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Examining the frequency of carbapenemase genes *bla*$_{KPC}$, *bla*$_{IMP}$, *bla*$_{OXA-48}$, *bla*$_{SPM}$, *bla*$_{NDM}$, *bla*$_{VIM}$, *bla*$_{GES}$, *bla*$_{BIC}$, *bla*$_{AIM}$, *bla*$_{GIM}$, *bla*$_{SIM}$, and *bla*$_{DIM}$ in *Pseudomonas aeruginosa* strains isolated from patients hospitalized in Northwest Iran hospitals

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Abstract

Pseudomonas aeruginosa (*P. aeruginosa*) is one of the most common bacteria isolated from clinical samples, with a rising incidence in hospital infections. This pathogen is inherently resistant to many antibacterial agents. This study aimed to investigate the frequency of carbapenemase genes in *P. aeruginosa* strains isolated from patients admitted to hospitals in northwestern Iran. A total of 500 *P. aeruginosa* samples were collected from different clinical samples. Antibiotic susceptibility testing was performed according to the Clinical and Laboratory Standards Institute (CLSI) guidelines, and the frequency of the target genes was assessed using polymerase chain reaction (PCR). The antibiotic resistance results of the samples by disc diffusion method showed that imipenem 98.4%, gentamicin 98%, meropenem 91.8%, amikacin 91.6% and cefepime 91% had the highest resistance; also, out of 500 *P. aeruginosa* isolates, 309 (61.8%) samples were carbapenemase producers. Using the PCR method, it was determined that the *bla*$_{OXA-48}$ (39.16%), *bla*$_{GES}$ (31.72%), and *bla*$_{IMP}$ (22.01%) genes were the dominant genes. Our results showed that the prevalence of carbapenemase genes in *P. aeruginosa* strains isolated from patients admitted to hospitals in northwestern Iran is very high; indicating a need for effective infection control measures to prevent the spread of *P. aeruginosa* in hospitals.

Keywords: *Pseudomonas aeruginosa*, drug resistance, carbapenemase, hospital infection

1. Introduction

Pseudomonas aeruginosa (*P. aeruginosa*) is one of the 5 opportunistic human pathogens, especially in immunocompromised patients, which is the cause of hospital-acquired opportunistic infections and carries multiple drug resistance (1-5). Hospital-acquired infections are one of the significant medical problems in developed and developing countries which cause the spread of infectious diseases in the society (6-12). Infection with this bacterium can cause septicemia, pneumonia, meningitis, and other fatal diseases (13-15). Antibiotic resistance in this bacterium is caused by intrinsic resistance due to low permeability, the presence of exudation systems, acquisition of resistance genes through plasmids, transposons, and integrons (16, 17). Over many years, the indiscriminate use of antibiotics, not paying attention to the doctor's prescription, the massive use of antibiotics in the food industry, and factors like this have effectively increased the antibiotic resistance of many bacteria (18-24). Today carbapenem drugs are used to treat antibiotic-resistant strains, but recently, the prevalence of carbapenem resistance is one of the most essential types of antibiotic resistance in *P. aeruginosa*, which is increasing worldwide (25). One of the mechanisms of antibiotic resistance in this bacterium is the production of beta-lactamases, which can deactivate many beta-lactam antibiotics such as penicillin, cephalosporin, and carbapenem (except for monobactams) (26). Currently beta-lactamases are divided into four Ambler categories A, B, C, and D (27, 28). Metallo-beta-lactamase (MBLs) are among the beta-lactamase enzymes, which are one of the main mechanisms of bacterial resistance against beta-lactam antibiotics and are in group B of Ambler's classification and group 3 of Bush's classification (27). MBLs are generally divided into several families: Verona integron-encoded MBL

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(VIM), Dutch Imipenemase (DIM), Adelaide Imipenemase (AIM), Seoul Imipenemase (SIM), Germany Imipenmase MBL (GIM), Imipenmase (IMP) Sao Paulo MBL (SPM), New Delhi MBL (NDM) (29, 30). In the meantime, IMP and VIM have been widely studied and are coded by *bla*$_{IMP}$ and *bla*$_{VIM}$ genes (31). Many strains of *P. aeruginosa* can produce several types of broad-spectrum beta-lactamases, which enables them to be resistant to commonly used antibiotics (32). Beta-lactamases are inhibited by beta-lactam inhibitors such as clavulanic acid (33, 34). The study of these enzymes is essential for faster diagnosis and investigation of the prevalence rate to prevent the spread of this type of resistance. Therefore, considering the importance of carbapenems in the treatment of infections caused by *P. aeruginosa* and the need to know the prevalence of resistance and the methods of creating resistance to this group of antibiotics, the present study was conducted to investigate the frequency of carbapenemase genes in *P. aeruginosa* strains isolated from hospitals in the northwest of Iran.

2. Materials and Methods

2.1. Collection and identification of samples

In this descriptive cross-sectional study, a total of 500 samples of *P. aeruginosa* from different clinical samples, including blood, urine, burn wounds, tracheal tube secretions, and other body secretions from September 2022 to February 2023 from patients hospitalized in Tabriz hospitals, along with information Patients including gender and age were collected by random sampling method. To determine the phenotypic identity using gram staining, determining the microscopic shape of the microorganism, catalase, oxidase tests, and other biochemical tests including, the Simon citrate test, H2S production, indole production and mobility, nitrate reduction, urease, triple sugar iron agar, ornithine and lysine decarboxylation, DNase and oxidative fermentation tests were performed. Then tryptic soy broth (TSB) transfer medium was used to store all the samples, and they were kept at -70°C until the next tests.

2.2. Determining antibiotic sensitivity in the examined samples

To determine the antibiotic resistance pattern, a suspension equivalent to 0.5 McFarland turbidity was prepared from all the isolates and cultured in Mueller Hinton Agar culture medium (QUELAB, Canada). The antibiotic discs used in this study include: gentamicin (GEN: 10 µg), amikacin (AK: 30 µg), imipenem (IPM: 10 µg), cefotaxime (CTX: 30 µg), cefepime (FEP: 30 µg), meropenem (MEM: 10 µg), doripenem (DPM: 10 µg), ciprofloxacin (CIP: 5 µg), ticarcillin (TIC: 75 µg), piperacillin/tazobactam (PTZ: 10/100 µg), ceftazidime (CAZ: 30 µg), aztreonam (AZA: 30 µg) and piperacillin (PIP, 100 µg), and tobramycin (TOB, 10 µg) (Mast, UK). *P. aeruginosa* strain ATCC 27853 was used as control. To evaluate the antibiotic resistance of the isolates, disk diffusion method was used according to CLSI guidelines (35).

2.3. Phenotypic detection of carbapenemase- producing strains

The Modified Hodge test (MHT) method was used to isolate carbapenemase- producing strains. To perform this test, first, 0.5 McFarland's dilution of *Escherichia coli* (*E. coli*) ATCC25922 was prepared in 5 ml of broth or saline, and the suspension was diluted 1:10 and cultured on Mueller Hinton agar medium. A carbapenem disk was placed in the center of the plate, and the tested organism was drawn in a straight line from the edge of the disk to the side of the plate. Then, the plate was incubated for 24 hours at 37 degrees Celsius. MHT-positive isolates produce a clover-shaped incision of *E. coli* ATCC 25922 along the test organism in the growth inhibition zone of the disc after 24 hours. In negative MHT no growth of *E. coli* ATCC 25922 occurs along the test organism in the growth inhibition zone. The performance of carbapenemase phenotypic tests was evaluated using polymerase chain reaction (PCR) (36).

2.4. Identification of bla$_{KPC}$, bla$_{IMP}$, bla$_{OXA-48}$, bla$_{SPM}$, bla$_{NDM}$, bla$_{VIM}$, bla$_{GES}$, bla$_{BIC}$, bla$_{AIM}$, bla$_{GIM}$, bla$_{SIM}$, and bla$_{DIM}$ genes

The PCR method was used to evaluate the frequency of the target genes, and the samples were extracted using the Sinaclon kit protocol (Iran). To carry out the PCR process to identify the desired genes, the specific primer pairs listed in Table 1 were used together in a PCR reaction.

The volume and materials required to perform the PCR reaction were as follows: Master Mix 2x from Sinaclon Company (Iran) was used to perform PCR. In this way, 12.5 µl of master mix, 1 µl of forward primer (10 pmol), 1 µl of reverse primer (10 pmol), 3 µl of DNA, and 7.5 µl of injection water were added. The thermal cycler program contains 5 minutes of initial denaturation at 95°C for 3 minutes and 35 cycles with 50 seconds of denaturation at 95°C, annealing at 55°C for 40 seconds, the extension was done at 72°C for 120 seconds, and finally, the final extension at 72°C for 5 minutes. Then, the PCR product was evaluated on a 1.5% agarose gel by electrophoresis, and the gel containing the PCR products was placed in a tank containing DNA Safe Stain (V2) (Sinaclon, Iran) for 15-20 minutes after the end of the electrophoresis period, and then under UV light. Bands were observed by the GelDoc device (Germany) and finally photographed and printed. Then, the PCR product was evaluated on a 1.5% agarose gel by electrophoresis, and the gel containing the PCR products was placed in the tank containing DNA Safe Stain (V2) (Sinaclon, Iran) for 15-20 minutes after the completion of the electrophoresis period. Then, the bands were observed under UV light by the GelDoc device (Germany). In this study, the results of phenotypic and genotypic tests were analyzed with statistical package for the social sciences (SPSS) software version 23 (IBM) and using chi-square tests.

Table 1. Primers used in this research

Gene	Primer	Nucleotide sequence (5′ to 3′)	Size (bp)	Reference
bla_{GES}	GES-F	GTTTTGCAATGTGCTCAACG	371	(37)
	GES-R	TGCCATAGCAAATAGGCGTAG		
bla_{NDM}	NDM-F	ACCGCCTGGACCGATGACCA	263	(38)
	NDM-R	GCCAAAGTTGGGCGCGGTTG		
bla_{OXA48}	OXA-F	TTGGTGGCATCGATTATCGG	734	(39)
	OXA-R	GAGCACTTCTTTTGTGATGGC		
bla_{SPM}	SPM-F	GGGTGGCTAAGACTATGAAGCC	447	(40)
	SPM-R	GCCGCCGAGCTGAATCGG		
bla_{IMP}	IMP-F	ACCGCAGCAGAGTCTTTGCC	585	(41)
	IMP-R	ACAACCAGTTTTGCCTTACC		
bla_{VIM}	VIM-F	AGTGGTGAGTATCCGACA	261	(41)
	VIM-R	ATGAAAGTGCGTGGAGAC		
bla_{KPC}	KPC-F	CTTGCTGCCGCTGTGCTG	489	(42)
	KPC-R	GCAGGTTCCGGTTTTGTCTC		
bla_{BIC}	blaBIC-F	TATGCAGCTCCTTTAAAGGGC	537	(43)
	blaBIC-R	TCATTGGCGGTGCCGTACAC		
bla_{AIM}	blaAIM-F	CTGAAGGTGTACGGAAACAC	322	(43)
	blaAIM-R	GTTCGGCCACCTCGAATTG		
bla_{GIM}	blaGIM-F	TCGACACACCTTGGTCTGAA	447	(43)
	blaGIM-R	AACTTCCAACTTTGCCATGC		
bla_{SIM}	blaSIM-F	TACAAGGGATTCGGCATCG	570	(43)
	blaSIM-R	TAATGGCCTGTTCCCATGTG		
bla_{DIM}	blaDIM-F	GCTTGTCTTCGCTTGCTAACG	699	(43)
	blaDIM-R	CGTTCGGCTGGATTGATTTG		

3. Results

Out of a total of 500 isolates of *P. aeruginosa*, 291 (58.2%) isolates were from male samples and 209 (41.8%) isolates were from female samples. Most samples were *P. aeruginosa* positive, from urine culture and blood culture (Figure 1). The average age of the patients was 38.54 ± 16.42, ranging from a minimum of one year to a maximum of 70 years (Table 2). There was no statistically significant difference in the distribution of *P. aerogenosa* isolates between age groups ($p>0.05$). There was no statistically significant difference in the distribution of *P. aerogenosa* between male and female groups ($p>0.05$). The results of antibiotic resistance of the samples by disk diffusion method showed that imipenem 98.4%, gentamicin 98%, meropenem 91.8%, amikacin 91.6% and cefepime 91% had the highest resistance (Figure 2). Out of 500 *P. aeruginosa* isolates, 309 (61.8%) samples were carbapenemase producers. 181 samples (58.58%) were isolated from males, and 128 samples (41.42%) were isolated from females. All carbapenemase strains had 100% resistance to meropenem, imipenem, ciprofloxacin, cefotaxime, ceftazidime, amikacin, azetronam, and gentamicin antibiotics. Using the PCR method, it was determined that out of 309 carbapenemase- producing samples, 121 samples (39.16%) contained the bla_{OXA-48} gene, 98 samples (31.72%) contained the bla_{GES} gene, 68 samples (22.01%) contained the bla_{IMP} gene, 59 samples (19.09%) contain bla_{VIM} gene, 50 samples (16.18%) contain bla_{SPM} gene, 43 samples (13.91%) contain bla_{KPC} gene, 36 samples (11.65%) contain bla_{NDM} gene, 21 samples (6.80%) contain bla_{DIM} gene, 19 samples (6.15%) contained bla_{SIM} gene, 16 samples (5.18%) contained bla_{GIM} gene, 13 samples (4.21%) contained bla_{AIM} gene, 9 samples (2.91%) contained bla_{BIC} gene (Table 3). 181 samples (58.58%) with an average age of 40.38 ± 12.72 years belonged to males, and 128 samples (41.42%) with an average age of 41.95 ± 11.64 years belonged to females.

Table 2. Distribution of *P. aeruginosa* isolates in clinical samples according to age

Age category	Clinical samples					
	Urine (%)	Blood (%)	Burn wounds (%)	Tracheal tube secretions (%)	Other body secretions (%)	Total
≤10 years	10 (2)	10 (2)	4 (0.8)	4 (0.8)	1 (0.2)	29 (5.8)
11-25 years	44 (8.8)	40 (8)	15 (3)	10 (2)	3 (0.6)	112 (22.4)
26-40 years	54 (10.8)	50 (10)	18 (3.6)	12 (2.4)	4 (0.8)	138 (27.6)
41-55 years	50 (10)	46 (9.2)	16 (3.2)	11 (2.2)	4 (0.8)	127 (25.4)
56-70 years	37 (7.4)	34 (6.8)	12 (2.4)	8 (1.6)	3 (0.6)	94 (18.8)
Total	195 (39)	180 (36)	65 (13)	45 (9)	15 (3)	500 (100)

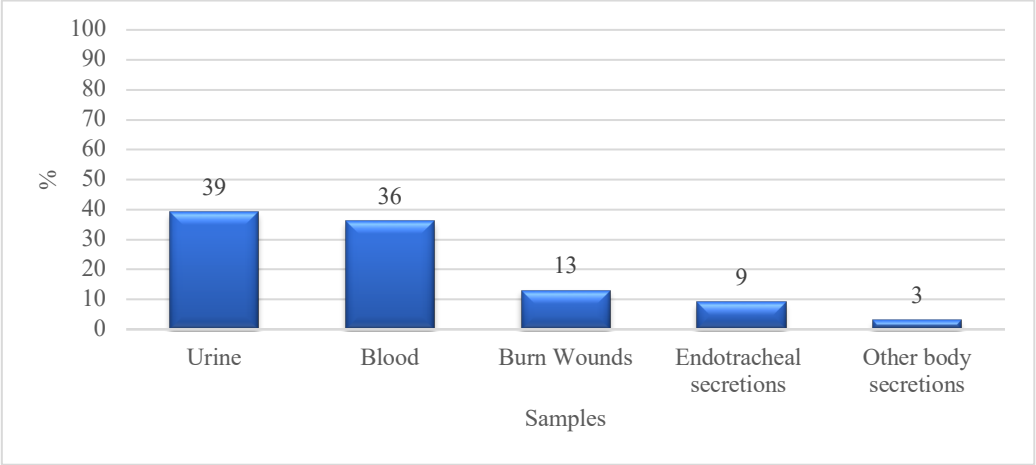

Fig. 1. Distribution of the relative abundance of *P. aeruginosa* isolated from different samples

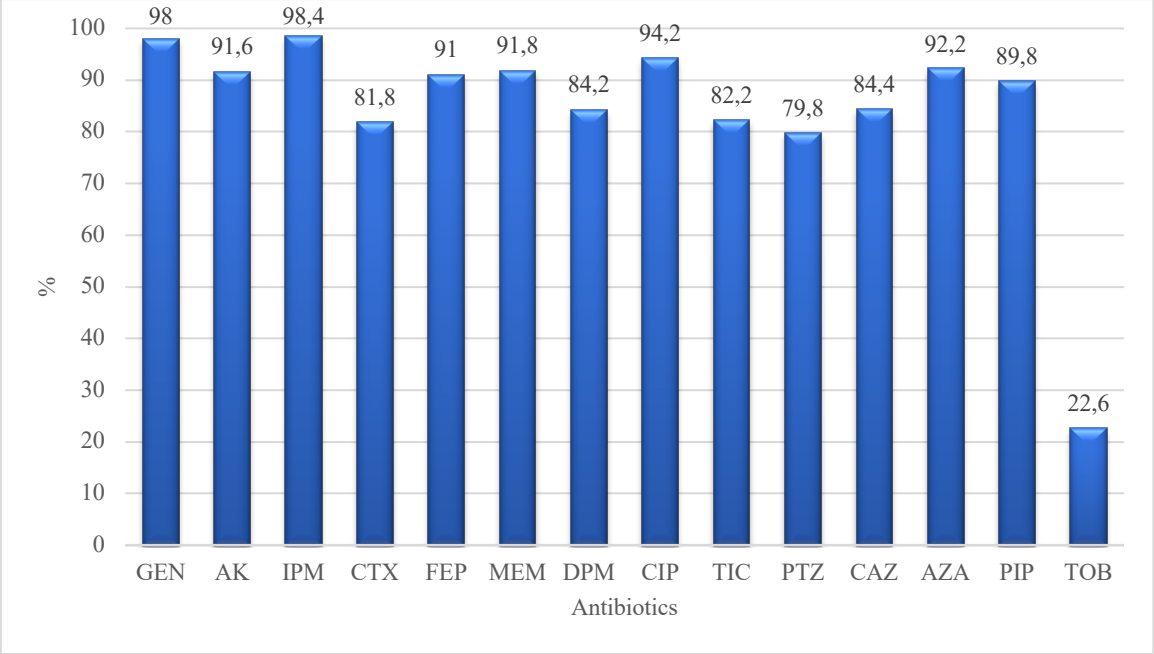

Fig. 2. Characteristics of antibiotic resistance in *P. aeruginosa* isolates

Gentamicin (GEN), amikacin (AK), imipenem (IPM), cefotaxime (CTX), cefepime (FEP), meropenem (MEM), doripenem (DPM), ciprofloxacin (CIP), ticarcillin (TIC), piperacillin/tazobactam (PTZ), ceftazidime (CAZ), aztreonam (AZA), piperacillin (PIP), tobramycin (TOB)

Table 3. Frequency of carbapemase-positive isolates based on clinical samples, and gender

Identified carbapenemase	n	Clinical specimens					Gender	
		Urine (%)	Blood (%)	Burn wounds (%)	Tracheal tube secretion (%)	Other body secretion (%)	Male (%)	Female (%)
***bla*$_{KPC}$**	43 (13.91%)	18 (41.86)	16 (37.21)	5 (11.63)	3 (6.98)	1 (2.32)	29 (67.44)	14 (32.56)
***bla*$_{IMP}$**	68 (22.01%)	27 (39.71)	26 (38.24)	8 (11.76)	6 (8.82)	1 (1.47)	41 (60.29)	27 (39.71)
***bla*$_{OXA-48}$**	121 (39.16%)	51 (42.15)	43 (35.54)	16 (13.22)	8 (6.61)	3 (2.48)	71 (58.68)	50 (41.32)
***bla*$_{SPM}$**	50 (16.18%)	20 (40)	17 (34)	5 (10)	4 (8)	4 (8)	31 (62)	19 (38)
***bla*$_{NDM}$**	36 (11.65%)	12 (33.33)	11 (30.56)	5 (13.89)	3 (8.33)	5 (13.89)	24 (66.67)	12 (33.33)
***bla*$_{VIM}$**	59 (19.09%)	23 (38.98)	22 (37.29)	7 (11.86)	4 (6.78)	3 (5.09)	34 (57.63)	25 (42.37)
***bla*$_{GES}$**	98 (31.72%)	40 (40.82)	33 (33.67)	11 (11.23)	7 (7.14)	7 (7.14)	59 (60.20)	39 (39.80)
***bla*$_{BIC}$**	9 (2.91%)	3 (33.33)	3 (33.33)	2 (22.22)	0 (0)	1 (11.11)	7 (77.78)	2 (22.22)
***bla*$_{AIM}$**	13 (4.21%)	5 (38.46)	5 (38.46)	3 (23.08)	0 (0)	0 (0)	9 (69.23)	4 (30.77)
***bla*$_{GIM}$**	16 (5.18%)	6 (37.5)	3 (18.75)	3 (18.75)	2 (12.5)	2 (12.5)	11 (68.75)	5 (31.25)
***bla*$_{SIM}$**	19 (6.15%)	8 (42.11)	7 (36.84)	3 (15.79)	0 (0)	1 (5.26)	15 (78.95)	4 (21.05)
***bla*$_{DIM}$**	21 (6.80%)	10 (47.62)	7 (33.33)	3 (14.29)	1 (4.76)	0 (0)	11 (52.38)	10 (47.62)

4. Discussion

Carbapenems are among the most important antibiotics used to treat infections caused by *P. aeruginos* (44). The emergence of MBL enzymes, which not only caused resistance to carbapenems but also caused resistance to other antibiotics, including aminoglycosides, created a new problem in treating infected patients (45). In the present study, the frequency of carbapenemase genes and the pattern of antibiotic resistance in *P. aeruginosa* isolates were investigated. In summary, a comparison has been made between the results of this study and similar studies. In a study conducted by Gomes et al. in 2010 to identify MBLs in imipenem-resistant *Pseudomonas*, it was shown that 34.5% of blood samples were resistant to imipenem. Among the samples containing MBL enzymes, 81% were reported to carry the bla_{SPM} gene (46); while this rate is 16.18% in the present study, the reason for this difference can be related to the specific geographical region. In 2008, a study was conducted in Taiwan on MBL enzymes, in which bla_{VIM}, bla_{IMP}, bla_{SIM}, bla_{SPM}, and bla_{GIM} genes were examined, and it was found that bla_{VIM} genes have the highest amount among the isolates (47). In Sader et al.'s study, the prevalence rate of the bla_{SPM} gene was reported to be 55.6%, and the prevalence rate of the bla_{IMP} was 8.3% (48). The frequency of IMP in the present study was high compared to the study conducted in Brazil, this study shows the high prevalence of this gene in this area. In the study by Mihani et al. an isolate carrying the IMP gene was not identified (49), but in our study, the frequency of this gene was 22%. These statistics show the emergence of resistant and MBL-producing bacteria and the treatment pattern dependent on beta-lactam antibiotics in the face of diseases leads to an increase in MBLs -producing bacteria. In Arunagir's study, out of 167 isolates of *P. aeruginosa*, 70.1% of the isolates were MBL producers; among these isolates, 2 (3%) isolates carrying the bla_{IMP} gene were reported (50), which was very low compared to our study. In the current study, this bacterium was also present in hospitalized patients in different departments, so it was more abundant in blood culture and urine culture samples. This indicates the role of *P. aeruginosa* in urinary tract diseases. The results obtained based on the most isolated samples are consistent with the studies of Tavajjohi et al. In this study, the prevalence of *P. aeruginosa* was the highest in blood and urine culture samples (51). In the above analysis, the most samples isolated from urine and blood were 39% and 36%, respectively; while in similar studies conducted in Tehran, Kermanshah, and Arak the most samples were wound infection and then urinary infection (52, 53). Based on the studies conducted in different countries, the rate of resistance to gentamicin in France is 50%, Spain 31%, Russia 96.6%, and America 19.3%. In the current study, the resistance to gentamicin is 98%, which shows the highest resistance compared to previous research (54, 55). Antibiogram results obtained from previous studies conducted in Iran indicate an increase in antibiotic resistance in recent years. During a study conducted by Saderi et al. in Tehran, the percentage of antibiotic resistance to amikacin, gentamicin, ceftazidime, and ciprofloxacin was reported as 73%, 86%, 73%, and 55%, respectively (56). In our study, the rate of resistance to these antibiotics was high, which is probably due to the difference in the way of treatment and prescribed antibiotics. In another study conducted by Foolad in Tehran, the rate of resistance to amikacin, cefotaxime, gentamicin, ciprofloxacin, and ceftazidime antibiotics was 43.6%, 25.5 %,20.9%, and 20.9% respectively (57), which is a significant difference compared to our study. Ceftazidime is another antibiotic investigated in the present study and showed a resistance rate of 84.4%. Shirani et al. (58), and Khosravi et al. (59), showed that *P. aeruginosa* samples have the highest resistance to ceftazidime (78.9% and 81%, respectively). The reason for the difference in these results can be seen in the type of sample, the test location, the time period and the discs used. In this study, 98.4% and 91.8% of the strains were resistant to imipenem and meropenem, respectively. Investigations show that the resistance level to imipenem is also increasing in the last few years due to the release and transfer of MBL coding genes. In 2004, Luzzaro et al. showed that 15% of *P. aeruginosa* strains resisted imipenem (60). This research showed similar results to Luzzaro, but it offered more resistance than other studies, the reason for this could be the type of *P. aeruginosa* samples that were isolated from hospitalized patients in the treatment center, and for this reason, they showed more resistance to imipenem. In 2010, Franco et al. observed that 100% of patients were resistant to imipenem in a study conducted in a Brazilian hospital (61). In Doosti et al.'s research, 55.1% of patients resisted imipenem (62). Antibiotic resistance occurs in most cases due to the indiscriminate and arbitrary use of antibiotics without conducting antibiogram tests and identifying the pathogen. This leads to the emergence of many cases of drug resistance, which in itself causes the failure of treatment and the emergence of many side effects that lead to high treatment costs. Drug resistances to antibiotics in different regions of Iran and the world are different due to genetic changes of the causative strains and differences in the amount of antibiotics used, and differences in the availability of broad-spectrum and new antibiotics (63).

Recent studies show an increase in the prevalence of antibiotic resistance by MBLs and since *P. aeruginosa* is considered as an opportunistic pathogen in hospital environments, resistant isolates should be detected as soon as possible in clinical laboratories in order to provide appropriate treatment for the resulting infections and prevent the spread of resistant strains as much as possible. According to the above results, prevention, control and treatment of infections caused by *P. aeruginosa* is of urgent significance due to the spread of resistant species. In this study, the resistance percentage of *P. aeruginosa* isolates used is high and the role of carbapenamase genes as one of the antibiotic resistance mechanisms is significance due to its presence in the isolates. In addition, the selective pressure caused by the widespread use of antibiotics

leads to the creation of bacteria with multidrug resistance and considering that resistant bacteria can transfer resistance genes to other Gram-negative bacilli, including *Enterobacteriaceae*, therefore, quick identification and tracking of isolates producing MBL enzymes can be considered an important and fundamental step in the treatment of infections caused by resistant isolates.

Ethical Statement

The ethics committee of Islamic Azad University of Tabriz branch approved this study (Number: IR.IAU.TABRIZ.REC.1403.209; Date: 11.09.2022). All ethical considerations have been observed during this research. The collection of isolates was conducted with the full consent of the patients and parents were legally authorized representatives of the minor subjects.

Conflict of interest

The authors declare that they have no competing interests.

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No to declare.

Authors' contributions

Concept: A.J.S, F.M., G.F., Design: A.J.S, F.M., G.F., Data Collection or Processing: A.J.S, F.M., Analysis or Interpretation: A.J.S, F.M., G.F., M.P., H.B.B., Literature Search: A.J.S, F.M., M.P., Writing: A.J.S, F.M., M.P.

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