

Evaluation of Metallo- β -Lactamase-Production and Carriage of *bla*-VIM Genes in *Pseudomonas aeruginosa* Isolated from Burn Wound Infections in Isfahan

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Abstract

Background: Metallo- β -lactamase-production among Gram-negative bacteria, including *Pseudomonas aeruginosa*, has become a challenge for treatment of infections due to these resistant bacteria.

Objectives: The aim of the current study was to evaluate the metallo- β -lactamase-production and carriage of *bla*-VIM genes among carbapenem-resistant *P. aeruginosa* isolated from burn wound infections.

Patients and Methods: A cross-sectional study was conducted from September 2014 to July 2015. One hundred and fifty *P. aeruginosa* isolates were recovered from 600 patients with burn wound infections treated at Imam-Musa-Kazem Hospital in Isfahan city, Iran. Carbapenem-resistant *P. aeruginosa* isolates were screened by disk diffusion using CLSI guidelines. Metallo- β -lactamase-producing *P. aeruginosa* isolates were identified using an imipenem-EDTA double disk synergy test (EDTA-IMP DDST). For detection of MBL genes including *bla*-VIM₁ and *bla*-VIM₂, polymerase chain reaction (PCR) methods and sequencing were used.

Results: Among the 150 *P. aeruginosa* isolates, 144 (96%) were resistant to imipenem by the disk diffusion method, all of which were identified as metallo- β -lactamase-producing *P. aeruginosa* isolates by EDTA-IMP DDST. Twenty-seven (18%) and 8 (5.5%) MBL-producing *P. aeruginosa* isolates harbored *bla*-VIM₁ and *bla*-VIM₂ genes, respectively.

Conclusions: Our findings showed a high occurrence of metallo- β -lactamase production among *P. aeruginosa* isolates in burn patient infections in our region. Also, there are *P. aeruginosa* isolates carrying the *bla*-VIM₁ and *bla*-VIM₂ genes in Isfahan province.

Keywords: Burn Patients, Metallo- β -Lactamase, *bla*-VIM₁, *bla*-VIM₂, *Pseudomonas aeruginosa*

1. Background

Burn wounds are major public health problems all over the world. Infection is one of the most complicated issues in burn patients, because the skin, a barrier against microbes, has been destroyed and the immunity agents cannot reach the sites of infection. There is a correlation between the severity of infection and the extent of the burn (1). It has been estimated that about 75% of burn-related mortality involves infections (2, 3). The rate of nosocomial infections among patients hospitalized in burn units is high, and *P. aeruginosa* has been documented as the most prevalent isolated bacteria in these patients (4, 5). *Pseudomonas aeruginosa* is characterized by an innate resistance to multiple antimicrobial agent as well as by acquired multidrug resistance ability. Due to multi-drug resistant *P. aeruginosa* isolates, treating these patients' burn wound infections is challenging (6). Carbapenems are a group of β -lactams that show resistance to hydrolysis by extended-spectrum β -lactamases (ESBLs), so are used as the drug of choice for treatment of infections caused by ESBL-positive Gram negative bacteria (7). Broad spectrum activity as well as these abilities have led to increased used

of carbapenems, especially imipenem, for treatment of nosocomial infections (8). Unfortunately, the emergence of metallo- β -lactamase (MBL), the enzyme that inactivates carbapenems, has prevented the use of these antibiotics (9). In a study conducted at Ghotbeddin Burn hospital in Shiraz, 55 (20.4%) MBL-producing *P. aeruginosa* were identified among 270 *P. aeruginosa* isolated patients hospitalized in the burn unit (1). MBLs are categorized as Ambler class B (10). These β -lactamase groups of enzymes have resistance to all β -lactamase inhibitors, and due to the presence of zinc ion in their active sites, are inactivated by chelating agents such as ethylene diamine tetra acetic acid (EDTA) (9). Metallo- β -lactamase-production is encoded by either chromosomal genes or genes are inserted in mobile genetic elements, which especially facilitate the spread of these genes among Gram negative bacteria (9). Recently, several new MBL codes such as VIM have been documented in *P. aeruginosa* isolates (7). The three main clusters of VIM MBL have been reported, including VIM-1, VIM-2, and VIM-7 (7), and the VIM type metallo- β -lactamase genes are shown to encode by inserted cassettes in the mobile integrons (7, 11). The rate of the infections caused by *P. aeruginosa* in the burn unit of Imam-Musa-Kazem hospital in Isfahan was

high; therefore rapid identification of MBL-producing *P. aeruginosa* strains is important.

2. Objectives

In the present study we determined the metallo- β -lactamase-production and carriage of *bla*_{VIM} genes among carbapenem-resistant *P. aeruginosa* isolates in burn patients in Isfahan Province.

3. Patients and Methods

3.1. Subjects

This cross-sectional study was conducted on 600 patients, who were hospitalized in the burn unit of Imam-Musa-Kazem university Hospital in Isfahan between September 2014 and July 2015.

3.2. Bacterial strains

For sampling, wound swabs were obtained from the burn patients and *P. aeruginosa* was identified by standard microbiological methods including Gram stains, culture on mediums such as blood agar, MacConkey agar, triple sugar iron agar (TSI), cetrinide agar (Merck, UK), oxidase (Sigma, USA), catalase, oxidative/fermentative (OF), indole, methyl red (MR), Voges-Proskauer (VP) tests (Merck, UK), and growth at 42°C.

3.3. Screening of Carbapenem-Resistant and MBL Producing *Pseudomonas aeruginosa* Isolates

The disk diffusion method was used according to the clinical and laboratory standards institute (CLSI) guidelines for detection of carbapenem-resistant *P. aeruginosa* isolates, and the imipenem (10 μ g) and meropenem (10 μ g) disks were purchased from Mast, UK Company (12). A *P. aeruginosa* standard strain (ATCC 27853) was used for quality control in susceptibility testing. Phenotypic screening for MBL producers *P. aeruginosa* isolates was performed using an EDTA double disk synergy test (EDTA-IMP DDST) in which imipenem resistant isolates selected by the disk diffusion method were examined. Briefly, a colony of each *P. aeruginosa* strain was suspended in Mueller-Hinton (MH) broth to 106 CFU/mL. after being spread on an MH agar plate with a cotton swab. Two commercial disks containing 10 μ g imipenem with (10 μ g/750 μ g) and without EDTA were placed on the center of the plate at a distance of 25 mm from each other, and were incubated at 37°C overnight. After overnight incubation, the presence of a ≥ 7 mm synergistic inhibition zone around the IMP-EDTA disk in comparison to the IMP disk was interpreted as positive (13).

3.4. Polymerase Chain Reaction Detection of Genes for MBLs

DNA from each *P. aeruginosa* isolate was extracted by the boiling method. Templates of the DNA were stored at -20°C until PCR amplification was performed. MBL-producing *P. aeruginosa* strains which were identified by a DDST confirmatory test were amplified by the PCR method, using *bla*_{VIM-1} and *bla*_{VIM-2} specific primers (Table 1) for amplification of 261 bp and 801 bp fragments, respectively (14).

Reactions were carried out using a thermo cycler (Eppendorf master cycler®, MA) in the PCR conditions previously described (14). For confirmation, some PCR products were sent elsewhere for sequencing (Bioneer, South Korea). The nucleotide sequences were analyzed by the program Chromas Pro version1.7.5 Technelysium (www.technelysium.com.au), and were aligned with GenBank to check the identity of each sequence against those deposited in GenBank.

3.5. Ethical Considerations

Each patient's demographic information was obtained by a survey questionnaire, and a written informed consent was received from all patients or their parents. The study design, including its ethical aspects, was reviewed and approved by the ethics committee of Kashan University of Medical Sciences (No. 4380).

3.6. Statistical Analysis

The statistical analysis of data was conducted using SPSS software version 16 (SPSS, Inc.). Differences by the χ^2 test were considered statistically significant if the P value was less than 0.05 ($P < 0.05$).

4. Results

Of 600 burn patients, who had been hospitalized in the burn unit of Imam-Musa-Kazem university hospital in Isfahan, a total of 150 *P. aeruginosa* isolates were recovered. The patients with isolates included 69 (46.0%) females and 81 (54.0%) males, and their ages ranged between 1 and 72 years old (Table 2). Of the 150 *P. aeruginosa* isolates identified by standard biochemical tests, 144 (96.0%) and 144 (96.0%) showed resistance to imipenem and meropenem, respectively, by the disk diffusion method. An EDTA-IMP DDST phenotypic confirmatory test revealed that 100% of the imipenem resistant *P. aeruginosa* isolates were metallo- β -lactamase producing (Figure 1).

The finding of the PCR assays showed that of 144 MBL-producing *P. aeruginosa* isolates, 27 (18%) and 8 (5.5%) were positive for the *bla*_{VIM-1} and *bla*_{VIM-2} genes, respectively (Figure 2). The nucleotide sequences of the PCR products of the *bla*_{VIM-1} and *bla*_{VIM-2} genes were identical to those deposited in GenBank.

Table 1. Primers Used for Polymerase Chain Reaction and Sequencing

Primer	Target Gene	Sequence (5' → 3')	Amplification Product, bp
VIM-1 F	<i>bla_{VIM-1}</i>	AGT GGT GAG TAT CCG ACA G	261
VIM-1 R	<i>bla_{VIM-1}</i>	ATG AAA GTG CGT GGA GAC	
VIM-2 F	<i>bla_{VIM-2}</i>	ATG TTC AAA CTT TTG AGT AAG	801
VIM-2 R	<i>bla_{VIM-2}</i>	CTA CTC AAC GAC TGA GCG	

Table 2. Association Between Burn Patients' Demographic Characteristics and *bla_{VIM}* Genes Carriage Among *Pseudomonas aeruginosa* Isolates

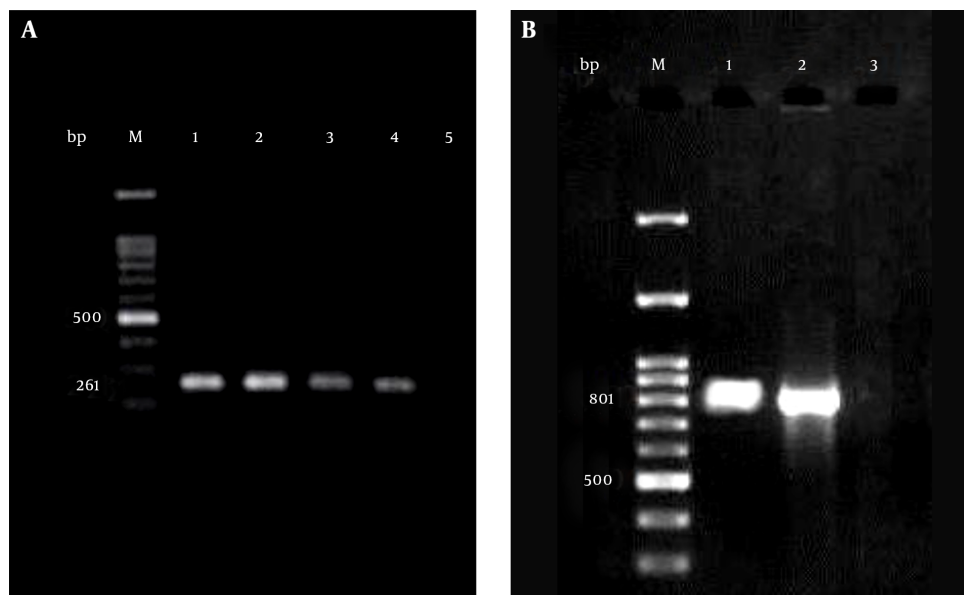
Factor	<i>bla_{VIM-1}</i>		P Value	<i>bla_{VIM-2}</i>		P Value	Total, No. (%)
	Positive, No. (%)	Negative, No. (%)		Positive, No. (%)	Negative, No. (%)		
Sex							
Male	20 (13.3)	61 (40.7)	0.374	4 (2.7)	77 (51.3)	0.449	81 (54.0)
Female	7 (4.7)	62 (41.3)		4 (2.7)	65 (43.3)		69 (46.0)
Age group, y							
Less than 30	5 (3.3)	25 (16.7)	0.812	1 (0.7)	29 (19.3)	1	30 (20.0)
30 - 49	21 (14.0)	90 (60.0)		3 (2.0)	108 (72.0)		111 (74.0)
More than 50	1 (0.7)	8 (5.3)		4 (2.7)	5 (3.3)		9 (6.0)
Burn level (38.85 ± 7.58)							
Equal and less than 35	7 (4.7)	24 (16.0)	0.041	3 (2.0)	28 (18.7)	0.032	31 (20.7)
More than 35	20 (13.3)	99 (66.0)		5 (3.3)	114 (76.0)		119 (79.3)
Burn cause							
Fire and flame	13 (8.7)	49 (32.6)	0.161	4 (2.7)	58 (38.6)	0.212	62 (41.3)
Chemical	5 (3.3)	14 (9.4)		0	19 (12.7)		19 (12.7)
Boiled water and hot liquids	9 (6.0)	60 (40.0)		4 (2.7)	65 (43.3)		69 (46.0)

**Figure 1.** Metallo- β -Lactamase Producing *Pseudomonas aeruginosa* Isolates Identified By a Imipenem-EDTA Double Disk Synergy Test (EDTA-IMP DDST) Phenotypic Confirmatory Test

5. Discussion

Pseudomonas aeruginosa is documented as one of the most frequently isolated bacteria in nosocomial infec-

tions, especially in burn units (3). Severe antimicrobial resistance in this bacterium has complicated the treatment of infections caused by drug-resistant *P. aeruginosa* strains among burn patients (15). Recently, the increase of carbapenem resistance in *P. aeruginosa* has been documented all over the world due to metallo- β -lactamase production, including the VIM type (16). This study revealed that the resistance rates to imipenem and meropenem were very high (96%). Farajzadeh Sheikh et al. (15) reported an imipenem resistance rate of 58.7% among *P. aeruginosa* isolates in Ahwaz, which was lower than our resistance rate. However, Anvarinejad and colleagues' (1) study, also conducted in Ahwaz, was in agreement with our study: 98.1% of MBL-producing *P. aeruginosa* strains, which were isolated from hospitalized patients in a burn unit showed resistance to imipenem. Another report, in contrast with our study, found a resistance rate of 21% to imipenem among *P. aeruginosa* samples from burn patients in Kurdistan (3). As carbapenems, including imipenem, are the

Figure 2. Amplification of *bla*_{VIM-1} and *bla*_{VIM-2} Genes in Imipenem Resistant MBL Positive *Pseudomonas aeruginosa* Isolates

M lanes, 100-bp DNA ladder as the molecular size marker; A, lane 1, positive control; lanes 2 - 4, *bla*_{VIM-1} positive *Pseudomonas aeruginosa* isolates; lane 5, negative control; B, lane 1, positive control; lane 2, *bla*_{VIM-2} positive *Pseudomonas aeruginosa* isolate; lane 3, negative control.

drugs of choice for treatment of infections caused by MDR-*P. aeruginosa* strains, this result in particularly alarming for burn patients with immune-compromised defenses. The high resistance rates to carbapenems may be relatively imprecise because they were not confirmed by confirmatory tests such as an E-test. The difference in resistance rates to imipenem in *P. aeruginosa* isolates from burn patients in Ahwaz, Isfahan, and Kurdistan could be due to frequent use of this antibiotic and the occurrence of high selective pressure in Ahwaz and Isfahan, and fewer patients' access to antibiotics due to economic problems in Kurdistan.

MBL-producing *P. aeruginosa* have been isolated from hospitalized patients increasingly, and isolates that produce these families of enzymes have been documented as one cause of prolonged nosocomial infections (17). Here we showed that all imipenem-resistant *P. aeruginosa* isolates had the ability of MBL-production. In Kurdistan and Ahwaz 22% and 19.5% of *P. aeruginosa* isolates among burn patients were identified as MBL-producing isolates (3). In a study conducted by Farajzadeh Sheikh et al. (15) in Ahwaz among *P. aeruginosa* isolates in burn and non-burn patients, all of the imipenem-resistant isolates were MBL E-test positive, which correlates with our results, although it would be better if the MBL-production among our imipenem resistant *P. aeruginosa* isolates were confirmed by an MBL E-test as well. The EDTA-IMP DDST test and sodium mercaptoacetic acid (SMA)-IMP DDST revealed

that 98.1% of imipenem-resistant *P. aeruginosa* and 78.9% of *P. aeruginosa* isolates were positive for production of metallo- β -lactamase enzymes in Brazil and Japan, respectively (8, 14). This finding shows that the prevalence of carbapenemases among nosocomial isolates of *P. aeruginosa* is high, especially in burn units.

It has been estimated that more than 40% of imipenem resistance in *P. aeruginosa* (IRPA) is due to MBL production, although enzyme types vary in different regions (18, 19). In the present study, 18% and 5.5% of MBL positive isolates were VIM-1 and VIM-2 MBL types, respectively. Similar to our finding, 19.3% of MBL-producing *P. aeruginosa* isolates in burn patients in Ahwaz were positive for *bla*_{VIM} genes (7). In a study in Brazil, 19% of IRPA isolates which were MBL-positive were shown to carry *bla*_{VIM-2}, whereas *bla*_{VIM-1} was not detected among them (8). The fact that the *bla*_{VIM-2} gene was detected in 48.8% of IRPA clinical isolates in Romania (20) shows that MBL type enzymes can vary geographically. Another interesting finding was that the remaining 109 IRPA isolates, which were MBL positive by a phenotypic test, proved to be negative for *bla*_{VIM-1} and *bla*_{VIM-2} genes. The presence of other types of MBL genes could be the reason.

Overall, the findings of the current study showed that the resistance rate to carbapenems, as the last-resort therapy in infections due to MDR *P. aeruginosa* strains among burn patients, is high. Therefore, in these patients a reg-

ular surveillance program for controlling infection and in vitro testing of antibiotics before empiric treatment could be helpful to control MDR-*Pseudomonas aeruginosa* infections in the burn units. Also, a high occurrence of MBL production among imipenem-resistant *P. aeruginosa* isolates in burn patients was seen. Further studies using more accurate confirmatory tests in our region are recommended.

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Footnote

Authors' Contributions: Farzaneh Firoozeh contributed to the study design, study management, supervision, and prepared and wrote the manuscript. Mohammad Pourbabaee performed the sampling, processing, and conventional and molecular procedures. Mohammad Zibaei provided advice, and read and arranged the final manuscript. Mahmood Saffari provided advice.

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