

Identification and Characterization of Metallo- β -Lactamases Producing *Pseudomonas aeruginosa* Clinical Isolates in University Hospital from Zanzan Province, Iran

Masoumeh Doosti¹, Ali Ramazani^{*2} and Maryam Garshabi³

¹Dept. of Microbiology, Faculty of Basic Sciences, Sciences and Research Branch, Islamic Azad University, Markazi, Iran; ²Dept. of Biotechnology, School of Pharmacy, Zanzan University of Medical Sciences, Zanzan, Iran; ³Dept. of Microbiology, Faculty of Basic Sciences, Islamic Azad University, Zanzan, Iran

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ABSTRACT

Background: Infectious by *Pseudomonas aeruginosa* has spread worldwide and metallo-beta-lactamases (MBL) are being reported with increasing frequency. The aim of this study was to investigate the antibiotic susceptibility and distribution of *bla*_{VIM} and *bla*_{IMP} genes in *P. aeruginosa* isolates from Zanzan Province of Iran. **Methods:** A total of 70 *P. aeruginosa* isolates were identified from patients admitted at intensive care units. The antimicrobial susceptibility was tested by disk diffusion (Kirby-Bauer) method and for production of MBL using double-disk synergy test (DDST). After DNA extraction, the presence of *bla*_{VIM} and *bla*_{IMP} genes and class 1 integron were detected by PCR. **RESULTS:** Most of the isolates were resistant to meropenem, cefotaxime and imipenem (IPM). Also, 44/70 (62.85%) IPM resistant isolates were confirmed by DDST. Of the 44 clinical isolates, 41 (93%) isolates showed MIC_{≥4} μg/ml for IPM. Based on the DDST results, 36 (87.8%) were confirmed to be MBL producers. PCR amplification showed that 23/41 (56%) carried *bla*_{VIM} and 10/41 (24.3%) possessed *bla*_{IMP} gene. Also, 31/44 (70.5%) isolates contained class 1 integron gene. **Conclusion:** Our results highlight that the genes for Verona integron-encoded metallo- β -lactamase, IPM β -lactamases and class 1 integrons were predominantly present among the IPM-resistant *P. aeruginosa* tested in our province and also the frequency of *bla*_{VIM} type is higher than *bla*_{IMP}. This is the first report of *P. aeruginosa* strains producing *bla*_{IMP} with high frequency from Zanzan province of Iran. *Iran. Biomed. J.* 17 (3): 129-133, 2013

Keywords: *Pseudomonas aeruginosa*, Beta-lactamases, PCR

INTRODUCTION

Pseudomonas aeruginosa is a non-fermenting Gram-negative rod of great clinical and epidemiological relevance in hospital-acquired infections. This bacterium is more frequently found in intensive care units and also in patients with cystic fibrosis, cancer, surgical wounds, trauma and severe burns [1]. There is an increase in occurrence of *P. aeruginosa* strains with resistance to multiple antibiotics worldwide [2].

Several mechanisms are involved in *P. aeruginosa* resistance to antimicrobial agents, such as chromosomal expression of resistance encoding genes, β -lactamase production, efflux pumps and decrease in membrane permeability [1]. One of the mechanisms of resistance to carbapenem antibiotics in *P. aeruginosa* is metallo- β -lactamases (MBL) production that hydrolyzes all carbapenems. The prevalence of carbapenem

resistance mediated by acquired MBL including imipenem (IPM) and Verona integron-encoded metallo- β -lactamase (VIM), are increasing from different parts of the world [3-6].

MBL genes are normally encoded in class 1 integrons along with other resistance determinants, such as the aminoglycoside-modifying enzymes. The integrons are frequently located in plasmids or transposons, which contributes to the global spread of this resistance mechanism [7]. Different types of MBL are known in *P. aeruginosa*, including VIM, IMP, German imipenemase, Sao Paulo metallo- β -lactamase, Seoul imipenemase, New Delhi metallo- β -lactamase and Adelaide imipenemase 1 [8-10]. The most common and widespread acquired MBL are those of the IMP and VIM types, which exhibit a worldwide distribution and for which several allelic variants are known.

*Corresponding Author; Tel.: (+98-241) 427 3636; Fax: (+98-241) 427 3639; E-mail: ramazania@zums.ac.ir

Table 1. PCR programs for amplification of target genes

Target gene	First denaturation	Extension	Cycle: 30 annealing	Denaturation	Final extension	Reference
<i>bla_{VIM}</i>	95°C 5 min	72°C 7 min	72°C 1 min	58°C 1 min	95°C 1 min	[11]
<i>bla_{IMP}</i>	95°C 5 min	72°C 10 min	72°C 1 min	54°C 40 s	95°C 1 min	[11]
<i>bla_{IMP-1}</i>	94°C 5 min	72°C 7 min	72°C 45 s	57°C 40 s	94°C 1 min	[11]
<i>Int1*</i>	94°C 5 min	72°C 7 min	72°C 2 min	52°C 1 min	94°C 1 min	[18]

*Class 1 integrons

Several previous studies from different parts of Iran showed a high frequency of MBL producing *P. aeruginosa* from different Hospital units [11-13]. The phenotypical and genotypical characterization of these isolates would be helpful for understanding the resistance mechanisms as well as its possible spread. We undertook this study to determine prevalence of MBL producing *P. aeruginosa* and to detect MBL-encoding genes (*bla_{IMP}*, and *bla_{VIM}*) and frequency of class 1 integron gene among clinical *P. aeruginosa* isolates in order to explore epidemiological approaches for understanding the distribution of resistant *P. aeruginosa* in hospital settings.

MATERIALS AND METHODS

Bacterial strains. A total of 300 various clinical specimens were obtained from Vali-E-Asr University Hospital in Zanjan during March 2011-January 2012. A number of 70/300 isolates were identified as *P. aeruginosa* by conventional bacteriological tests. The source of studied isolates was as follows: urine, 7 (10%); wounds, 2 (2.8%); respiratory tract, 54(77.1%); stool, 4(5.7%); sputum, 2(2.8%) and ocular, 1(1.4%). The isolates producing MBL were more prevalent in respiratory tract specimens.

Antibiotic susceptibility testing. Antimicrobial susceptibilities were determined by Kirby-Bauer disk diffusion according to the CLSI recommendation [14]. The antibiotic disks used were as follow: IPM (10 µg), ceftazidime (30 µg) gentamicin (10 µg), piperacillin (100 µg), ciprofloxacin (5 µg) and meropenem (10 µg) (Mast Diagnostic, Merseyside, U.K.). For quality control, *P. aeruginosa* standard strain (ATCC 27853) was used as a reference strain (MIC = 2.75). MIC for IPM was performed by micro broth dilution method.

Identification of metallo-beta lactamases. IPM-resistant isolates were evaluated for MBL production by double-disk synergy test (DDST) as described previously [11, 15]. DNA template preparation was performed as follows. A few colonies were removed from culture and suspended in 300 µL of sterile distilled water and boiled for 10 min. After centrifugation at 12000 × g for 10 min, the supernatant was used as a source of template for PCR amplification of *bla_{IMP}*, *bla_{VIM}* and class 1 integron genes. PCR amplification was performed in a solution containing 200 µM concentrations of dNTP, 10 pM of each primer, 1.5 mM MgCl₂, 0.5 U Taq polymerase and 50 ng DNA templates in a final volume of 25 µL. The mentioned genes were amplified under the conditions mentioned in Table 1. *Acinetobacter baumannii* AC54/97 producing *bla_{IMP}* gene and *P. aeruginosa* COL-1 producing *bla_{VIM-2}* were used as controls. The amplicon sizes and sequences of primers used in this study have been indicated in Table 2. The PCR products were analyzed by electrophoresis (70V, 30 min) in gels composed of 1.5% (w/v) agarose stained with ethidium bromide (5 µg/100 mL) and the PCR products were visualized under a UV light. For nucleotide sequence confirmation, several PCR products were also sequenced (Bioneer, South Korea). After revision with Chromas Lite software (version 2.01), DNA sequences were aligned with GenBank

Table 2. Primers used for detection of *bla_{IMP}*, *bla_{VIM}*, and *int1* genes and lengths of the PCR products

Target gene	Primer sequences (5' to 3')	Amplicon size (bp)
<i>bla_{VIM}</i>	Fw: CCGATGGTGTGGTTCGCAT Rv: GAATGCGCAGCACCAGGA	391
<i>bla_{IMP}</i>	Fw: CTACCGCAGCAGAGTCTTTG Rv: AACCAGTTTTGCCTTACCAT	587
<i>Int1</i>	Fw: GTTCGGTCAAGGTTCTG Rv: GCCAACTTTCAGCACATG	923

Fw, forward primer; Rv, reverse primer

Table 3. Antimicrobial susceptibility testing results of 70 *P. aeruginosa* clinical isolates

Antibiotic	Antibacterial class	Susceptible (%)	Intermediate (%)	Resistant (%)
Imipenam	Carbapenemes	36.2	0	63.8
Meropenem		1.4	0	98.6
Piperacillin	Extended-spectrum β -lactams	50.7	4.3	44.9
Ciprofloxacin	Fluoroquinolone	58.0	1.4	40.6
Gentamicin	Amino glycosides	42.0	2.9	55.1
Ceftazidime	Third generation Cephalosporin	17.5	3.5	78.9

database and then assessed for *P. aeruginosa* assignment using BLAST software [16].

RESULTS

During the period of study, 300 isolates from different clinical samples were collected, of which 70 isolates of *P. aeruginosa* were identified by conventional bacteriological tests.

Of the 70 *P. aeruginosa* clinical isolates included in this study, 44.9% were resistant to piperacillin, 78.9% to ceftazidime, 55.1% to gentamicin, 40.6% to ciprofloxacin, 98.6% to meropenem and 63.8% to IPM. The isolates producing MBL were more resistant to ceftazidime, meropenem, and IPM and less resistant to piperacillin and ciprofloxacin. Table 3 shows the antibiotic resistance pattern of these strains.

In this study, 44 (63.8%) isolates were found resistant to IPM by disk diffusion test. Of these 44 IPM resistant isolates, 41 (93%) isolates showed MIC \geq 4 μ g/ml for IPM. Based on the DDST results 36 (87.8%) were confirmed to be MBL producers.

Among the 41 IPM-resistant isolates detected, PCR screening for the presence of *bla*_{IMP} gene revealed that 10 (24.4%) isolates were positive for this gene (Fig. 1). Also, PCR amplification showed that 23 (56%) isolates carried *bla*_{VIM} gene (Fig. 2). Screening for class I integron gene revealed that 31/41 (75.6%) of IPM-non-

susceptible isolates carried class I integron, while only 42.3% (11/26) of IPM-sensitive isolates were positive for class I integrons (Fig. 3).

The sequencing of the PCR products confirmed that the nucleotide sequences obtained were identical to genes for *bla*_{IMP} and *bla*_{VIM} for *P. aeruginosa* (GenBank accession number: JQ766528, JQ766529 and JQ766530).

DISCUSSION

This study illustrates screening for MBL producing *P. aeruginosa* isolates by DDST and molecular approach. Results showed that 36/41 (87.8%) of isolates were MBL positive, but PCR results confirmed presence of MBL genes only in 33/41 (80%) of isolates. The differences between phenotypic and genotypic detection of MBL producing *P. aeruginosa* isolates have been reported in previous investigations [11, 17, 18]. In the present study, we found a high frequency of *P. aeruginosa* strains carrying *bla*_{IMP} gene that significantly was different ($P < 0.05$) from other parts of our country [11-13, 19]. In the studies from Ahwaz and Tehran Provinces, the scientists could not find any IMP-type MBL-producing *P. aeruginosa* strains [12, 20]. Prevalence of VIM-type MBL producing *P. aeruginosa* strains in

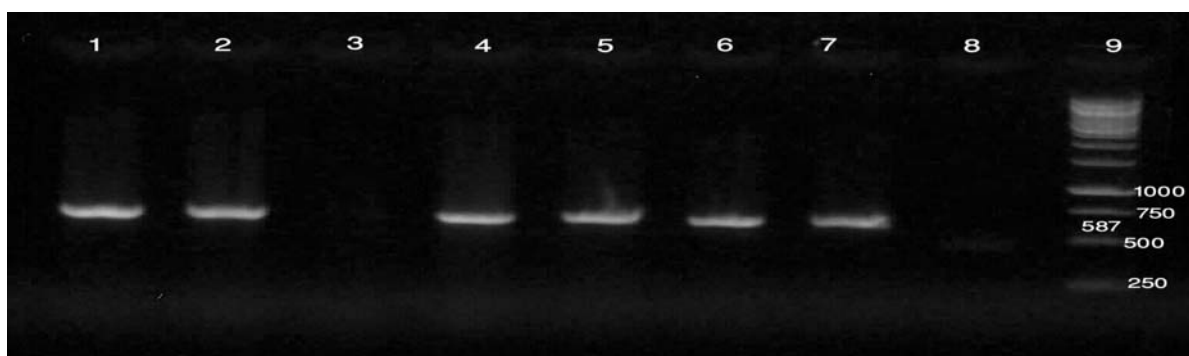


Fig. 1. Gel electrophoresis of PCR products following amplification with specific primers for *bla*_{IMP} gene (587 bp). Lanes: 1, IMP-1 positive control; 2, 4, 5, 6 and 7, clinical isolates for *bla*_{IMP} gene; 3, negative isolate; 8, negative control; 9, 1 kb DNA Ladder.

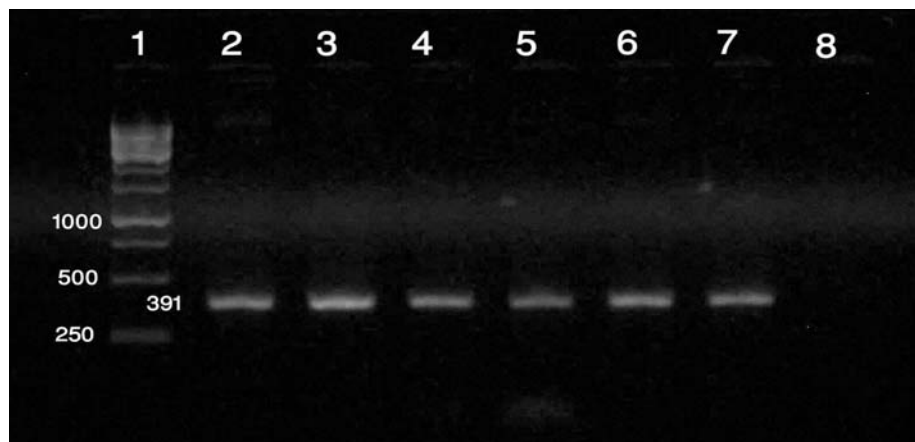


Fig. 2. Gel electrophoresis of PCR products following amplification with specific primers for *bla_{VIM}* gene (391bp). Lanes: 1, 1 kb DNA ladder; 2, *bla_{VIM}* gene positive control; 3-7, clinical isolates for *bla_{VIM}* gene; 8, negative control.

the present study was 23 (56%) isolates that also significantly was different ($P < 0.05$) from other provinces of Iran [11, 12, 20] and it seems that this is the first report of IMP- and VIM- type MBL producing *P. aeruginosa* strains with a high frequency from Zanjan Province of Iran. It was demonstrated by several previous reports that the genes of both IMP- and VIM-type MBL are often encoded on mobile gene cassettes inserted into class 1 integrons [18]. Most of MBL-producing isolates (70.45%) carried class 1 integron gene, which can easily spread the resistance encoding genes among these isolates. Several studies have also reported different frequencies of MBL positive isolates carrying class 1 integrons [11, 21, 22]. While many underlying mechanisms may account for carbapenem resistance, the possession of MBL genes is of particular concern because they are able to hydrolyze most beta-lactams, including imipenem and meropenem, drugs considered of reserve for the treatment of Gram-negative pathogens especially in *P. aeruginosa* multidrug-resistant strains [23]. Therefore, the reliable detection of the MBL-producing strains is

essential for the optimal treatment of infected patients and to control the nosocomial spread of resistance [7].

In conclusion, our results showed that the prevalence of antibiotic resistance and also both IMP and VIM-type MBL producing *P. aeruginosa* strains is higher than other parts of our country and hospital managers should emphasize on screening of clinically important isolates for MBL genes and implementation of quality assurance management for infectious control.

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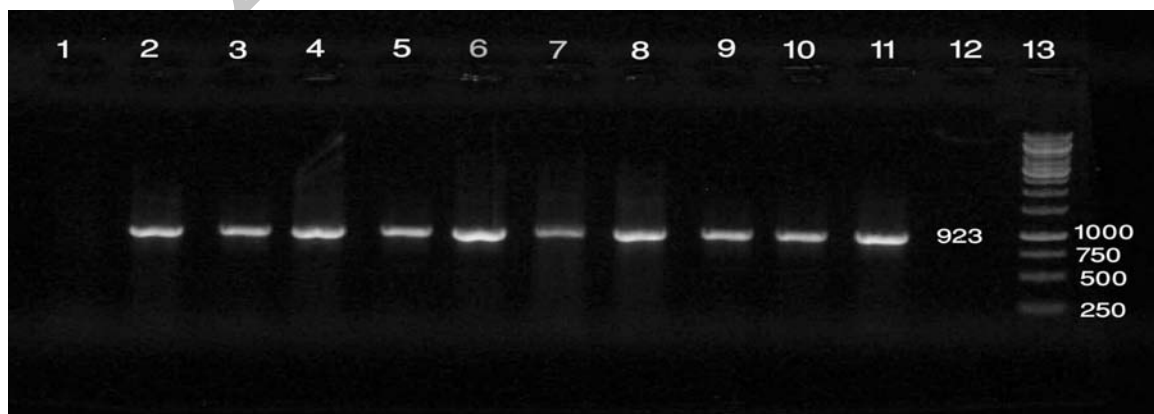


Fig. 3. Gel electrophoresis of PCR products following amplification with specific primers for *int1* gene (923 bp). Lanes: 1, negative control; 2-10, clinical isolates for *int1* gene; 11, *int1* positive control; 12, negative isolate; 13, 1 kb DNA Ladder.

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