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Determination rates of antibiotic resistance, inducible beta-lactamase, and metallo beta-lactamase ratios in *Pseudomonas aeruginosa* isolates in a university hospital in Turkey.

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ABSTRACT

Objective: This study aimed to determine the antibiotic resistance, inducible beta-lactamase (IBL), and Metallo beta-lactamase (MBL) rates in *P. aeruginosa* isolates.

Material and Methods: In our study, 100 *P. aeruginosa* isolates obtained from various clinical samples were used. Antibiotic susceptibility was performed by using the Kirby-Bauer disk diffusion method. Carbapenem resistance to imipenem and meropenem was verified by the E test. The disk induction method was used to determine the IBL production while the Modified Hodge test, MBL E test, and combined imipenem/ EDTA disk were used to determine the production of MBL.

Results: According to the results of antibiotic susceptibility tests, 58% of *P. aeruginosa* isolates were susceptible to all antipseudomonal drugs, while resistance rates to other drugs were as follows; ceftazidime 7%, cefoperazone sulbactam 8%, cefepime 13%, piperacillin 14%, piperacillin-tazobactam 12%, imipenem 9%, meropenem 11%, aztreonam 8%, amikacin 8%, gentamicin 13%, tobramycin 12%, netilmicin 19%, There was a 10% resistance to ciprofloxacin. 8% of the isolates were resistant to at least three drugs, of which two isolates were positive for MBL enzyme production. IBL production was detected in 86% of the isolates with the disk induction method.

Conclusion: The results we obtained in our study are consistent with other researchers globally and in Turkey. It was concluded that there is a need for well-standardized phenotypic tests with defined evaluation criteria and further studies to verify these tests genotypically.

Keywords: Antibiotic susceptibility, inducible beta-lactamase, Metallo beta-lactamase, *Pseudomonas aeruginosa*, multidrug-resistant

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INTRODUCTION

Pseudomonas aeruginosa, the most pathogenic species in the *Pseudomonadaceae* family, is gram-negative, non-spore, straight or slightly curved, 1.5-5.0 µm long, and 0.5-1.0 µm wide (1,2). The characteristic grape scent from tryptophan 2-aminoacetophenone production is used to differentiate *P.aeruginosa* (3,4).

P. aeruginosa is considered an opportunistic pathogen that causes nosocomial and ventilator-associated infections. It rarely causes infections in healthy individuals, but cysts are observed with high morbidity and mortality in patients with fibrosis and immunocompromised individuals. *P. aeruginosa* is an important infectious agent thanks to its ability to reproduce under minimum conditions, the presence of various virulence factors, and the resistance mechanisms it has developed (5,6). Today, the prevalence of multidrug-resistant *P. aeruginosa* strains is reported to be between 15-30% in different regions, while resistance rates of more than 10% are reported for all antimicrobial groups used (7). The resistance profile of *P. aeruginosa* varies between hospitals and even clinics in the same hospital.

The susceptibility profile of the bacterium should be well known and regularly monitored in the application of appropriate empirical therapy. In this context, determining the resistance status of isolated *P. aeruginosa* strains to antibiotics will help clinicians have an empirical approach and promote rational drug use thus, ensuring that resistance profiles that may develop during the treatment process will be prevented (**8,9**). It has been reported that *P. aeruginosa* is a factor in 10% of hospital infections (**10**).

The present study aimed to determine the resistance status in *P. aeruginosa* infections, to contribute to empirical treatment options and effective treatment approaches. For this purpose, antibiotic resistance, inducible beta-lactamase (IBL), and Metallo beta-lactamase (MBL) ratios were determined in *P. aeruginosa* isolates isolated from various clinical samples in Sanlıurfa, Turkey.

MATERIAL and METHODS

This study was approved by the Ethical Committee of Harran University Faculty of Medicine (Protocol no.05/2015).

1. Sample collection

P. aeruginosa isolates were isolated from various clinical samples sent to the Harran Research and Application Hospital microbiology laboratory. Repeating samples belonging to the same patient were excluded from the study. The isolates were taken into a Tryptic Soy Broth storage media and stocked at -70°C until the study day.

2. Identification of isolates

Isolates were identified by standard microbiological identification techniques. Non-fermentative, oxidase-positive, aerobic, motile, blood agar and eosin methylene blue agar grew at 37° C and 42° C with a characteristic odor, and blue-green pigmentary strains were evaluated as *P. aeruginosa*.

3. Antibiotic susceptibility test

Antibiotic susceptibility profiles of the isolates were determined using a Kirby-Bauer disk diffusion method as per the guidelines of the Clinical Laboratory Standard Institute (CLSI) (11).

3.1. Disc diffusion method

In antibiotic disc diffusion tests, imipenem 10µg (IP), meropenem 10µg (MP), aztreonam 30µg (ATM), ceftazidime 30µg (CAZ), cefepime 30µg (CEP), gentamicin 10µg (GN), amikacin 30µg (AK), tobramycin 30µg (TOB), netilmicin 30µg (NET), ciprofloxacin 5µg (CIP), piperacillin 100µg (PRL), piperacillin/ tazobactam 110µg (TZP), and cefoperazone/ sulbactam 105 µg (CFS) antibiotic discs were used. Antibiotic discs were placed in the plates of Mueller-Hinton agar (MHA) (adjusted to 0.5 McFarland standards). Plates were incubated for 18-20 hours at 35 °C. The inhibition zone diameter was recorded for each isolate and reported as per the CLSI guidelines (**11**). Carbapenem resistance was confirmed by determining the MIC value with the E test method according to disk diffusion test results.

3.2. E-test

E test strips (OXOID, UK) containing 0.02-32 µg/ml IP and 0.02-32 µg/ml MP were used. Briefly, bacterial suspensions with 0.5 McFarland turbidity were prepared from *P. aeruginosa* cultures grown on the MHA plate. Imipenem and meropenem strips were placed on plates and left to incubate for 18-20 hours at 35°C. At the end of the incubation, the E test strips were evaluated according to the manufacturer's recommendations and the MIC values were determined (**12**).

3.3. IBL detection test

IBL production was detected by the disc induction method. To determine the IBL activity on the MHA plate, the IP (10µg) was placed in the middle of the plates containing CAZ (30µg) and ATM (30µg) discs with a distance of 20 mm between the disc centers. After 18-20 hours of incubation at 35° C, the narrowing of the inhibition zone diameters of the CAZ and ATM discs in the MHA plate facing the IP disc (⩾ 4 mm) was evaluated as positive. IBL positivity was evaluated for each isolate relative to IP. The *P. aeruginosa* ATCC 27853 strain was used as a negative control (**13**).

3.4. Determination of MBL production in isolates

MBL production was evaluated by using a Modified Hodge test, IP/ EDTA Combined Disk test, and MBL E test.

3.5. Modified hodge test

For the modified Hodge test, bacterial suspension was prepared according to the 0.5 McFarland of E. coli ATCC 25922 strain (indicator strain) and was left to incubate overnight on an MHA plate. After the plate was dried, the IP (10µg) disk was placed in the center of the plate. IP and/ or MP MIC values were determined by E test, and the cultivation of resistant *P. aeruginosa* isolates was done mutually, linearly from the center to the periphery, and incubated at 35° C for 16-18 hours. The MBL production was evaluated as positive when a clover leaf-shaped deterioration of the IP disc inhibition diameter was sighted. *P. aeruginosa* ATCC 27853 was used as a negative control in the assay (**14,15**).

3.6. IP/ EDTA combined disk test

EDTA was used as an MBL enzyme inhibitor in the IP/ EDTA combined disk test. For 0.5 M EDTA (pH=8.0); 18.61g disodium EDTA.H_2O powder was dissolved in 100 ml of sterile distilled water, and stored at + 4° C.Two IP discs were placed 25 mm apart on one-half of the plate of *P. aeruginosa* isolates. 10 µl of 0.5 M EDTA solution was dropped onto one of the IP discs and then incubated at 35 ^{0}C for 18 hours. When the inhibition zone diameter around the IP/ EDTA disc was ⩾7 mm from the inhibition zone diameter around the IP disc, the MBL was considered positive. *P. aeruginosa* ATCC 27853 was used as the negative control (**16,17**).

3.7. MBL E test

MBL E test strips containing 4-256 µg/ml IP on one side and IP (1-64 µg/ml) –EDTA (IPE) on the other were used. IP/ IPE test strips were placed on the plates containing *P.aeruginosa* and incubated at 35 °C for 18 hours. According to the manufacturer's recommendations, the MIC value for IP was proportioned to the MIC value for IPE. Isolates with ⩾ 8 MIC ratio obtained on both the reagent side of the

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"phantom" zone or deformation in both ellipses was evaluated as MBL positive. *P. aeruginosa* ATCC 27853 was used as the negative control (**18**).

4. Statistical analysis

Statistical analysis of the data obtained from antibiotic susceptibility results was performed using the chi-square test in the SPSS (Incorporated, Chicago) program. One-way Annova was used between multiple groups. The Student T and Pearson Correlation tests were used between single groups.

RESULTS

The most isolated sample type of *P. aeruginosa* was urine with 26.7%, and the least sample type was determined as Cerebrospinal fluid (CSF) with 2%. The distribution of isolates according to clinical samples is shown in Table 1.

Table 1. Distribution of clinical samples in which *P.aeruginosa* was isolated.

Sample type	n	%
Trachea	20	19.8
Sputum	19	18.8
Urine	27	26.7
Throat	4	4.0
Blood	6	5.9
Wound	15	14.9
CSF	2	2.0
Aspiration sample	3	3.0
Swab	5	5.0
Total	**100**	**100.0**

P. aeruginosa isolates were studied with 51 females and 49 males. The ages of these patients were between 1-87 and the mean age was 35 ± 2.8. When the patients from whom *P. aeruginosa* isolates were produced are classified according to their age, they are divided into four groups. Accordingly, the prevalence of *P. aeruginosa* isolates was found to be in 38% of children aged 0-14 (Table 2).

Table 2. The age range of patients from whom *P. aeruginosa* strains were isolated (n: 100)

Clinics	0-5 years	5-14 years	14-50 years	>50 years
ICU	36.7	6.7	10	46.7
Urology	28.6	42.9	21.4	7.1
Endocrine	0	16.7	33.3	50
Chest disease	0	0	56.3	43.8
Dermatology	0	0	100	0
Otolaryngology	0	0	100	0
Pediatric service	75	25	0	0
General surgery	33.3	0	50	16.7
Orthopedics	0	0	50	50
Infection	0	0	57.1	42.9

In our study in which 100 *P. aeruginosa* isolates were evaluated, *P. aeruginosa* isolates were most frequently (30%) isolated from the ICU. The distribution of the isolates according to the clinics where they were isolated is shown in figure 1.

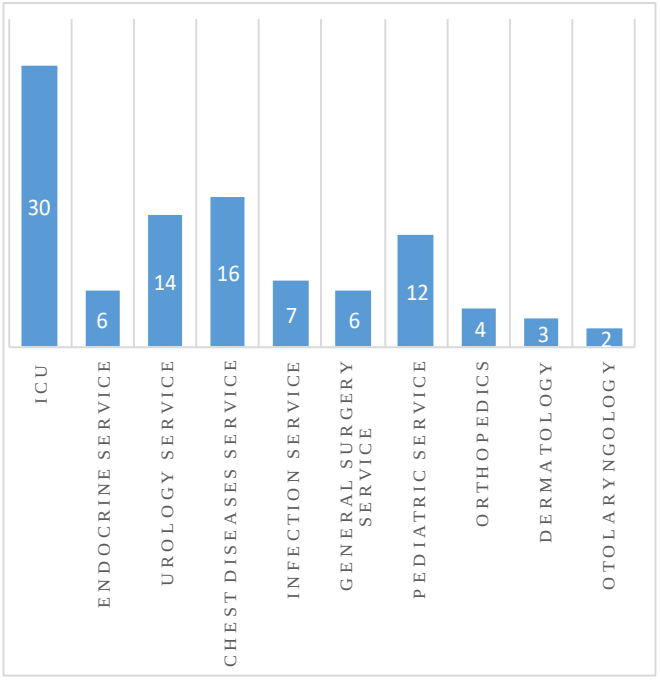

Figure 1. Distribution of *P. aeruginosa* isolates according to the clinics where they were isolated.

1.Antibiotic susceptibility test

According to the results of antibiotic susceptibility tests, 58% of *P. aeruginosa* isolates were susceptible to all antipseudomonal drugs studied; CAZ 7%; CFS 9%; CFP 11%; PRL 12%; TZP 8%; IP 9%; MP 11%; ATM 9%; AK 8%; GN 11%; TOB 9%; NET 19%; and 10% resistance to CIP. The sensitivity of *P. aeruginosa* isolates to antibiotics is shown in Table 3. It was found that 8% of the isolates were multidrug resistance (MDR) to at least three antipseudomonal drug groups. MBL enzyme production was determined in two of these eight MDR isolates. It has been observed that the rate of MBL enzyme production is higher in bacteria with MDR. As a result, colistin with a sensitivity rate of 100% was seen as the most effective antibiotic in *P. aeruginosa* isolates. Apart from colistin, the least resistance was determined in CAZ, followed by AK, IP, and CFS.

Table 3. Sensitivity of *P. aeruginosa* isolates to antimicrobial agents.

Antibiotic agents	Sensitive		Resistant	
	n	%	n	%
Imipenem	91	91	9	9
Meropenem	89	89	11	11
Ceftazidime	93	93	7	7
Cefepime	87	87	13	13
Aztreonam	92	92	8	8
Piperacillin	86	86	14	14
Piperacillin/tazobactam	88	88	12	12
Amikacin	92	92	8	8
Gentamycin	87	87	13	13
Tobramycin	88	88	12	12
Netilmicin	81	81	17	17
Ciprofloxacin	90	90	10	10
Cefoperazone/Sulbactam	92	92	8	8
Colistin	100	100	0	0

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The difference between the resistance rates of colistin and beta-lactam antibiotics IP, MP, CAZ, CEP, CFS, and PRL, was found to be statistically significant ($p < 0.05$). Again, the difference between the resistance rates of beta-lactam antibiotics within themselves was found to be statistically significant also ($p < 0.05$). The sensitivity of nine *P. aeruginosa* isolates resistant to carbapenems to other antibiotics is shown in Table 4.

Table 4. Sensitivity of 9 isolates of *P. aeruginosa* resistant to carbapenems to antibiotics other than carbapenem.

Antibiotic agents	Sensitive		Resistant	
	n	%	n	%
Ceftazidime	6	66.7	3	33.3
Cefepime	3	33.3	6	66.7
Aztreonam	4	44.4	5	55.6
Piperacillin	3	33.3	6	66.7
Piperacillin/tazobactam	4	44.4	5	55.6
Amikacin	5	55.6	4	44.4
Gentamycin	4	44.4	5	55.6
Tobramycin	1	11.1	8	88.9
Netilmicin	1	11.1	8	88.9
Ciprofloxacin	3	33.3	6	66.7
Cefoperazone/Sulbactam	4	44.4	5	55.6
Colistin	9	100	0	0

2. Disk induction test

IBL production in *P.aeruginosa* isolates was determined by the disc induction method as 86% of the isolates were IBL positive and 14% as IBL negative (Fig. 2). All carbapenem-resistant isolates were identified as IBL negative. When the relationship between IBL positivity and color was investigated, it was found to be significant ($p < 0.05$); the green pigment ratio of IBL producing *P. aeruginosa* strains was 68.9%, and the green pigment ratio of *P. aeruginosa* strains that did not produce IBL was 31.1%.

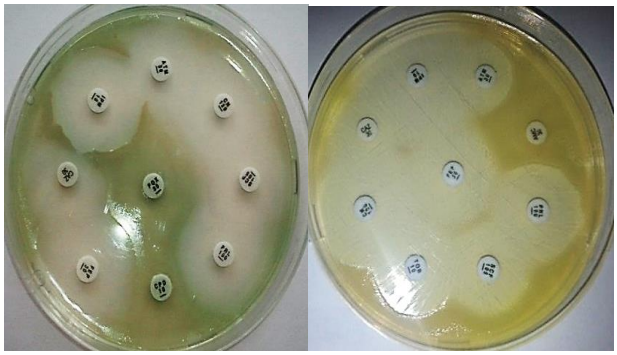

Figure 2. IBL positive (left) and negative (right) *P. aeruginosa* isolates.

3. MBL E combined disc and Modified Hodge tests

MBL production in *P. aeruginosa* isolates was investigated by three different phenotypic methods. MBL production was determined in 2 isolates out of 9 carbapenem-resistant isolates with the MBL E test (Fig. 3).

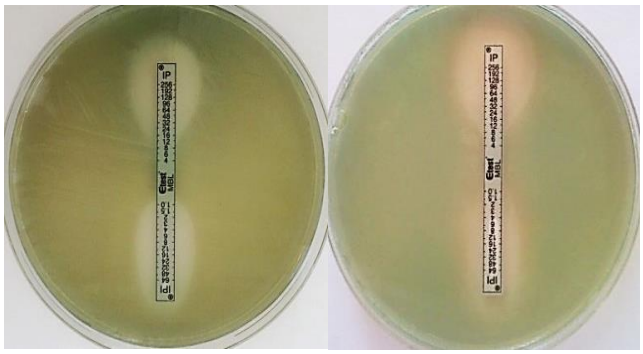

Figure 3. MBL positivity (left) and negativity (right) with the MBL E test.

MBL uremia was again determined in two isolates with the Combined disc test. Two isolates that were found positive with the MBL E test also gave positive results with the Combined disc test. Combined disc test results were also negative in all isolates with negative results with the MBL E test (Fig. 4). With the modified Hodge test, one of the 9 carbapenem-resistant isolates was detected as MBL positive and 8 as MBL negative (Fig. 4). One isolate was detected as MBL positive by all three tests and one isolate detected as MBL positive by the MBL E test, and the Combined disk test was accepted as MBL producing isolates.

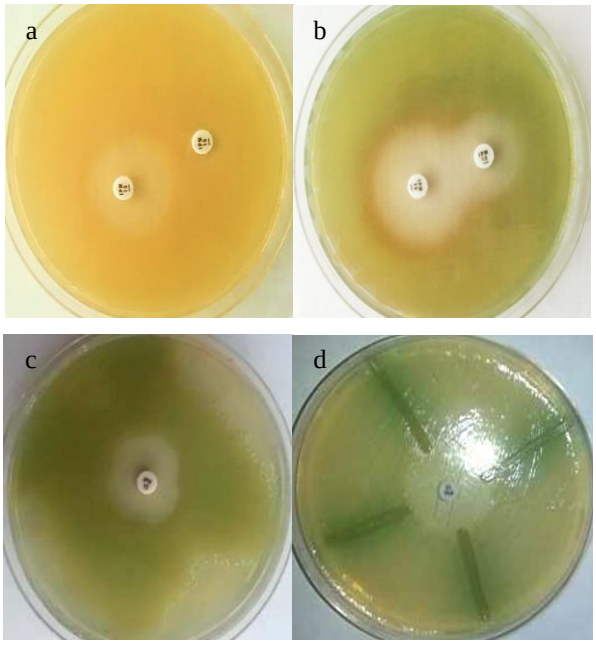

Figure 4. Detection of MBL production with the Combined disc test (**a, b**) and modified Hodge test **(c, d**). **a,c**: MBL positive, **b,d**: MBL negative.

Table 5. Test results of two MBL positive *P. aeruginosa* isolate by MBL E test and Combined disc test.

MBL positive isolates	MBL E test (MIC value/µg/ml)		MBL with Combined Disk test (zone diameter/mm)	
	IP	IP/EDTA	IP	IP/EDTA
1	12	1	9	19
2	9	1	11	28

Table 6. Results of MBL detection by phenotypic tests in 9 carbapenem-resistant *P. aeruginosa* isolates and standard strains.

Isolates	Phenotypic Methods		
	Modified Hodge Test	Combined Disk Test	Etest
1	1 (11.1%)	2 (22.2%)	2 (22.2%)
2	1 (11.1%)	7 (77.7%)	7 (77.7%)
***P. aeruginosa*ATCC 27853**	Negative	Negative	Negative

The difference between the IP/ EDTA and IP disc inhibition zone diameters of two isolates with MBL production positive with the Combined disk test was19 mm in the first isolate and 28 mm in the second isolate (Table 5).

It was determined that the difference between inhibition zone diameters in 7 isolates determined as MBL negative with the Combined Disk test varied between 1-6 mm.

Among the carbapenem-resistant *P. aeruginosa* isolates, the rate of MBL positive isolates was determined as 22.2%, while the rate of MBL among all isolates was determined as 2%. The results of three phenotypic methods used in detecting the MBL enzyme are shown in Table 6.

The compatibility of MBL E, Combined Disc, and Modified Hodge tests with each other was calculated according to the formula below:

The compatibility rate (%) = a + b / c x 100; where

a: Number of isolates giving positive results with both tests: 1

b: Number of isolates that gave negative results with both tests: 7

c: Total number of isolates: 9

According to this; the compatibility between the MBL E test and the Combined Disc test was 100%, the compatibility between the MBL E and the Modified Hodge tests was 88.8%, and the agreement between the IP/ EDTA Combined Disc and the Modified Hodge tests was 88.8%.

The sensitivity of *P. aeruginosa* isolates positive for MBL by E test and Combined Disc test to other antibiotic agents was evaluated in this study. Accordingly, the MBL positive 1st isolate was resistant to CEP, TZP, GN, TOB, and NET, while the 2nd isolate was resistant to CAZ, CEP, TZP, GN, TOB, NET, and CFS (Data have not shown). Two MBL positive isolates were isolated from the tracheal aspirate of patients hospitalized in the ICU.

DISCUSSION

P. aeruginosa is an opportunistic pathogen and it has been reported to frequently cause nosocomial infections in immunocompromised patients (**19**). It is responsible for 10-25% of hospital infections in the world (**20,21**). *P. aeruginosa* is the most commonly isolated non-fermentative bacterium among nosocomial pneumonia, urinary tract infection, wound infections, and septicemia.

The ability of *P. aeruginosa* to reproduce easily, the β-lactamase enzymes it produces, and its natural and acquired resistance capabilities emerge as an important problem worldwide (**22**). In most of the studies conducted in our country, *P. aeruginosa* strains were most frequently isolated from the ICU (**23**).

In a study conducted between 2010 and 2016, it was observed that the *P. aeruginosa* strain isolated was mostly associated with patients admitted to the pediatric outpatient clinic and hospitalized in the ICU. Outpatient isolates were 58% urine, 24.2% wound, 11.5% external auditory canal, and 7.5% sputum. 37.8% of the patients hospitalized in the ICU had respiratory tract, 25.4% wound, 22.7% urine, and 9.7% blood (**24**).

In our study, *P. aeruginosa* strains were isolated most frequently (30%) from the ICU. These strains were isolated as 26.72% urine, 19.8% tracheal aspirate, 18.8% sputum, 14.9% wound, 5.9% blood, 5% swab, 4% throat, 3% aspiration fluid and 2% CSF. *P. aeruginosa* can develop resistance to antibiotics through various resistance mechanisms. Production of chromosomal and plasmid-derived beta-lactamases, changes in antibiotic targets, decrease in outer membrane permeability as a result of changes in porin proteins, and expulsion of the antibiotic by the efflux pump system are the main resistance mechanisms (**25**). One of the mechanisms to develop resistance during antibiotic therapy is to produce AmpC inducible chromosomal beta-lactamase and OXA type chromosomal beta-lactamase enzymes (**26,27**).

Resistance rates have been reported in various studies conducted in our country. In one study, all 195 *P. aeruginosa* strains (100%) had resistance to CAZ, 90.8% to TZP, 60.5% ATM, 50.2% CEP, 48.2% IP, 47.2% MP, 44.1% PRL, 31.3% levofloxacin (LVX), and 26.2% to CIP, It was also reported that 11.8% was resistant to GN, 8.7% to AK and 6.2% to TOB (**28**).

In a study conducted in our country, IP resistance was detected in 20 (18.5%) of 108 *P. aeruginosa* strains isolated from ICU patients, and MBL production was 14 (70%) in 20 IP resistant strains. The highest MBL production was observed in isolates obtained from sputum (**29**).

In a study in which 72 *P. aeruginosa* isolates from 1443 stool samples were evaluated, ceftazidime resistance was 8%, CEP 7%, ATM 7%, GN 3%, CIPas 1% and IPresistance as 1% (**30**). While 58% of *P. aeruginosa* isolates were sensitive to all antipseudomonal drugs in our study; IP 9%, MP11%,

ATM 8%, CAZ 7%, CFS 8%, CEP 13%, PRL 14%, TZP 12%, AK 8%, GN 13%, TOB 12%, NET 19%, and 10% resistance to CIP was found.

Antimicrobial resistance rates in *P. aeruginosa* isolates in our hospital were determined similar to other studies in our country. The most effective antibiotic has been determined as colistin with a sensitivity rate of 100%. Less resistance except for colistin, beta-lactam antibiotics CAZ; AK was determined from the aminoglycosides Determination of MBL production in Pseudomonas strains in hospitals by phenotypic methods will contribute to infection control with early control measures. In a study conducted in Italy, MBL positivity was found in 12.6% of *P. aeruginosa* isolates by E-test (**31,32**).In our study, MBL production was detected in 22.2% of 9 carbapenem-resistant *P. aeruginosa* isolates. The MBL positivity rate was determined as 2% among all isolates. The rate of MBL was found to be lower than many studies in our country and abroad. Carbapenem-resistant isolates, which we found to be MBL negative, could not be determined by phenotypic methods other than the MBL enzyme or efflux due to OprD porin protein loss. However, it has been concluded that molecular studies are required to confirm this. Various studies on IBL production have been carried out abroad and in our country. In a study conducted in Turkey isolated from various clinical specimens of 87 *P. aeruginosa* strains, 64 (74%) was the determined IBL production rate (**33**).

In our hospital, no research has been conducted on the prevalence of IBL in *P. aeruginosa* isolates in previous years. In our study, IBL production was detected in 86% of *P. aeruginosa* isolates by the disc induction method. While this rate is parallel to IBL rates in studies abroad, it was higher than many studies conducted in our country. Although the production of IBL in the treatment was not specified by the laboratory due to the high rate of IBL in our hospital, it should be accepted that *P. aeruginosa* isolates have this feature. Also, the rate of green pigment was 68.9% in the IBL producing *P. aeruginosa* isolates and 31.1% in the isolates that did not produce IBL. It has been determined that most of the carbapenem-resistant isolates produce green pigment. These results suggest that there may be a relationship between the pigment color produced by *P. aeruginosa* and antibiotic resistance. It was thought that other studies should be conducted on this subject.

There are some limitations to this study. All isolates were obtained from one center in Turkey (Sanlıurfa). The low number in the centers led to limited the spread of the results across the country. Also, resistance mechanisms were not investigated in the study. As future perspectives, further studies with the molecular methods are needed to the identification of the resistance mechanisms.

CONCLUSIONS

In conclusion, for the treatment of cases caused by *P. aeruginosa*, the resistance profile of these isolates should be well known. Accordingly, necessary precautions should be taken to prevent the increase in resistance rates, effective policies regarding the use of antibiotics in hospitals should be developed, and an appropriate infection control program should be implemented. In summary, the study will contribute to the treatment of *P. aeruginosa* infections by determining its resistance profile, inducible beta-lactamase, and Metallo beta-lactamase ratios.

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