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Molecular Epidemiology and Clinical Characteristics of Metallo-beta-lactamase Producing *Pseudomonas aeruginosa* Isolates

Metallo-Beta Laktamaz Üreten *Pseudomonas aeruginosa* Suşlarının Moleküler Epidemiyolojisi ve Klinik Özellikleri

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Abstract

Introduction: In this study, we aimed to determine the epidemiological properties of metallo-beta-lactamase-producing *Pseudomonas aeruginosa* (MBL-PA) isolates and to investigate the relationship between the presence of MBL-PA and patient morbidity and mortality.
Materials and Methods: The study included carbapenem-resistant *P. aeruginosa* isolates recovered from various clinical specimens of 334 patients in Karadeniz Technical University Faculty of Medicine Hospital, a 900-bed university hospital in Trabzon, Turkey. MBL-related carbapenem-resistant PA strains were phenotypically investigated using the Modified Hodge test and the imipenem/imipenem-ethylene diamine tetra acetic acid combined disc tests. Multiplex polymerase chain reaction was used to investigate the presence of bla_{IMP}, bla_{VIM}, bla_{GIM}, bla_{SIM}, and bla_{SPM} genes, which are responsible for MBL production. Clonal relationships among MBL-PA isolates were analyzed by pulsed-field gel electrophoresis. The patients' hospital records were retrospectively examined. Various demographic and clinical characteristics were evaluated in relation to MBL-PA.
Results: Thirty-two (9.6%) of the carbapenem-resistant PA isolates were found to carry bla_{VIM} and/or bla_{IMP}, with three strains harboring both bla_{VIM} and bla_{IMP}. MBL-PA isolates were more resistant to aminoglycosides and quinolones. Eight Verona integron-encoded metallo-beta-lactamase-type MBL-PA isolates were found to be identical in adults, while several clonally-related clusters were observed among MBL-PA isolates in both the pediatric and adult inpatients. Compared to non-MBL carbapenem-resistant PA, the risk factors evaluated were found to have no association with MBL-PA. In addition, there was no statistically significant difference in mortality between patients from whom MBL-PA or non-MBL-PA was isolated.
Conclusion: Although MBL-PA has been implicated in various healthcare-related outbreaks, no specific risk factor has been identified in association with MBL-PA isolation. To our knowledge, this is the first study in Turkey to detect *P. aeruginosa* isolates carrying both bla_{VIM} and bla_{IMP}.
Keywords: *Pseudomonas aeruginosa*, carbapenem resistance, metallo-beta-lactamase, bla_{IMP}, bla_{VIM}, pulsed-field gel electrophoresis

Öz

Giriş: Bu çalışmada metallo-beta-laktamaz üreten *Pseudomonas aeruginosa* (MBL-PA) suşlarının moleküler epidemiyolojik özelliklerinin belirlenmesi ve MBL-PA varlığının hasta mortalitesi ve morbiditesi ile ilişkisinin değerlendirilmesi amaçlandı.
Gereç ve Yöntem: Karadeniz Teknik Üniversitesi Tıp Fakültesi Hastanesi'nde 2009-2010 yıllarında bir, çeşitli klinik örneklerden izole edilmiş 334 karbapenem dirençli *P. aeruginosa* kökeni çalışmaya dahil edildi. Karbapenem dirençli PA izolatlarında MBL varlığı fenotipik olarak Modified Hodge ve imipenem/imipenem-etilen diamin tetra asetik asit kombine disk testleri ile araştırıldı. MBL üretiminden sorumlu genlerden olan bla_{IMP}, bla_{VIM}, bla_{GIM}, bla_{SIM} ve bla_{SPM} genlerinin varlığı çoklu polimeraz zincir reaksiyonu yöntemi ile araştırıldı. MBL-PA olduğu belirlenen suşların klonal ilişkisi pulsed-field jel elektroforez ile analiz edildi. Hastaların tıbbi kayıtları geriye dönük olarak MBL-PA ile ilişkisi açısından incelendi. Metallo-beta-laktamaz üreten *Pseudomonas aeruginosa* ve non-MBL-PA izole edilen hastaların çeşitli demografik ve klinik özellikleri istatistiksel olarak değerlendirildi.
Bulgular: Karbapenemlere dirençli PA suşlarının 32'sinde (%9,6) bla_{VIM} ve/veya bla_{IMP} olumlu saptandı. Bunların üç tanesinin aynı anda bla_{VIM} ve bla_{IMP} geni taşıdığı tespit edildi. MBL-PA suşlarının aminoglikozidlere ve kinolonlara direnç oranlarının daha yüksek olduğu görüldü. Erişkinlerde

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saptanan VIM tipi MBL üreten PA suşlarından sekizinin identik olduğu görüldü ve gerek erişkin gerekse çocuk hastalardan izole edilen MBL-PA'lar arasında klonal kümelenmeler saptandı. Karbapenem dirençli PA izole edilenler hastalardan, MBL-PA ve non-MBL-PA grupları arasında incelenen risk faktörleri ve mortalite açısından fark bulunmamıştır.

Sonuç: Metallo-beta-laktamaz üreten *Pseudomonas aeruginosa* izolasyonu ile ilişkili özellikli bir risk faktörü saptanmamış olsa da MBL-PA'nın sağlık bakımı kaynaklı çeşitli salgınlara neden olduğu gösterilmiştir. Ayrıca bildiğimiz kadarıyla çalışmamız, Türkiye'de bla_{VIM} ve bla_{IMP} genlerinin aynı anda saptandığı PA izolatlarını rapor eden ilk çalışmadır.

Anahtar Kelimeler: *Pseudomonas aeruginosa*, karbapenem direnci, metallo-beta-laktamaz, bla_{IMP}, bla_{VIM}, pulsed-field jel elektroforez

Introduction

Metallo-beta-lactamase-producing *Pseudomonas aeruginosa* (MBL-PA) is a microorganism of crucial importance. It is resistant to all beta-lactam antibiotics except monobactams[1]. Metallo-beta-lactamases identified in carbapenem-resistant *P. aeruginosa* include imipenemase (IMP), Verona integron-encoded metallo-beta-lactamase (VIM), São Paulo metallo-beta-lactamase (SPM), Germany imipenemase (GIM), New Delhi metallo-beta-lactamase, and Florence imipenemase[2]. These MBL genes are carried by specific plasmids, along with other genes encoding regions responsible for resistance to carbapenems and other antibiotics. Therefore, MBL-PA isolates are usually multidrug-resistant, and these genetic elements also cause increased resistance since resistance can be transferred to other Gram-negative species[1].

There is a need to assess the current epidemiological status of MBL-PA in the local setting and to delineate the mechanisms which lead to resistance. By precisely determining the clinical and molecular characteristics of the isolates involved, we may better understand their epidemiology, since these characteristics form the basis for effective epidemiological surveillance. The surveillance in turn helps us to gauge the effectiveness of infection control measures and to guide correctly targeted and timely treatment measures[1-3]. According to the hitherto limited research conducted in this field in Turkey, VIM-type MBLs are the most commonly reported MBL types in *P. aeruginosa* isolates[3-8]. More comprehensive research on this subject is required to reveal the absolute epidemiology of resistance and the clinical and molecular characteristics of MBL-PA in Turkey[3].

In order to assess the molecular epidemiology in terms of dissemination of MBL-PA isolates and the clinical characteristics related to MBLs in our hospital, we aimed to detect MBL-PA isolates, demonstrate the molecular epidemiology of MBL-PA, and determine any clinical risk factors associated with MBL-PA and the clinical outcomes of MBL-PA isolation among inpatients.

Materials and Methods

Study Design

This single-center retrospective study was conducted at Karadeniz Technical University Faculty of Medicine Hospital, a 900-bed university hospital in Trabzon, Turkey. A non-duplicate carbapenem-resistant *P. aeruginosa* strain from each patient was included to the study. If >1 isolates were obtained from a single patient, only the first isolate was included the study. Carbapenem-resistant isolates (intermediate or resistant to imipenem and/or meropenem) had been previously isolated from a total of 356 patients in 2009-2010, and the 334 samples that could be recovered from the frozen stocks were included in this study. All isolates were stored at -80 °C in cryopreservation vials (Thermo Scientific, USA) until analysis with Modified Hodge test (MHT), imipenem/imipenem-ethylene diamine tetra acetic acid (EDTA) combined disc test (CDT), and molecular tests.

Medical records of the patients were retrospectively reviewed. Hence, owing to the retrospective nature of this part of the study, it was not deemed necessary to obtain written consent from the patients. Due to inadequacy of the patients' hospital records, it could not be ascertained whether the *P. aeruginosa* isolates represented colonization or were the causative agents of an infection.

Phenotypic investigation of MBL producers was performed by both the MHT and the imipenem/imipenem-EDTA CDT. Multiplex polymerase chain reaction (PCR) was performed to investigate carriage of the bla_{IMP}, bla_{VIM}, bla_{GIM}, $bla_{SIM,}$ and bla_{SPM} genes. The molecular epidemiology and microbiological characteristics of the MBL-PA isolates were evaluated in two groups: a pediatric inpatient group and an adult inpatient group.The genetic relatedness of carbapenem-resistant MBL-PA isolates was investigated by pulsed-field gel electrophoresis (PFGE) for each group[13-18].

Retrospective Case-Control Study

A retrospective case-control study was performed to evaluate risk factors related to MBL-PA. The risk factors analyzed were: age, sex, underlying diseases, comorbidities, source of the carbapenem-resistant *P. aeruginosa* isolate, previous surgical operations, invasive device usage (central venous catheter, endotracheal tube, and urinary catheter), mechanical ventilation, immunosuppression lasting longer than 14 days (e.g. chemotherapy, corticoids), and antimicrobial usage (for at least 48 h over the preceding 14 days). A total of 158 patients (from a potential 334 patients) with available hospital records were retrospectively reviewed

from the pediatric (n=23) and adult (n=135) inpatient groups. Outpatients (n=42) were excluded from the risk assessment and the clinical outcome evaluation. Patients whose samples yielded MBL-PA were defined as the case group, while the patients with non-MBL-PA isolates were defined as the control group. Clinical outcome was assessed based on the length of hospital stay and whether the final outcome was discharge or death. MB-PA-related mortality was defined as death occurring within ten days of MBL-PA being isolated[9,10].

Bacterial Identification and Susceptibility Tests

Identification and susceptibility testing of the isolates was performed using standard laboratory methods and Phoenix NMIC/ID-55 panels (Becton Dickinson Bioscience automatic system; USA) in accordance with the manufacturer's instructions. *P. aeruginosa* ATCC 27853 was used as a quality control strain. Antimicrobial susceptibility test results were interpreted according to the recommendations of the Clinical and Laboratory Standards Institute[11].

Modified Hodge Test

A suspension of *Escherichia coli* ATCC 25922 was inoculated on a Mueller-Hinton agar after the density was adjusted to McFarland 0.5 standard, after which a 10-µg imipenem disc (Oxoid Thermo Fisher, UK) was placed at the center of the agar plate. The test isolates, positive control strain (*Klebsiella pneumoniae* ATCC BAA-1705), and negative control strain (*K. pneumoniae* ATCC BAA-1706) were streaked in a straight line from the edge of the disc to the edge of the plate, in different directions for each isolate. In case of *E. coli* ATCC 25922 growth on the test isolate streak line towards the imipenem disc after overnight incubation, the so-called "*clover leaf*" pattern, the test isolate was interpreted as MHT-positive[11].

Imipenem/Imipenem-Ethylene Diamine Tetra Acetic Acid Combined Disc Test

Suspensions of the test isolates and control strains were inoculated on Mueller-Hinton agar after adjusting the density to McFarland 0.5 standard. Two 10-µg imipenem discs (Oxoid Thermo Fisher, UK) were placed on the agar plate with a distance of 20 mm between their centers. Ten µL of 0.5 M EDTA was added onto one of the imipenem discs. After overnight incubation, the discs were examined. If the zone of inhibition surrounding the disc impregnated with both imipenem and EDTA was at least 7 mm greater in size than the zone around the disc containing imipenem alone, the isolate was considered MBL-positive[12]. IMP-positive 587585 *P. aeruginosa* and VIM-positive 670448 *P. aeruginosa* isolates, which Ozgumus et al.[5] reported to be producers of MBL, were used as positive controls, while *P. aeruginosa* ATCC 27853 was used as a negative control[5].

Polymerase Chain Reaction Detection of Metallo-beta-lactamase Genes

Bacterial DNA was extracted by the boiling method[13]. Polymerase chain reaction was performed using a bacterial DNA template together with the specific primers listed in Table 1[14,15]. IMP-positive 587585 *P. aeruginosa* and VIM-positive 670448 *P. aeruginosa* isolates were used as positive controls. *P. aeruginosa* ATCC 27853 and distilled water were used as negative controls[5]. For the bla_{IMP} (A), bla_{VIM} (A), $bla_{GIM,}$ $bla_{SIM,}$ and bla_{SPM} genes, the PCR amplification conditions were as follows: initial DNA denaturation at 94 °C for 5 min,

Table 1. List of primers used in this study[14,15]

Primer		Sequence (5'-3')	Amplicon size (bp)	Reference
bla_{IMP} (A)	F-1	GAATAG(A/G)(A/G)TGGCTTAA(C/T)TCTC	188	14
	R-1	CCAAAC(C/T)ACTA(G/C)GTTATC		
bla_{IMP} (B)	F-2	ATG AGC AAG TTA TCT TAG TAT TC	765	15
	R-2	GCT GCA ACG GAC TTG TTA G		
bla_{VIM} (A)	F-3	GTTTGGTCGCATATCGCAAC	382	14
	R-3	AATGCGCAGCACCAGGATAG		
bla_{VIM} (B)	F-4	AGT GGT GAG TAT CCGACA G	261	15
	R-4	ATG AAA GTG CGT GGA GAC		
bla_{GIM}	F-5	TCAATTAGCTCTTGGGCTGAC	72	14
	R-5	CGGAACGACCATTTGAATGG		
bla_{SIM}	F-6	GTACAAGGGATTCGGCATCG	569	14
	R-6	TGGCCTGTTCCCATGTGAG		
bla_{SPM}	F-7	CTAAATCGAGAGCCCTGCTTG	798	14
	R-7	CCTTTTCCGCGACCTTGATC		

bp: Base pair

followed by 35 cycles of denaturation at 94 °C for 20 sec, annealing at 53 °C for 45 sec, and extension at 72 °C for 30 sec, followed by final extension at 72 °C for 6 min[16]. For the bla_{IMP} (B) and bla_{VIM} (B) genes, PCR amplification was done using the following conditions: initial DNA denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 25 sec, annealing at 57 °C for 40 sec, and extension at 72 °C for 50 sec, followed by final extension at 72 °C for 6 min[15].

Pulsed-field Gel Electrophoresis

Chromosomal DNA was prepared as previously described. SpeI (Promega Corp., USA) was used to digest the genomic DNA. Lambda phage concatemers (Bio-Rad Laboratories, USA) were used as a size marker. Electrophoresis was carried out under the following conditions using the CHEF-DR III system (Bio-Rad Laboratories, USA): an initial switch time of 5 seconds, final switch time of 35 seconds, gradient of 6 V/cm, and included angle of 120° for a 24-hour run[17]. Bio-Rad Gel Doc System (Bio-Rad Laboratories, USA) was utilized to document the PGFE patterns, and the clonal relationship among isolates was analyzed by Molecular Analyst Software (Bio-Rad Laboratories, USA) using the Dice similarity coefficient. Isolates with ≥95% genetic similarity in PFGE profiles were defined as being from the same clone, while clusters were defined as DNA patterns with ≥85% similarity[18].

Statistical Analyses

The SPSS 13.0 program (SPSS Inc., USA) was used for all statistical analyses. The Kolmogorov-Smirnov (K-S) test was used to determine normality. For binomial comparisons of numerical data, Student's t-test was used for normal distributions and the Mann-Whitney U test was used for non-normal distributions. The independent-samples t-test was used to compare means of the data. The χ^2 test was used for qualitative comparisons. The Kaplan-Meier test was used for survival analysis. Statistical tests with $p<0.05$ were considered statistically significant.

Results

Detection of Metallo-beta-lactamase-producing *P. aeruginosa* Isolates

The evaluation of MHT and CDT in the detection of MBL-PA isolates is summarized in Table 2. The bla_{VIM} (A) and bla_{IMP} (A) primer sets were more effective for PCR detection of VIM- and IMP-type MBL genes. However, the strains used as positive controls (IMP-positive 587585 *P. aeruginosa*, and VIM-positive 670448 isolates *P. aeruginosa*) were positive when using either of the specific primer sets (Table 3). The bla_{SPM}, bla_{SIM}, and bla_{GIM} genes were not found in any of the 334 *P. aeruginosa* isolates.

Table 2. Evaluation of Modified Hodge test and imipenem/imipenem-ethylene diamine tetra acetic acid combined disc test for detection of metallo-beta-lactamase-producing *Pseudomonas aeruginosa* isolates

		PCR			PPV	NPV	Sensitivity	Specificity
		Positive	Negative	Total				
Modified Hodge test	Positive	6[a]	15[b]	21	28.6%	5.7%	40.0%	86.2%
	Negative	9[c]	150[d]	159				
	Undetermined	17	137	154				
Imipenem/imipenem-EDTA combined disc test	Positive	29[a]	80[b]	109	26.6%	98.7%	90.6%	73.5%
	Negative	3[c]	222[d]	225				
Total		32	302	334				

PCR: Polymerase chain reaction for detection of bla_{IMP}, bla_{VIM}, bla_{GIM}, bla_{SIM}, and bla_{SPM}, PPV: Positive predictive value, NPV: Negative predictive value, EDTA: Ethylene diamine tetra acetic acid, [a]Number of true positive isolates, [b]Number of false positive isolates, [c]Number of false negative isolates, [d]Number of true negative isolates

Table 3. Polymerase chain reaction detection of bla_{VIM} and bla_{IMP} genes regions using different specific primers

Primer	Amplificon size	Positive isolates		
		Isolate code	n	(%)
bla_{VIM} (A)	382 bp	PA14, PA19, PA43, PA143, PA146, PA152, PA156, PA170, PA206, PA230, PA236, PA237, PA238, PA240, PA241, PA254, PA259, PA272, PA277, PA282, PA298, PA306, PA328	23	6.9
bla_{VIM} (B)	261 bp	PA14, PA19, PA170, PA206, PA237, PA254	6	1.8
bla_{IMP} (A)	188 bp	PA31, PA33, PA35, PA136, PA139, PA140, PA143, PA145, PA146, PA156, PA157, PA166	12	3.6
bla_{IMP} (B)	765 bp	PA31, PA33, PA35	3	2.5

bp: base pair. n: number

Microbiological Characteristics of Carbapenem-resistant *P. aeruginosa* Isolates

Carbapenem-resistant *P. aeruginosa* organisms were isolated from several clinical specimens: 165 (49.4%) were isolated from respiratory tract samples, 63 (18.9%) from genitourinary system samples, 59 (17.6%) from skin and soft tissue samples, 28 (8.4%) from blood samples, and 19 (5.7%) from other samples.

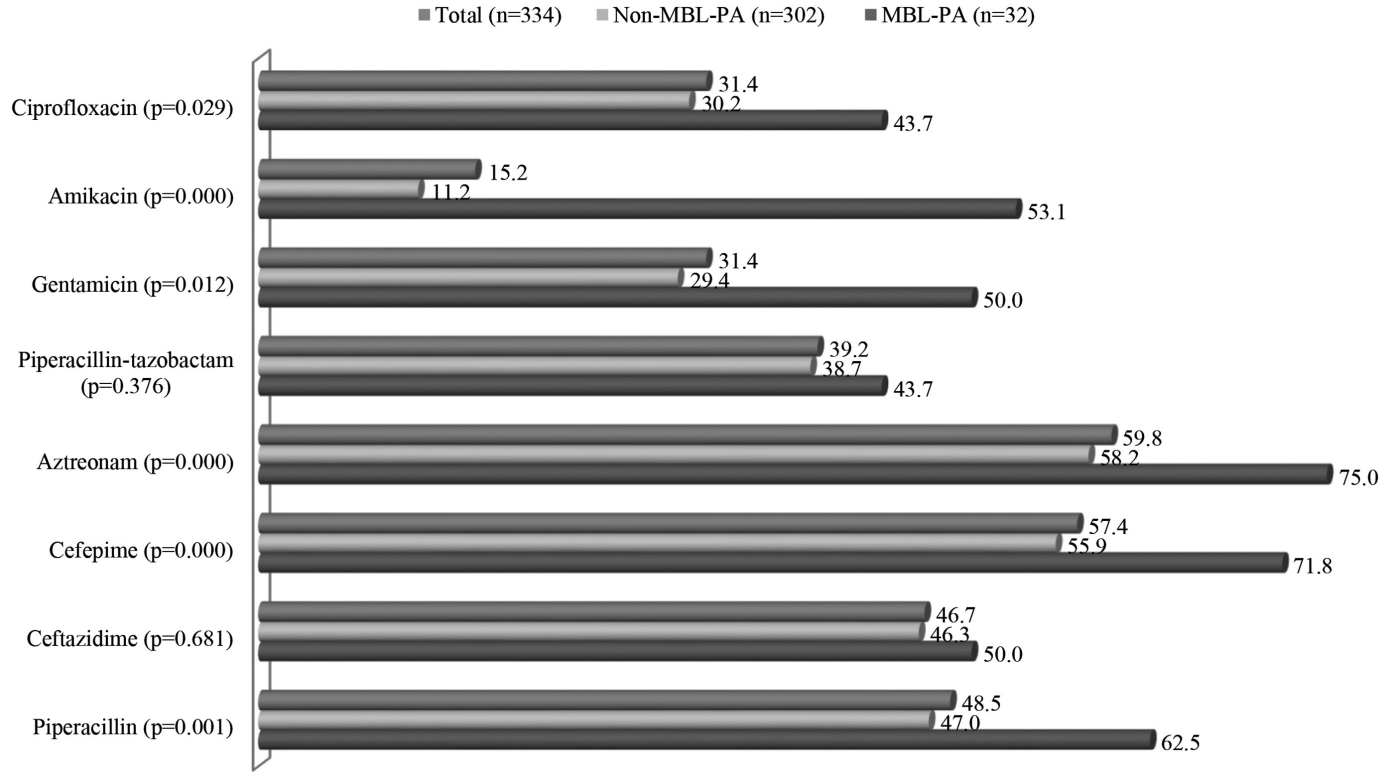
Carbapenemase production was detected by MHT in 21 (6.3%) of the 334 *P. aeruginosa* isolates. MBL production was found in 109 (32.6%) isolates with CDT (Table 2). Among 334 carbapenem-resistant *P. aeruginosa* isolates, 32 (9.6%) were positive for bla_{VIM} or/and bla_{IMP}, and 3 of these isolates were harboring both bla_{VIM} and bla_{IMP} (Table 3).

Eight of the MBL-PA were isolated from pediatric inpatients. Three of them were bla_{VIM}-positive, four were bla_{IMP}-positive, and one was both bla_{VIM}- and bla_{IMP}-positive. Among *P. aeruginosa* isolates found to be MBL-positive, 17 were isolated from the adult inpatient group, of which 13 were VIM-positive only and four were IMP-positive only. Seven of the MBL-PA were isolated from outpatients, of which four were bla_{VIM}-positive, one was bla_{IMP}-positive, and two were found to be positive for both bla_{VIM} and bla_{IMP}.

Antibiotic resistance patterns of the carbapenem-resistant *P. aeruginosa* isolates are summarized in Graphic 1. Compared to non-MBL-PA, MBL-PA isolates showed higher rates of resistance for antibacterial agents such as piperacillin, ceftazidime, cefepime, piperacillin-tazobactam, aztreonam, gentamicin, amikacin, and ciprofloxacin.

Molecular Epidemiology of Metallo-beta-lactamase-producing *P. aeruginosa* Isolates in the Pediatric Inpatient Group

All the bla_{VIM} or/and bla_{IMP}-harboring PA strains (n=8) isolated from the pediatric inpatient group were compared by PFGE. Three different pulsotypes were obtained among the isolates of PA harboring bla_{VIM} (n=3) and PA harboring bla_{VIM} and $blaI_{MP}$ (n=1), while five different pulsotypes were obtained from isolates of PA harboring bla_{IMP} (n=4) and PA harboring bla_{VIM} and bla_{IMP} (n=1) (Table 4). Three particular bla_{VIM}-harboring PA isolates (PA206, PA14, and PA19) were considered to form a cluster, with similarity over 85%. Furthermore, two of them (PA14, and PA19) were effectively identical, with similarity over 95%. With similarity over 85%, two bla_{IMP}-harboring PA isolates (PA31, and PA35) were considered as a cluster, whereas a bla_{IMP}-harboring PA isolate (PA140) and the bla_{VIM} and bla_{IMP}-harboring PA isolate (PA146) were defined as another cluster.



Graphic 1. Evaluation of antibiotic resistance rates (%) of metallo-beta-lactamase-producing *Pseudomonas aeruginosa* and non-metallo-beta-lactamase-producing *Pseudomonas aeruginosa*

MBL-PA: Metallo-beta-lactamase-producing Pseudomonas aeruginosa

Molecular Epidemiology of Metallo-beta-lactamase-producing *P. aeruginosa* Isolates in the Adult Inpatient Group

All bla_{VIM} or bla_{IMP}-harboring PA isolates (n=17) isolated from adult inpatients were compared by PFGE (Table 5). Six different pulsotypes were obtained in bla_{VIM}-harboring PA (n=13), while four different pulsotypes were obtained in bla_{IMP}-harboring PA (=4). Eleven bla_{VIM}-harboring PA isolates (PA230, PA237, PA250, PA282, PA238, PA254, PA43, PA328, PA241, and PA236) were considered a cluster, with over 85% similarity. In addition, eight of those strains (PA282, PA238, PA298, PA254, PA43, PA328, PA241, and PA236) were identical, with similarity exceeding 95%. Pulsotypes obtained from bla_{IMP}-harboring PA isolates (PA166, PA139, PA145, and PA136) were less than 75% similar.

Clinical Characteristics of Metallo-beta-lactamase-producing *P. aeruginosa* Isolates

From a total of 334, 158 patients' hospital records were available. Of these, MBL-PA was isolated from 16 patients and non-MBL-PA was isolated from 142 (Figure 1). No statistical difference was found between patients with MBL-PA and those with non-MBL-PA in terms of age, gender, stay in the intensive care unit (ICU), length of stay in hospital before infection, site of infection, whether or not the infection was polymicrobial, presence of any other accompanying infection, underlying disease, or invasive procedure, history of surgery or trauma, hospitalization within the last 30 days, length of hospital stay, administration of antibiotherapy, or immunosuppressive therapy (Table 6). Nine (43.8%) of the patients with MBL-PA

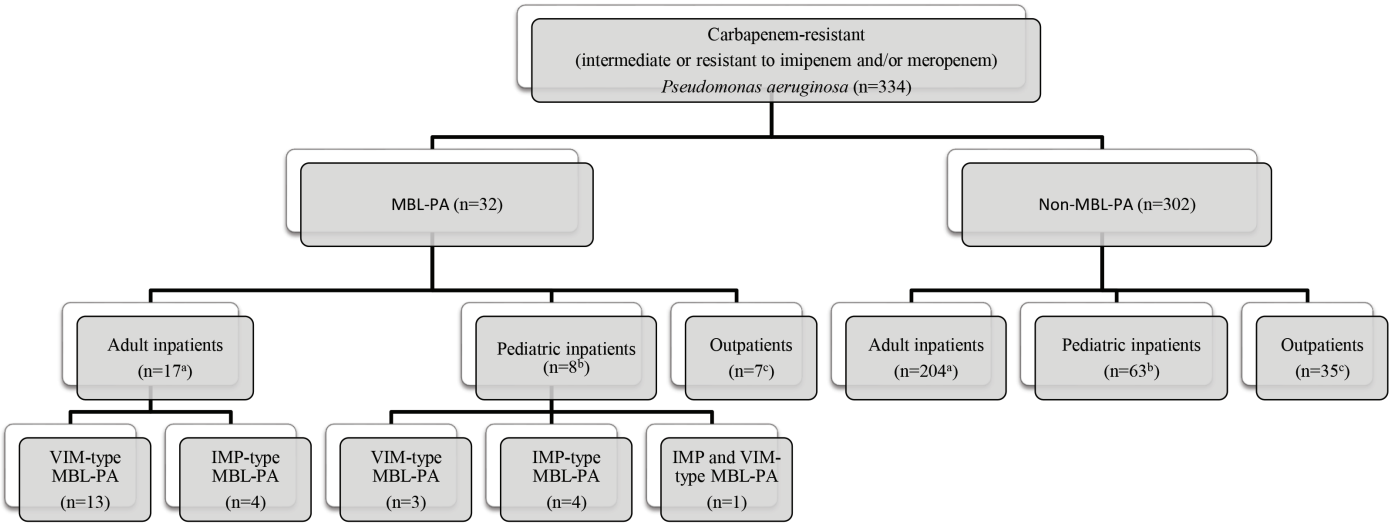


Figure 1. Summary of study findings for MBL-PA and non-MBL-PA

[a]Of 221 adult inpatients, hospital records were available for 135 patients, 11 of whom had MBL-PA isolates and 124 with non-MBL-PA isolates. bOf 71 pediatric inpatients, hospital records were available for 23 patients, 5 of whom had MBL-PA isolates and 18 with non-MBL-PA isolates. cA total of 42 outpatients were excluded from the PFGE analysis, risk assessment, and clinical outcome evaluation.

MBL-PA: Metallo-beta-lactamase-producing Pseudomonas aeruginosa, PFGE: Pulsed-field gel electrophoresis, IMP: P. aeruginosa include imipenemase, VIM: Verona integron-encoded metallo-beta-lactamase

Table 4. Pulsed-field gel electrophoresis patterns of the bla_{VIM}-positive *Pseudomonas aeruginosa* and bla_{IMP}-positive Pseudomonas aeruginosa isolates in the pediatric inpatient group

Isolate code	Hospital unit	Source	Month/year of isolation	MBL type	PFGE pattern
PA140	Neonatal ICU	Tracheal aspirate	02/2009	bla_{IMP}	A1
PA146	Pediatric Ward	Urine	03/2009	bla_{VIM} and bla_{IMP}	A2
PA206	Pediatric Ward	Tracheal aspirate	08/2009	bla_{VIM}	B1
PA14	Pediatric Ward	Tracheal aspirate	02/2010	bla_{VIM}	B2
PA19	Pediatric Ward	Tracheal aspirate	02/2010	bla_{VIM}	B2
PA33	Pediatric ICU	Tracheal aspirate	04/2010	bla_{IMP}	C
PA35	Pediatric ICU	Tracheal aspirate	04/2010	bla_{IMP}	D1
PA31	Pediatric ICU	Tracheal aspirate	04/2010	bla_{IMP}	D2

PFGE: Pulsed-field gel electrophoresis, ICU: Intensive care unit, MBL: Metallo-beta-lactamase

Table 5. Pulsed-field gel electrophoresis patterns of the bla_{VIM}-positive *Pseudomonas aeruginosa* and bla_{IMP}-positive *Pseudomonas aeruginosa* isolates in the adult inpatient group

Isolate code	Hospital unit	Source	Month/year of isolation	MBL type	PFGE pattern
PA272	Surgical ICU	Tracheal aspirate	05/2008	bla_{VIM}	A
PA306	Surgical ICU	Tracheal aspirate	10/2008	bla_{VIM}	B
PA230	Surgical ICU	Tracheal aspirate	12/2009	bla_{VIM}	C1
PA237	Neurology-Neurosurgery ICU	Tracheal aspirate	01/2008	bla_{VIM}	C2
PA259	Surgical ICU	Catheter	05/2008	bla_{VIM}	C3
PA282	Cardiology Ward	Urine	07/2008	bla_{VIM}	C4
PA238	Internal Medicine ICU	Urine	01/2008	bla_{VIM}	C4
PA298	Surgical ICU	Blood	09/2008	bla_{VIM}	C4
PA254	Neurology-Neurosurgery ICU	Tracheal aspirate	04/2008	bla_{VIM}	C4
PA43	Burn Unit	Burn	05/2010	bla_{VIM}	C4
PA328	Surgical ICU	Tracheal aspirate	12/2008	bla_{VIM}	C4
PA241	Plastic Surgery Ward	Wound	02/2008	bla_{VIM}	C4
PA236	Orthopedic Ward	Surgical material	01/2008	bla_{VIM}	C4
PA166	Surgical ICU	Catheter	10/2009	bla_{IMP}	D
PA139	Neurology-Neurosurgery	Tracheal aspirate	02/2009	bla_{IMP}	E
PA145	Internal Medicine ICU	Urine	03/2009	bla_{IMP}	F
PA136	Plastic Surgery Ward	Wound	02/2009	bla_{IMP}	F

PFGE: Pulsed-field gel electrophoresis, ICU: Intensive care unit, MBL: Metallo-beta-lactamase

Table 6. Statistical analysis of risk factors for metallo-beta-lactamase-producing *Pseudomonas aeruginosa* infections

		MBL-PA		nonMBL-PA		p value
		n	%	n	%	
Age (years); Mean±SD		37.1±29.4		47.9±25.4		0.116
Length of hospital stay before PA isolation (days); Mean±SD		33.3±34.9		23.3±20.9		0.279
Duration of hospitalization after PA was isolated (days); Mean±SD		32.3±24.5		24.9±41.2		0.488
Group	Pediatric	5	3.2	18	11.4	0.610
	Adult	11	78.5	124	78.5	
Sex	Female	3	1.9	52	32.9	0.850
	Male	13	8.2	84	53.2	
Stay in ICU 48 hours before PA isolation	No	9	5.7	62	39.2	0.337
	Yes	7	4.4	80	50.6	
Stay in ICU 48 hours after PA isolation	No	9	5.7	63	39.9	0.366
	Yes	7	4.4	79	50.0	
Sample source						
LRT	Negative	10	6.3	74	46.8	0.430
	Positive	6	3.8	68	43.0	
UT	Negative	13	8.2	124	78.5	0.449
	Positive	3	1.9	18	11.4	
Skin and soft tissue	Negative	12	7.6	119	75.3	0.480
	Positive	4	2.5	23	14.6	

Table 6. Continued

		MBL-PA		nonMBL-PA		p value
		n	%	n	%	
Blood	Negative	16	10.1	131	82.9	0.605
	Positive	0	0.0	11	7.0	
Intra-abdominal	Negative	15	9.5	138	87.3	0.418
	Positive	1	0.6	4	2.5	
Multisite	Negative	14	8.9	132	83.5	0.348
	Positive	2	1.3	10	6.3	
Other[a]	Negative	16	10.1	134	84.8	1.000
	Positive	0	0.0	8	5.1	
Polymicrobial growth	Negative	12	7.6	71	44.9	0.058
	Positive	4	2.5	71	44.9	
Accompanying infection	No	10	6.3	66	41.8	0.224
	Yes	6	3.8	76	48.1	
Sepsis or septic shock associated with PA	No	11	7.0	123	77.8	0.072
	Yes	5	3.2	19	12.0	
Underlying disease						
Any (≥1 disease)	Absent	12	7.6	117	74.1	0.497
	Present	4	2.5	25	15.8	
Pregnancy	Absent	16	10.1	140	88.6	1.000
	Present	0	0.0	2	1.3	
Metabolic disease	Absent	14	8.9	134	84.8	0.268
	Present	2	1.3	8	5.1	
Diabetes mellitus	Absent	14	8.9	128	81.0	0.667
	Present	2	1.3	14	8.9	
Liver disease	Absent	15	9.5	136	86.1	0.534
	Present	1	0.6	6	3.8	
Renal insufficiency	Absent	13	8.2	116	73.4	1.000
	Present	3	1.9	26	16.5	
Cardiovascular diseases	Absent	12	7.6	108	68.4	1.000
	Present	4	2.5	34	21.5	
Pulmonary disease	Absent	13	8.2	92	58.2	0.186
	Present	3	1.9	50	31.6	
Neurological disease	Absent	13	8.2	82	51.9	0.069
	Present	3	1.9	60	38.0	
Malignancy	Absent	14	8.9	118	74.7	1.000
	Present	2	1.3	24	15.2	
Invasive procedure						
Any	No	4	2.5	27	17.1	0.521
	Yes	12	7.6	115	72.8	
Central venous catheterization	No	11	7.0	99	62.7	1.000
	Yes	5	3.2	43	27.2	

Table 6. Continued

		MBL-PA		nonMBL-PA		p value
		n	%	n	%	
Urinary catheterization	No	5	3.2	34	21.5	0.545
	Yes	11	7.0	108	68.4	
Mechanical ventilation	No	7	4.4	48	30.4	0.428
	Yes	9	5.7	94	59.5	
Total parenteral nutrition	No	10	6.3	121	76.6	0.034
	Yes	6	3.8	21	13.3	
Hemodialysis	No	13	8.2	129	81.6	0.209
	Yes	3	1.9	13	8.2	
Other invasive procedures b	No	12	7.6	118	74.7	0.488
	Yes	4	2.5	24	15.2	
Hospitalization within the last 30 days	No	2	1.3	12	7.6	0.637
	Yes	14	8.9	130	82.3	
Surgical operation within the last 30 days	No	8	5.1	83	52.5	0.517
	Yes	8	5.1	59	37.3	
Trauma within the last 30 days	No	12	7.6	107	67.7	1.000
	Yes	4	2.5	35	22.2	
Immunosuppressive therapy within the last 30 days	No	14	8.9	87	55.1	0.038
	Yes	2	1.3	55	34.8	
Antibiotherapy within the last 30 days						
Use of any antibiotics	No	2	1.3	12	7.6	0.637
	Yes	14	8.9	130	82.3	
Penicillins	No	13	8.2	119	75.3	0.729
	Yes	3	1.9	23	14.6	
$1^{st}/2^{nd}$ generation cephalosporins	No	13	8.2	112	70.9	1.000
	Yes	3	1.9	30	19.0	
3^{rd} generation cephalosporins	No	9	5.7	72	45.6	0.674
	Yes	7	4.4	70	44.3	
Carbapenems	No	10	6.3	78	49.4	0.563
	Yes	6	3.8	64	40.5	
Aminoglycosides	No	9	5.7	113	71.5	0.055
	Yes	7	4.4	29	18.4	
Quinolones	No	14	8.9	126	79.7	1.000
	Yes	2	1.3	16	10.1	
SXT	No	14	8.9	130	82.3	0.637
	Yes	2	1.3	12	7.6	
Tigecycline	No	16	10.1	128	81.0	0.364
	Yes	0	0.0	14	8.9	

MBL-PA: Metallo-beta-lactamase-producing *Pseudomonas aeruginosa*, nonMBL-PA: Non-metallo-beta-lactamase-producing *Pseudomonas aeruginosa*, n: Number of patients, Mean: arithmetic mean, SD: Standard deviation, LRT: Lower respiratory tract, UT: Urinary tract, SXT: Trimethoprim-sulfamethoxazole, [a]: Ear, conjunctiva, vagina, [b]: Ventriculoperitoneal shunt, chest tube, nephrostomy, colostomy, peritoneal dialysis catheter

died, while 70 (50.7%) of the patients with non-MBL-PA died. No significant difference was found between patients with MBL-PA and non-MBL-PA in terms of mortality (Log Rank: 0.536, p=0.384).

Discussion

MBL-PA is a critical pathogen due to its pathogenicity and antimicrobial resistance characteristics. In order to prevent and manage infections caused by MBL-PA, studies focused on epidemiology, mechanisms of antimicrobial resistance, antimicrobial stewardship, clinical risk factors for MBL-PA infection, and the development of diagnostic tools for rapid detection of MBL-PA are needed[2]. In the present study, approximately 9.6% (32/334) of the carbapenem-resistant PA were found to be bla_{VIM} and/or bla_{IMP}-positive, and three of them carried both of the bla_{VIM} and bla_{IMP} genes. Different clonally related clusters were identified among MBL-PA isolates in the pediatric inpatient group as well as in the adult inpatient group by PFGE fingerprinting. In addition, eight of the MBL-PA strains isolated from adult inpatients were found to be from the same clone. Comparing carbapenem-resistant *P. aeruginosa* isolates with non-MBL-PA isolates, higher resistance rates were observed in the MBL-PA isolates.

Carbapenemases are responsible for an important part of carbapenem resistance and are a source of concern worldwide. Various types of carbapenemases have been increasingly reported in *P. aeruginosa* strains over the years[2,3,19,20]. The distribution of carbapenemases varies according to geographical region, but the most prevalent MBL enzyme types are VIM and IMP[2]. Verona integron-encoded metallo-beta-lactamase-type MBLs in *P. aeruginosa* have been the most commonly reported carbapenemases in Turkey to date, whereas *P. aeruginosa* producing IMP-type MBLs have only been reported from Trabzon and Muş[3-8]. Include imipenemase-type MBLs have not yet been reported in *Enterobacteriaceae* or *Acinetobacter baumannii* isolates in Turkey[21-25]. In the present study, 32 MBL-PA were detected among 334 carbapenem resistant *P. aeruginosa* isolates (9.6%), and bla_{VIM} was more prevalent than bla_{IMP}. Additionally, considering that our study was conducted at the same hospital from which the first IMP-type MBL was detected, additional studies should be performed in different regions of the country in order to ascertain whether the VIM-type MBLs are limited to these regions[5-8]. Our study appears to be the first from Turkey reporting PA isolates with both bla_{VIM} and bla_{IMP} carriage.

Compared with non-MBL-PA isolates, MBL-PA isolates were found to be more resistant to aminoglycosides, quinolones, and even aztreonam in this study. Although aztreonam is not a substrate for MBLs, it is often ineffective against these strains due to additional mechanisms of resistance[1,26]. Additional resistance mechanisms in MBL-PA commonly include cephalosporinase, efflux pumps, or low intrinsic outer membrane permeability. Furthermore, MBL genes and genes encoding other antibiotic resistance determinants may be located on the same plasmids[27]. Therefore, additional resistance mechanisms could be acquired simultaneously with MBL genes and lead to resistance to antibiotics other than beta-lactams such as aminoglycosides and quinolones[1,26]. Depending on the factors listed, it is possible to detect high resistance rates and multidrug resistance in MBL-PA isolates, as demonstrated by this study.

PFGE analysis demonstrated several clonally-related genotype clusters among the VIM-type MBL-PA and IMP-type MBL-PA isolates in the pediatric inpatient group, while eight of the VIM-type MBL-PA isolates from the adult inpatient group were identical. These findings indicate that cross-transmission is an important mechanism for dissemination of MBL-PA, resulting in multidrug resistance in *P. aeruginosa* isolates.

Potential risk factors identified for infection or colonization with MBL-PA include ICU stay, long-term hospitalization, use of indwelling urinary catheters, urinary tract diseases, hemodialysis, and hospitalization within the preceding year, administration of antineoplastic agents or corticosteroids, fluoroquinolone usage, and long-term antibiotic use (especially beta-lactams)[9,28-31]. Nonetheless, we found no statistically significant difference between MBL-PA and non-MBL-PA in terms of demographic features, stay in ICU, length of hospital stay before isolation, sample source, underlying disease, invasive procedure, or history of antibiotic therapy, hospitalization, surgery, trauma, or immunosuppressive therapy within the last 30 days. While some researchers have reported that MBL-PA infections result in a higher mortality rate than non-MBL-PA infection, others have found no association between MBL-PA and mortality[28,30,31]. We also found no statistically significant differences in terms of mortality. This finding may be because of our limited data about the patients. Differences of mortality rates may also be related to the virulence properties of the infecting strains.

One of the limitations of this study is that because of its retrospective design, records were not available for all patients. In addition, the MBL-PA groups were significantly smaller in number than the non-MBL-PA groups due to the prevalence of MBLs.

Conclusion

MBL-PA isolates are more resistant than non-MBL-PA, and the high clonality among the MBL-PA strains indicates that cross-transmission is an important mechanism of dissemination for MBL-PA isolates. Therefore, key factors in the management of antibiotic-resistant bacteria and preventing the spread

of these resistance mechanisms are proper antibiotic usage and applicable infection control strategies. To the best of our knowledge, this is the first study in Turkey to detect isolates with co-existing bla_{VIM} and bla_{IMP} genes.

Ethics

Ethics Committee Approval: The study protocol was approved by Karadeniz Technical University Faculty of Medicine Ethic Council (decision date-number: 2011/7-2).

Informed Consent: Written informed consent of the patient was not obtained due to the retrospective nature of this study but hospital records were reviewed by the approval of the hospital management.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Y.B., G.B., Concept: G.B., F.A., Design: Y.B., G.B., Data Collection or Processing: Y.B., N.K., Analysis or Interpretation: G.B., İ.T., Literature Search: Y.B., G.B., Writing: Y.B.

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