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ORIGINAL ARTICLE

Study on imipenem resistance and prevalence of blaVIM1 and blaVIM2 metallo-beta lactamases among clinical isolates of *Pseudomonas aeruginosa* from Mashhad, Northeast of Iran

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ABSTRACT

Background and Objectives: The main cause of serious nosocomial infections is a Gram-negative pathogen known as *Pseudomonas aeruginosa* (*P. aeruginosa).* Carbapenems are widely used as an appropriate treatment for these infections, however resistance to these agents has been observed and is increasing. Metallo beta-lactamase (MBLs) enzyme is one of the main causes of resistance to carbapenem. In the current study the frequency and production of VIM1 and VIM2 by imipenem-resistant *P. aeruginosa* isolates of patients hospitalized in Imam Reza hospital were evaluated.

Materials and Methods: In this study, 131 clinical samples were collected from patients hospitalized in Imam Reza hospital in Mashhad during a 15-month period from May 2011 to November 2012. After verification of *P. aeruginosa* isolates, antibiotic resistance patterns of isolates were determined for 14 antibiotics by Kirby-Bauer standard disk diffusion according to the CLSI guidelines. Combined-disk test was used for phenotypic determination of MBLs-producing isolates and after DNA extraction, genotypic determination of VIM1 and VIM2 metallo beta-lactamase genes was carried out using Multiplex-PCR.

Results: Of 63 imipenem-resistant isolates (48.5%), 56 (88.8%) were MBL-producing in phenotypic assessments. Also amongst imipenem-resistant isolates, the frequency of VIM1 and VIM2 genes were 58.7 and 3.17%, respectively.

Conclusion: The results of the current study along with the results of the other conducted studies in Iran in recent years demonstrate that the average resistance to imipenem in *P. aeruginosa* isolates was 51.3% which has increased in comparison with the results in 2006 (32.9%). It was also determined that the frequency of VIM1 gene was more than VIM2 gene. In phenotypic assessment by using CD method, 49.6% of isolates were determined as MBLs-producing. The sensitivity and specificity of this method were verified in comparison with the results of PCR test.

Key words: *Pseudomonas aeruginosa*; Imipenem; Metallobeta-lactamase genes

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INTRODUCTION

Pseudomonas aeruginosa (P. aeruginosa) is responsible for a wide spectrum of nosocomial infections including pneumonia, urinary infections, postoperative bacteremia, and wound infection.

According to several reports published from 1997 to 2003, the prevalence of *P. aeruginosa*-induced nosocomial infections has been increased. *P. aeruginosa* is the second most prevalent pathogen isolated from patients hospitalized in ICU, after *Staphylococcus aureus* (1). Due to the low permeability of the outer membrane of *P. aeruginosa*, it is sensitive to a limited number of antimicrobials. As a result, the infections caused by them could not be easily treated. Resistant strains of *P. aeruginosa* are a great threat to the health of patients (2).

Amongst betalactam anitibiotics which are suitable for the treatment of those infections caused by Gram-negative bacteria carbapenems including imipenem and meropenem are considered as effective antibiotics for the treatment of these infections due to its' broad-spectrum effect and resistance to beta-lactamase enzymes. However, resistance to these antibiotics is increasing (3).

Various mechanisms contribute to drug resistance in *P. aeruginosa* (2). The main mechanism responsible for resistance to imipenem in *P. aeruginosa*, is losing porin D gene in outer membrane of bacterium due to mutations. Mutations in porin D gene following the treatment with imipenem is prevalent and a 25% increase in imipenem resistance has been observed in patients (4, 5).

Another reason for resistance to carbapenem is the production of metallo beta-lactamase enzymes which can be either chromosomal or plasmid mediated. The most prevalent types of these enzymes are IMP, VIM, GIM, and SPM. Amongst metallo beta-lactamase, IMP and VIM are prevalent worldwide. Also MBL genes are located on plasmids which can be transferred to other Gram-negative bacteria (6-8). Therefore, using phenotypic and genotypic methods, which rapidly detect metallo beta-lactamase-producing bacteria, can help physicians to begin the most suitable treatment as soon as possible and to prevent the transfer of resistance to other bacteria. Currently there is no available data regarding the prevalence of MBL-producing *P. aeruginosa* among patients hospitalized in Imam Reza hospital. Therefore, this study was conducted to determine the frequency of VIM1 and VIM2 metallo beta-lactamase produced by imipenem-resistant *P. aeruginosa*.

MATERIALS AND METHODS

In the current study, 131 clinical samples were collected from May 2011 to November 2012 from hospitalized patients at Imam Reza hospital in Mashhad, Iran. *P. aeruginosa* was isolated by using standard microbiological methods including Gram staining, culturing on MacConkey agar medium, oxidase tests, catalase tests, MRVP, SIM, TSI, OF, culturing at 42˚C, and production of green-blue pigments. For further analysis, isolates were inoculated into glycerol-containing BHI culture and were stored at -70 ˚C (9).

Antibiotic resistance pattern of isolates were determined for 14 antibiotics (MAST-UK) using standard Kirby-Bauer Disk Diffusion method. Medium was prepared and sterilized by autoclaving at 121° for 15 min. 25 ml of media was poured in 90 mm sterile Petri dishes and incubated at 37ºC overnight in order to check sterility. Antibiotics used in the current study were as following: ceftriaxone, ciprofloxacin, gentamycin, cefixime, meropenem, cephtazidiom, tetracycline, imipenem, colistin, amikacin, cefepime, carbenicillin, piperacilline, and Tobramycin. Then, the plates were incubated at 37ºC for 24h and finally inhibition zone surrounding the disk were measured and sensitive, intermediate, resistant isolates were assigned according to the CLSI guideline (10).

Phenotypic detection of MBL-producing isolates by using Combined-Disk (CD) method. In this study, combined-disk test was used in order to phenotypically distinguish MBL-producing isolates. First, 18.61 gr of disodium EDTA.2H_2O was dissolved in 100 ml of distilled water. Then its pH was adjusted to 8 by adding Sodium Hydroxide and was then autoclaved to prepare 0.5 M EDTA (Sigma, USA). Microbial suspension with the turbidity of equal to 0.5 McFarland was prepared on Brain Heart Infusion (BHI) medium and then the suspension was cultured on Muller Hinton agar medium after incubation for 20 minutes. After that, an imipenem disk and an imipenem disk containing 10µl of 0.5 M EDTA was located on the plate with 2 cm distance from each other and were incubated for 16-18 h at 35 ˚C. More than 7mm increase in the diameter of inhibition zone around the EDTA-containing imipenem disk in comparison with imipenem disk demonstrated the production of MBL by bacterium (11, 12).

P. aeruginosa ATCC27853 was used as a control

during antibiogram and production of MBL tests.

Determining MBL gene by using Multiplex PCR. DNA of bacterial isolates were extracted by simple boiling method in order to perform genotypic tests (13). VIM1 and VIM2 primers were designed in accordance with nucleotide sequence mentioned in Table 1. Optimized PCR was performed in order to determine bla_{VIM1} (261 bp) and bla_{VIM2} (798 bp) genes. The mixture of the main solution with the volume of 20 µl was as following: 10X PCR buffer, 0.4 µl dNTP, 1.25 µl Magnesium Chloride (50Mm), 0.2 µl Taq DNA polymerase, 0.8 µl of each 10 pM primers, 1 µl of DNA template, and 13.55 µl distilled water. PCR program was performed for 35 cycles which was as following: The first step was denaturation for 5 min at 94 ˚C, then 35 cycles including 30 sec at 94 ˚C, 40 sec at 55 ˚C, and 50 seconds at 72 ˚C, and final elongation step for 5 min at 72 ˚C.

PCR products were assessed by performing electrophoresis on 1.5% agarose gel in TBE buffer. Finally, gels were stained with Ethidium Bromide and UV light was used to visualize PCR products.

Table 1. Nucleotide sequences of primers blaVIM1 and blaVIM2

Primers	(Nucleotide) sequence
VIM1-F	5̓ -AGTGGTGAGTATCCGACAG-3̓
VIM1-R	5̓ -ATGAAAGTGCGTGGAGAC-3̓
VIM2-F	5̓ -ATGTTCAAACTTTTGAGTAAG-3̓
VIM2-R	5̓ -CTCAACGACTGAGCGATTG-3̓

RESULTS

Of 131 patients hospitalized from whom *P. aeruginosa* isolates were cultured in Imam Reza hospital, 46.6% (n= 61) and 53.4% (n= 70) were male and female, respectively. Age distribution of patients is given in diagram (Fig. 1). Also the frequency of patients in different wards are listed in Table 2. The majority of patients were hospitalized in the burn ward.

Results of drug susceptibility testing of isolates is demonstrated in Table 3.

In phenotypic assessment of MBL production, 49.6 % (n= 65) of isolates were MBL-producing. In genotypic assessment performed by PCR, 55% (n= 72) of isolates were determined as MBL-producing isolates and frequency distribution of VIM1 and VIM2 genes are shown in Table 4.

Increase in diameter of inhibitory zone (≥7mm) in EDTA-containing imipenem disks demonstrates the production of MBL by the bacterium. Of 63 (48.5%) imipenem-resistant strains, 56 (88.8%) strains were MBL-producing in phenotypic assessments. Furthermore, amongst imipenem-resistant strains, the presence of VIM1 and VIM 2 were 58.7 and 3.17%, respectively.

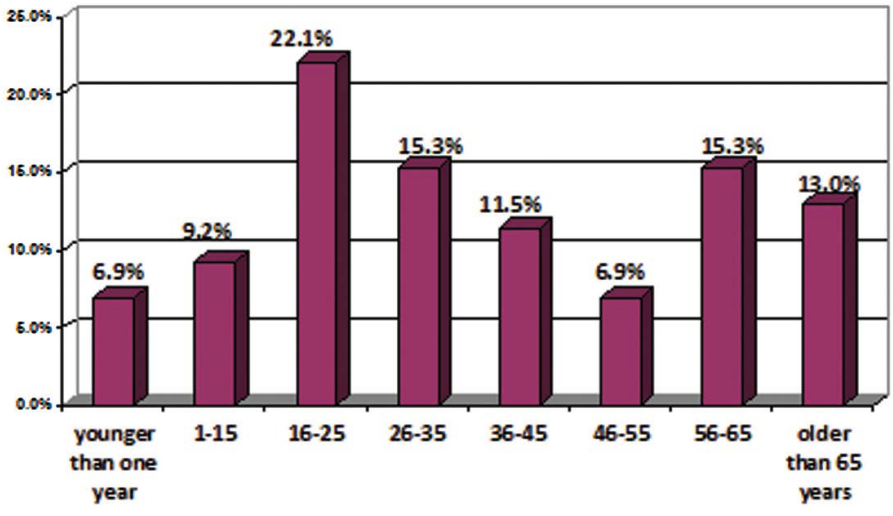

Fig.1. Age distribution of 131 hospitalized patients of Imam Reza hospital, Mashhad, Iran

Table 2. Prevalence of distribution of samples from patients of different wards of Imam Reza hospital, Mashhad, Iran

		Sample					Total
		Urine	Bronchi	Wound	Other	Blood culture	
ICU	Number	1	12	4	8	1	26
	Percentage	3.8%	46.2%	15.4%	30.8%	3.8%	100.0%
Other Ward	Number	3	2	3	7	9	24
	Percentage	12.5%	8.3%	12.5%	29.2%	37.5%	100.0%
Iutera Disease	Number	3	1	5	7	4	20
	Percentage	15.0%	5.0%	25.0%	35.0%	20.0%	100.0%
Burn	Number	1	0	57	0	3	61
	Percentage	1.6%	0.0%	93.4%	0.0%	4.9%	100.0%
Total	Number	8	15	69	22	17	131
	Percentage	6.1%	11.5%	52.7%	16.8%	13.0%	100.0%

Table 3. Results of susceptibility testing on isolates cultured from patients with nosocomial infection at Imam Reza Hospital

Antibiotic	Sensitive		Semi-sensitive		Resistance		Total	
	Number	%	Number	%	Number	%	Number	%
Amikacin	55	51.4%	16	15.0%	36	33.6%	107	100.0%
Ceftriaxon	14	23.7%	7	11.9%	38	64.4%	59	100.0%
Cephtazideme	22	22.2%	15	15.2%	62	62.6%	99	100.0%
Cefepime	16	13.4%	11	9.2%	92	77.3%	119	100.0%
Cefixime	3	4.4%	4	5.9%	61	89.7%	68	100.0%
Ciprofloxacin	79	63.2%	7	5.6%	39	31.2%	125	100.0%
Gentamicin	47	37.0%	11	8.7%	69	54.3%	127	100.0%
Meropenem	7	8.1%	3	3.5%	76	88.4%	86	100.0%
Piperacillin	55	47.8%	13	11.3%	47	40.9%	115	100.0%
Tetracyclin	9	22.5%	5	12.5%	26	65.0%	40	100.0%
Imipenem	61	46.9%	6	4.6%	63	48.5%	130	100.0%
Carbenicillin	1	25.0%	0	.0%	3	75.0%	4	100.0%
Tobramycin	0	.0%	0	.0%	2	100.0%	2	100.0%
Colistin	61	78.2%	17	21.8%	0	.0%	78	100.0%

Table 4. Distribution of metallo-beta-lactamase genes VIM1 and VIM2 among isolated *P. aeruginosa* samples of hospitalized patients of Imam Reza hospital in Mashhad, Iran

VIM	Number	Percentage
VIM1(-) ---- VIM2(-)	59	45.0%
VIM1(-) ---- VIM2(+)	2	1.5%
VIM1(+) ---- VIM2(-)	64	48.9%
VIM1(+) ---- VIM2(+)	6	4.6%
Total	131	100.0%

DISCUSSION

Infection with Gram-negative bacteria is one of the main causes of nosocomial infections. *P. aeruginosa* is the most noticeable causes of these infections which contribute to high rates of mortality rate due to its resistance to the broad spectrum antibiotics. Since metallo beta-lactamase (MBL) is one of the most important beta lactamase-hydrolyzing enzymes and could be easily transferred to other bacteria, using appropriate methods to rapid determination of MBL-producing bacteria will help physicians to choose the most appropriate treatment and hence to prevent the transmission of resistance to other bacteria (14).

In the current study, 48.5% of isolates were resistant to imipenem with 88.8% being MBL-producing . In comparison of these results with the study of Khosravi et al., the rate of resistance to imipenem was relatively similar (48.5% in the city of Mashhad and 41% in the city of Ahwaz in Iran) but MBL-producing strains in Mashhad was significantly higher than that of Ahvaz (in the city of Mashhad: 88.8%, and in the city of Ahwaz in Iran: 19.5%) indicating that the production of MBL is one of the main mechanisms for resistance to imipenem in *P. aeruginosa* strains isolated from patients in Mashhad (15).

In a study by Saderi *et al*., in Tehran, among 100 isolates of *P. aeruginosa*, 69 (69%) were resistant to imipenem, of which, 65 (94.2%) had metallo beta lactamase activity (determined by phenotypic detection of metallo beta lactamase production). Using PCR, 13 (18.84%) of isolates had VIM2 gene and none of them had VIM1 gene (11). Compared to our results, a similar frequency of imipenem resistance was observed for *P. aeruginosa*. However, metallo beta lactamase production in their study was lower than that of the current study. In the present study, VIM1 gene was observed in 58.7% of isolates, however, that of Sadri *et al* ., did not find the presence of this gene in any of the isolates. In a study by Euh-jee Oh in South Korea in 2003 on 99 isolates of *P. aeruginosa,* CD phenotypic test was shown to have a high specificity and sensitivity for detection of metallo beta lactamase production. Using PCR, VIM2 gene was detected in 29 (29.3%) of isolates. This study showed a higher frequency of VIM2 than that of ours (16).

The results of the current study along with results of other conducted studies in Iran suggest that the mean resistance to imipenem in *P. aeruginosa* strains isolated from patients is 51.3% which has increased in comparison with 2006 which was reported to be 32.9% (9, 13, 15, 17).

Ting-ting Qu *et al*. performed a study in 2009 to evaluate phonotypic methods of *P. aeruginosa* isolates capable of producing metallobeta Lactamase. Three phenotypic methods of Etest, DDST and CD were used to detect metallobeta lactamase production among 264 isolates of imipenem-resistant strains. Of which, 24 (9.1%) showed positive results. This was confirmed by PCR and DNA sequencing procedures. The study showed that CD was the most suitable phenotypic test for detection of *P. aeruginosa* isolates capable of producing metallobeta lactamase (18).

In phenotypic assessment with CD method, 49.6% of samples were MBL-producing. The sensitivity and specificity of this method were verified in comparison with the results of PCR method in Table 1.

Results of current study suggests increase in MBL-producing *P. aeruginosa* isolates and also increase in drug resistance among these strains. Therefore, prescribing appropriate antibiotics and also quick determination of MBL-producing strains are vital in order to prevent the transfer of drug resistance to other bacteria. According to comparison of CD phenotypic test and PCR in evaluation of MBL-producing strains, CD phenotypic test could be used as a simple and cost-effective method in laboratory experiments. Molecular typing of these strains based on MBL genes is one of the aims of this similar projects in future.

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