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Original Article

Detection of *bla*$_{VIM}$ and *bla*$_{IMP}$ Carbapenemase-Producing *Pseudomonas aeruginosa* from Clinical Samples in Iran

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ABSTRACT

Background and Aims: The prevalence of carbapenemase-producing *Pseudomonas aeruginosa* (*P. aeruginosa*) strains has been recently reported worldwide. Therefore, accurate and rapid detection of carbapenemase-producing isolates is essential. So, this study aimed to detect *bla*$_{VIM}$ and *bla*$_{IMP}$ carbapenemase-producing strains using the modified Hodge test (MHT) and reverse transcription-polymerase chain reaction (RT-PCR).

Materials and Methods: In this cross-sectional study, *P. aeruginosa* strains were collected from clinical samples (blood, urine, wound, and other liquids body) in Firoozgar and Shahid Motahari Hospitals in Tehran and Velayat Hospital in Rasht Province, from May to December 2018. After identifying the isolates using the standard microbial tests, carbapenemase-producing strains were isolated by the modified hodge test. After that, the detection of *bla*$_{VIM}$ and *bla*$_{IMP}$ genes was performed by RT-PCR technique.

Results: One hundred *P. aeruginosa* were isolated from different clinical samples. Among these, 74 (74%) isolates were considered as carbapenemase positive using MHT. The frequencies of *bla*$_{VIM}$ and *bla*$_{IMP}$ genes were obtained as 83% and 11%, respectively.

Conclusions: The results of this study indicate a high level of resistance to most of the antibiotics tested and a high prevalence of *bla*$_{VIM}$ gene in *P. aeruginosa* strains.

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Introduction

Pseudomonadsae aeruginosa (*P. aeruginosa*) is a gram-negative, aerobic, rod-shaped bacterium. Moreover, *P. aeruginosa* is an opportunistic pathogen causing many infections such as urinary tract infections (UTI), respiratory infections, wounds, and nosocomial infections in humans [1]. Beta-lactam antibiotics are the first drugs used to treat these infections, among which carbapenems are the most effective ones against *P. aeruginosa* that can be used to treat infections caused by this bacterium. However, unfortunately, we are currently witnessing widespread resistance to various types of beta-lactam antibiotics worldwide [2-4].

The mechanism of bacterial resistance to antibiotics is very diverse. Mechanisms involved in developing resistance to carbapenems are integrin or plasmid-mediated carbapenemases, the reduced porin expression, the increased chromosomal cephalosporins activity, and the increased expression of efflux systems [5]. The common carbapenemases in *P. aeruginosa* are mainly Metallo-β-lactamases of Verona integron-encoded Metallo-β-lactamase, active on imipenem (IMP), São Paulo metalo-beta-lactamase, German imipenemase, Adelaide imipenemase, Dutch imipenemase, and New Delhi metallo-beta-lactamase types. Accordingly, IMP was firstly identified in Japan, and VIM was found in Italy. However, recently, the prevalence of these genes is reported worldwide [6-9].

The presence of carbapenemase genes on plasmids transfers them between different species and bacterial genera [7]. The accurate detection of carbapenemases in bacteria is not easy. Although the Modified Hodge Test (MHT) is a relatively easy and simple phenotypic test, it is not recommended due to its low accuracy and sensitivity. The identification of carbapenemase genes using molecular methods, e.g., polymerase chain reaction (PCR), is usually reliable and accurate; however, it requires specialized knowledge in the epidemiology of carbapenemases [10].

This study aimed to detect bla_{IMP} and bla_{VIM} carbapenemases by MHT and reverse transcription-polymerase chani reaction (RT-PCR) among *P. aeruginosa* isolated in some hospitals in Iran.

Materials and Methods

Sample collection

In this cross-sectional study, *P. aeruginosa* strains were collected from different clinical samples such as burn, blood, wound, and other liquids body in Firoozgar and Shahid Motahari Hospitals in Tehran and Velayat Hospital Rasht Province, from May to December 2018. The initial identification was performed based on conventional bacteriological and biochemical methods such as colony morphology, oxidase, catalase, Indol, Motility, citrate, and triple sugar iron [11].

Antibiotic resistance pattern

The antibiotic susceptibility test was determined using the Kirby-Bauer method in terms of the Clinical Laboratory Standard Institute guidelines (CLSI 2017). The antibiotic discs use as follows: ceftazidime (30 µg), cefotaxime (30 µg), cefepime (30 µg), ceftriaxone (30 µg), imipenem

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(10 µg), meropenem (10 µg), ertapenem (10 µg), gentamicin (10 µg), piperacillin (100 µg), and aztreonam (30 µg) (MAST Company, UK) [12]. *P. aeruginosa* ATCC 27853 was used as the positive control strain in the antimicrobial susceptibility test [13].

MHT for the detection of carbapenemase

The MHT was performed according to CLSI using *E. coli* ATCC 25922 and meropenem at the dose of 10 µg (Oxoid, UK). In order to perform this test, *E. coli* ATCC 25922 *was* cultured on Mueller-Hinton agar, and 0.5 McFarland turbidity of *P. aeruginosa* isolates were then inoculated on the surfaces of plates. Afterward, a disc of meropenem at 10 µg (Oxoid, UK) was placed in the center. After the incubation for 18 hours at 37^{0}C, a positive result was observed by the enhanced growth of the indicator strain towards a meropenem disc, clover leaf-type indentation at the point of intersection of the isolate with the indicator strain [14]. *P. aeruginosa* ATCC 15442 and *P. aeruginosa* ATCC 27853 *were* used as negative and positive controls, respectively.

Detection of *bla*$_{IMP}$ and *bla*$_{VIM}$ genes

According to the manufacturer's guidelines, total RNA isolation was done, and DNA was removed using 20 U of RQ1 *DNase I* (Sinaclon, Iran). After that, cDNA was synthesized by Pars Tous kit and then stored at -20 ºC.

In this study, RT-PCR was used due to time- and cost-effectiveness and less error due to less contamination. *Bla*$_{IMP}$ and *bla*$_{VIM}$ were detected in all the isolates by special primers designed via IDT software and then synthesized by Macrogen Company (Korea) (Table 1). Finally, PCR was performed on the RT reaction product [15]. Positive control isolates were obtained from Rouhi et al., who studied Metallo-beta-lactamase genes in Kurdistan Province, Iran [16]. This research was approved by the Ethics Committee of Islamic Azad University, Qom, Iran.

Statistical analysis

T-test and Pearson's chi-square test were used for data analyses by SPSS version 23.0 (SPSS, Chicago, IL). The level of significance in the current study was considered as <0.05.

Results

One hundred *P. aeruginosa* were isolated in Tehran. These isolates had the following properties: Gram-negative bacilli, oxidase-positive, catalase-positive, Indol negative, motile, citrate positive, and triple sugar iron Alk/Alk. In addition, the specimens included burn (N = 55, 55%), blood (N = 2, 2%), wound (N = 16, 16%), and other liquids body (N = 27, 27%). Resistance rates of all the clinical isolates against the tested antibiotics were as follows: ceftazidime 39%, cefotaxime 51%, cefepime 28%, ceftriaxone 34%,imipenem 46%, meropenem 32%, ertapenem 41%, gentamicin 41%, piperacillin 35%, and aztreonam 30%. Therefore, the highest and lowest resistance rates were related to cefotaxime and cefepime, respectively. The results of the antibiotic resistance pattern of *P. aeruginosa* isolates in the different clinical specimens of this study are shown in Table 2. In this study, those semi-sensitive isolates were considered resistant isolates. To note, the burn samples had the highest resistance to ertapenem and the lowest resistance to piperacillin. Among the wound samples, the highest and lowest rates of antibiotic resistance were related to imipenem

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and ertapenem, respectively. However, among *P. aeruginosa* strains isolated from other body fluids, the highest and lowest resistance rates were related to cefotaxime and gentamicin, respectively.

Table 1. Primer sequences of bla_{IMP} and bla_{VIM} genes and reaction setup for RT-PCR and PCR

Primer sequences	RT-PCR		PCR		
	Conditions	Volume reactions	Conditions	Volume reactions	Fragment size
blaIMP			1cycle	10X PCR Buffer:2.5 µl	
F: 5′-AAA GAT ACT GAA AAG TTA GT-3'	1 cycle	Total RNA	94 ˚C 5 min	10 mM dNTPs: 0.5 µl	
R: 5' -TCY CCA AYT TCA CTR TGA CT-3'	25 ˚C 10min	5µl	30 cycle:	10 mM $MgCl_2$:0.75 µl	391bp
blaVIM	47 ˚C 60min	Random	94 ˚C 30s	10 pmol F+R Primer: 1.25 µl	
F: 5'-CCGATGGTGTTTGGTCGCAT-3´	70 ˚C 10min	hexamer 2µl	58 ˚C 30s	Taq DNA polymerase (5u µl): 0.2 µl	
R: 5´-GAATGCGLAGCACCAGGAT-3´		H_2O up to 10µl	72 ˚C 30s		
			1cycle: 72 ˚C 8 min	Template DNA: 1 µl	446bp
				H_2O up to 25 µl	

Table 2. Antibiotic resistance pattern in the different clinical specimens

Antibiotics	Burn		Blood		Wound		Other liquites	
	Resistance N	Susceptible N	Resistance N	Susceptible N	Resistance N	Susceptible N	Resistance N	Susceptible N
Ceftazidime	23	32	1	1	6	10	9	18
Cefotaxime	20	35	2	0	11	5	18	9
Cefepime	13	42	0	2	5	11	10	17
Ceftriaxone	16	39	1	1	4	12	13	14
Imipenem	18	37	1	1	12	4	15	12
Meropenem	18	37	0	2	5	11	9	18
Ertapenem	29	26	1	1	4	12	7	20
Gentamicin	31	24	0	2	6	10	4	23
Piperacillin	12	43	1	1	5	11	17	10
Aztreonam	8	47	1	1	8	8	13	14

Table 3. Distribution of carbapenemase genes in the different clinical specimens

	Source of sample				
Gene (s)	Burn	Blood	Wound	Other liquids	Total
blaVIM	55	1	16	11	83
blaIMP	7	0	2	2	11
blaVIM/ blaIMP	7	0	2	2	11

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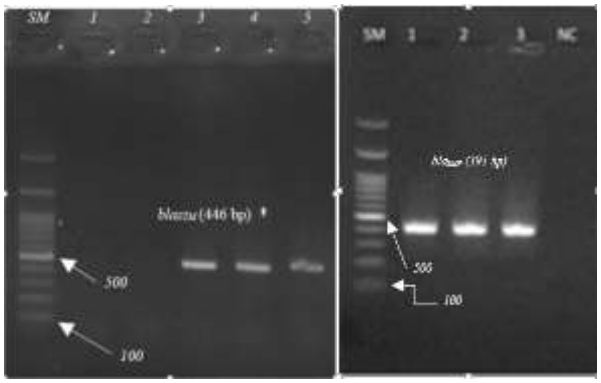
Fig. 1. MHT: All isolates showed a positive result.



A **B**

Fig. 2. Gel electrophoresis shows the PCR products of *bla*$_{IPM}$ and *bla*$_{VIM}$: **A:** Lane SM. DNA Ladder 100 bp, Lane 1. Control negative, lane 2. *bla*$_{VIM}$ negative clinical isolate, lane 3. Control positive of *bla*$_{VIM}$, lane4 and 5. Clinical isolates carrying of *bla*$_{VIM}$, **B:** Lane1. Control positive of *bla*$_{VIM}$, lanes 2 and 3. Clinical isolates carrying of *bla*$_{IMP, lane}$, lane 4 (NC). Control negative

The result of MHT showed that 74 isolates (74%) were positive in this research. In addition, the results of the standard reference MIC tests were in fair agreement with those of the MHT (N = 74) (Figure 1). *Bla*$_{VIM}$ and *bla*$_{IMP}$ genes were detected in 83% and 11% of the samples, respectively (Figure 2). The results of the distribution of carbapenemase genes in the different clinical specimens are shown in Table 3.

Discussion

Recently, we observed the increasing prevalence of high antibiotic resistance in bacteria causing various infections. Since treating these infections is very difficult and has raised concerns such as the reduced treatment choices and the increased costs and mortality, it is currently considered a problem in health centers [17-19]. One of the causes of antibiotic resistance is carbapenemase enzymes, which can be easily transferred to bacteria due to being transported by integrons and then increase resistance. So, identifying these types of resistance and treating these patients as soon as possible can help control the infection [19]. Therefore, it was attempted to isolate these isolates in the studied hospitals using an accurate and reliable molecular technique in this study.

Studying the frequency of antibiotic resistance of the tested isolates showed that the resistance

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of the strains against therapeutic antibiotics is high. The highest resistance rate was found to be related to cefotaxime (51%). Correspondingly, in another study conducted by Bagheri Bejestani et al., the highest resistance rate was detected against cefotaxime (36.6%) [20]. In the current study, the resistance rate to imipenem was 46% using the disc diffusion method, similar to Saberi's study (33 %) in Rasht [21]. However, this rate was higher than those of Bagheri Bejestani's study (15.5%) in Iran [20] and Rouhi's study (19.23%) in Iran [16] and lower than those of Wang's study (77.5%) in China [22], Abaza's study (78.3%) in Egypt [23], and Li's study (73.3%) in China [24]. Differences in the results of various studies may be due to different antibiotic therapy methods. In our study, 74% of the strains were phenotypically carbapenemase positive. In Othman and Shahcheraghi's studies, 28.1% and 54% of the isolates were positive for carbapenemases by MHT, respectively [25, 26]. MHT is an easy, cost-effective, and fast method to identify carbapenemase strains. The accuracy and sensitivity of this test are less than molecular techniques, but due to its high-speed capability, it is recommended to identify and isolate more carbapenemase-producing isolates.

The difference among the frequencies of carbapenemase-producing isolates by the MHT phenotypic method in different studies may be due to regional differences and various patterns of antibiotic use [27].

The result of the RT-PCR method related to bla_{IMP} and bla_{VIM} genes indicated that 83% of *P. aeruginosa* strains harbored the bla_{VIM} gene, and 11% of them carried bla_{IMP} gene. Other studies performed in Portugal and China confirmed 19.4% and 43.5% of bla_{VIM} gene in *P. aeruginosa* strains, respectively [28, 29]. Additionally, the rate of bla_{IMP} gene in the tested isolates was similar to those of the Cheng et al. and Othman et al.'s studies (26, 29). Alkhudhairy in Iraq reported 33.3% and 25% strains carrying bla_{VIM} and bla_{IMP} genes, respectively [30].

Differences in the results of different studies can be due to differences in time, geographical area, and even the test methods used. The pattern of antibiotic use varies at different times and geographical locations, which can affect the prevalence of carbapenemase genes. Besides, the differences in the results related to the frequency of genes can be due to the increasing resistance to cephalosporins of the third generation, thereby more carbapenems consumption in recent years [31].

According to previous reports performed in this regard, *the* bla_{VIM} gene in *P. aeruginosa* strains is more abundant than other Metallo-beta-lactamase genes, which also is the dominant Metallo-beta-lactamase gene in Iran [25, 32].

In the current study, the isolates that did not carry the genes tested, but resulted as positive for carbapenemase using phenotype test, were likely to carry other carbapenemase genes that were not tested due to the cost and time required to perform these tests.

Conclusions

As the bla_{VIM} and bla_{IMP} genes are carried by integrons, which can carry other antibiotic resistance genes, they make it difficult to treat infections resulted from these strains.

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Therefore, the detection of strains carrying these genes can be an effective step in treating these infections in different rejoins.

Consequently, performing phenotypic tests to evaluate antibiotic susceptibility and resistance before antibiotic administration is unavoidable. Moreover, molecularly studying carbapenemase-producing enzyme genes due to their wide variety and prevalence rates in different geographical areas to control this strain is essential.

Conflicts of Interest

There are no conflicts of interest.

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