by multiplex PCR method in 51 *P. aeruginosa* strains of imipenem resistant or intermediate resistant isolated from various samples, and found no blaIMP and blaVIM genes. Our study is the first one that detects MBL gene in *Pseudomonas* in our hospital.

In our country, other MBL genes except VIM and IMP also need to be investigated. Because the NDM-1 type MBL enzyme was detected in *K. pneumoniae* that was isolated from the blood culture of a patient coming from Iraq to Türkiye.[23] Until last years India, and more recently the Balkan countries were reservoirs for NDM production.[24] In our study, IMP and NDM type enzymes were not detected in any of the isolates.

Carbapenem resistance in carbapenem-resistant but MBL-negative isolates is thought to originate from other carbapenemases except MBL (oxacillinases such as OXA-20, OXA-23, OXA-24) or other mechanisms such as OprD loss and the efflux pump system. Extensive studies are needed to investigate other mechanisms that cause carbapenem resistance.

Other mechanisms leading to carbapenem resistance in *P. aeruginosa* isolates have also been reported in the studies.[25] In Mac Aogáin et al's study,[26] OprD loss was detected in about 85% of IPM-resistant *P. aeruginosa* strains, but the MBL genes were negative. In another study, it was found that 50 (39%) of the 129 carbapenem-resistant *P. aeruginosa* isolates produced carbapenemase: 3 GES-5, 5 VIM, 10 KPC, and 41 SPM genes.[27]

In our study, like other ones in our country and the world, intensive care units are one of the most clinics in which *Pseudomonas* strains isolated.[8] Therefore, it is important to monitor the antibiotic resistance profile, to ensure the usage of appropriate antibiotics and to take "infection control measures" in the intensive care units. The most important precautions to be taken in preventing the spread of MBL are to determine whether MBL-producing microorganisms are colonized in the patients in intensive care units.

In our study, in line with other conducted studies in our country[28] colistin was found to be the most effective antibiotic with 100% susceptibility. The second most effective antibiotic was amikacin, and the third one was gentamicin.

If genes coding for the MBL enzyme are found side by side with genes that cause resistance to aminoglycosides, it means aminoglycoside resistance may also develop.[29] However, in our study, three of the isolates that we detected as MBL positive were susceptible to gentamicin, and also one of them was also susceptible to amikacin at the same time.

Conclusion

In our study, the VIM-type MBL gene was first shown in *Pseudomonas* strains at our hospital. In cases in which PCR is not applicable, the E-test can be considered a convenient phenotypic method in MBL diagnosis. In strains where MBL genes are undetectable, we thought the result of resistance to carbapenem may be due to various resistance mechanisms except for MBL enzymes. Therefore, the identification of carbapenemase genes that are restricting treatment options and their types will contribute to the rational use of antibiotics and also to implement 'infection prevention measures' to reduce their spreading.

Availability of Data and Materials: The data that support the findings of this study are available on request from the corresponding author.

Ethics Committee Approval: The study was carried out with the permission of Istanbul University Cerrahpasa Faculty of Medicine Clinical Investigations Ethics Committee (Approval no: A-21, Date: March 3, 2015). All procedures were carried out in accordance with the ethical rules and the principles of the Declaration of Helsinki.

Informed Consent: Due to the nature of the study, because it is a retrospective study using patient samples coming to routine laboratory, informed consent is not required.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept – F.Y.Y., F.K.Ç., Design – F.Y.Y., F.K.Ç.; Supervision – F.K.Ç.; Resource – F.Y.Y., F.K.Ç.; Materials – F.Y.Y.; Data Collection and/or Processing – F.Y.Y.; Analysis and/or Interpretation – F.Y.Y., F.K.Ç.; Literature Search – F.Y.Y., F.K.Ç.; Writing – F.Y.Y.; Critical Review – F.K.Ç.

Acknowledgments: We thank Recep Öztürk (Department of Infectious Diseases, İstanbul University-Cerrahpaşa, İstanbul, Türkiye) and Esma Akkoyun Bilgi (Department of Medical Microbiology, İstanbul University-Cerrahpaşa, İstanbul, Türkiye).

Declaration of Interests: The authors have no conflict of interest to declare.

Funding: This work was supported by the Scientific Research Projects Coordination Unit of İstanbul University-Cerrahpaşa (Grant no: 53433/2846).

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