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Detection and Genetic Characterization of Metallo-β-Lactamase *IMP-1* and *VIM-2* in *Pseudomonas aeruginosa* Strains From Different Hospitals in Kermanshah, Iran

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Background: *Pseudomonas aeruginosa*is a frequent nosocomial pathogen that causes severe diseases in many settings. Carbapenems, including meropenem and imipenem, are effective antibiotics against this organism. However, the use of carbapenems has been hampered by the emergence of strains resistant to carbapenemsvia different mechanisms such as the production of metallo-β-lactamases (MBLs), which hydrolyze all carbapenems. Several kinds of MBLs have been reported, among them *VIM* and *IMP* types being the most clinically significant carbapenemases.
Objectives: We aimed to determine the distribution of bla_{VIM-2} and bla_{IMP-1} transferable genes encoding MBLs in *P. aeruginosa* isolated from three academic hospitals in Kermanshah.
Patients and Methods: From 22nd June to 22nd September 2012, 225 isolates of *P. aeruginosa* were collected. These isolates were tested for antibiotic susceptibility with the Kirby-Bauer disk-diffusion method, and the MBLs were assessed using the imipenem-EDTA double-disk synergy test. The isolates were investigated for bla_{VIM-2} and bla_{IMP-1} genes using polymerase chain reaction.
Results: Among the 225 isolates, 33.7% (76/225) and 18.1% (41/225) were resistant to imipenem and meropenem, respectively. Of the 76 imipenem-resistant *P. aeruginosa* strains, 45 (59.2%) were positive for MBLs, 34 (75%) strains carried the bla_{IMP-1} gene, and 1 (2.2%) strain carried the bla_{VIM-2} gene.
Conclusions: Our results showed that there was a high frequency of *IMP-1* positive *P. aeruginosa* in the different wards of the hospitals.

Keywords: β-Lactamases; Carbapenems; Polymerase Chain Reaction; *Pseudomonas aeruginosa*

1. Background

*Pseudomonas aeruginosa*is a frequent nosocomial pathogen that causes severe diseases in many settings, particularly in immune-compromised patients such as those with cancer, burns, and cystic fibrosis. Infections are frequently severe, and two recent studies have indicated that the rate of mortality attributable to *P. aeruginosa* bacteremia is approximately 34% (1, 2). This organism is clinically important since it possesses several virulence factors and is intrinsically resistant to many antimicrobial and disinfectant agents (3). Carbapenems such as meropenem and imipenem are effective antibiotics against this organism since they present a good spectrum of activity and are stable to hydrolysis by most β-lactamases, including the extended spectrum β-lactamases (3, 4). However, the use of carbapenems has been hampered by the emergence of strains resistant to carbapenems via different mechanisms such as impermeability to the drug due to loss of OprD porin, upregulation of the active efflux pump systems, and production of metallo-β-lactamases (MBLs), which hydrolyze all carbapenems (5-7).

MBLs are divided into two categories: chromosomally mediated enzymes and those encoded by movable genetic elements such as plasmids and transposons (8-10). Several kinds of MBLs have been reported, including *IMP*, *VIM*, *SPM*, *GIM*, and *SIM-1* (11). The *VIM* and *IMP* types are the most clinically significant carbapenemases coded by bla_{VIM} and bla_{IMP} genes. At least 14 different *VIM* and 23 different *IMP* MBLs have been identified so far (7, 8). There are many reports which indicate an increase in the prevalence of carbapenem-resistance mediated by acquired MBLs; however, the prevalence of the MBL-producing isolates of *P. aeruginosa* varies in Asian countries (8, 12, 13). In Iran, the first isolation of bla_{IMP-1} was reported in 2011 by Peymani et al. (14), who collected *Acinetobacter baumannii* isolates from several hospitals in Tabriz (Northwest of Iran).

The growing rate of resistance against the traditional antibiotics has rendered the treatment of patients with infections caused by MBL-producing *P. aeruginosa* critical. Previous studies have shown a high prevalence rate of multidrug-resistant *P. aeruginosa* isolated from differ-

ent clinical specimens, especially in burn wards in Iran (15, 16); nevertheless, little information is available on the distribution of MBL-producing isolates in the country. For example, in Markazi province, 37% of the *P. aeruginosa* isolates obtained from the hospitalized patients were shown to be resistant to imipenem, and 50% of these strains were detected to have bla_{VIM-1}, 56.6% bla_{VIM-2}, and 6.6% bla_{IMP-1} (17). In another study, from 240 *P. aeruginosa* isolates, 34.16% were imipenem-resistant, and 23.3% were screened as MBL-positive strains using the double-disk synergy test. Additionally, a specific polymerase chain reaction (PCR) test confirmed the presence of 8 (9.75%) and 10 (12.19%) *IMP-1* and *VIM-2* types, respectively (18).

2. Objectives

The current study was performed to examine the prevalence of MBL-producing *P. aeruginosa* isolates obtained from clinical specimens at different hospitals in Kermanshah (Imam Khomeini, Imam Reza, and Taleghani hospitals) and also the prevalence of those harboring the bla_{VIM-2} and bla_{IMP-1} genes, which are the most clinically significant carbapenemases.

3. Patients and Methods

3.1. Samples

From 22nd June to 22nd September 2012, 225 isolates of *P. aeruginosa* were collected from three hospitals in Kermanshah. The isolates were taken from urine (n = 79, 35%), blood (n = 14, 6.2%), wound (n = 29, 12.8%), respiratory tract (n = 81, 36%), burned tissue (n = 17, 7.5%), and eye (n = 5, 2.2%). The isolates were collected from different wards, including ICU, CCU, urology, emergency, burn, respiratory, and surgery. *P. aeruginosa* was identified by Gram staining, ability to produce oxidase and catalase, oxidative-fermentative test, resistance to cetrimide, and growth at 42°C (19).

3.2. Antimicrobial Susceptibility Testing

The disk diffusion test was conducted in accordance with the clinical and laboratory standards institute (CLSI) methodology. The antimicrobial agents included comprised aztreonam (10 μg), cefepime (10 μg), ceftazidime (30 μg), ciprofloxacin (5 μg), gentamicin (10 μg), imipenem (10 μg), and piperacillin/tazobactam (10 μg, 100 μg) (MAST, U.K.). *P. aeruginosa* ATCC 27853 was used as quality control in each run of antimicrobial susceptibility testing.

3.3. Identification of Metallo-β-Lactamases

Among β-lactamases, MBLs are unique in requiring the presence of zinc ion in the active site of the enzyme, and are, thus, inhibited by chelating agents such as ethylenediaminetetraacetic acid (EDTA) (20). The imipenem-EDTA double-disk synergy test was used in order to identify the *P. aeruginosa* producing MBLs. Therefore, a 0.50-M EDTA (Merck, Germany) solution was prepared by dissolving 186.1 g of EDTA in 1000 mL of distilled water, and the pH was adjusted to 8 by adding NaOH (21). Then, 4 μL of the prepared solution was added to the imipenem disk and dried in an incubator. The prepared EDTA/imipenem disk and the imipenem disk itself were placed in a plate containing the Müller-Hinton agar (Merck, Germany) with cultured *P. aeruginosa*. After 16 - 18 hours of incubation at 37°C, an increase of ≥ 7 mm in the inhibition zone diameter in the presence of EDTA compared to imipenem tested alone was considered to be a positive test for the presence of an MBL (4, 18, 22).

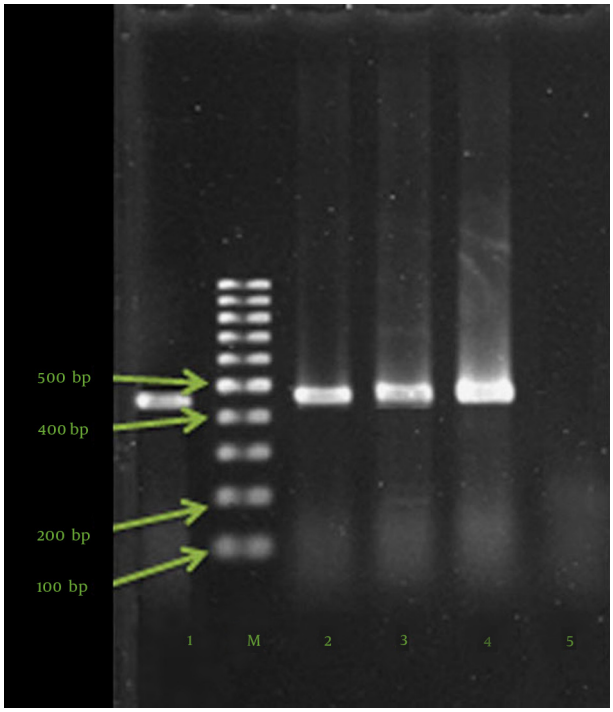
PCR was performed to detect the bla_{VIM-2} and bla_{IMP-1} genes. The DNA templates from all the isolates were extracted by boiling (10 minutes in 95°C), and PCR was carried out in a thermal cycler (Bio Rad, USA) using 25-μL volumes containing 12.5 μL of PCR Master Mix (500 mM of Tris-HCl pH 8.55, 1.5 mM of $MgCl_2$, 0.2 mM of dNTPs, and 0.04 units/uL of Taq DNA polymerase) (SinaClon Inc., Tehran, Iran) and 0.5 μM of each of the primers (SinaClon Inc., Iran). The primers and the cycling condition are listed in Table 1 (23, 24).

Following amplification, 10 μL of each sample was analyzed with 1.5% agarose gel electrophoresis for the detection of positive samples. For all the amplification reactions, the mixture was heated at 96°C for 4 minutes prior to thermocycling. The mixture was held at 72°C for 7 minutes after the final cycle before cooling at 4°C. The agarose gel electrophoresis of bla_{VIM-2} and bla_{IMP-1} PCR products is depicted in Figure 1. The positive controls (clinical *P. aeruginosa* isolates with sequenced bla_{IMP-1} and bla_{VIM-2} genes) were kindly dedicated by Dr. Najjar Pirayeh (Tarbiat Modarres University), and the negative control contained water in place of DNA.

Table 1. Primers and Cycling Condition

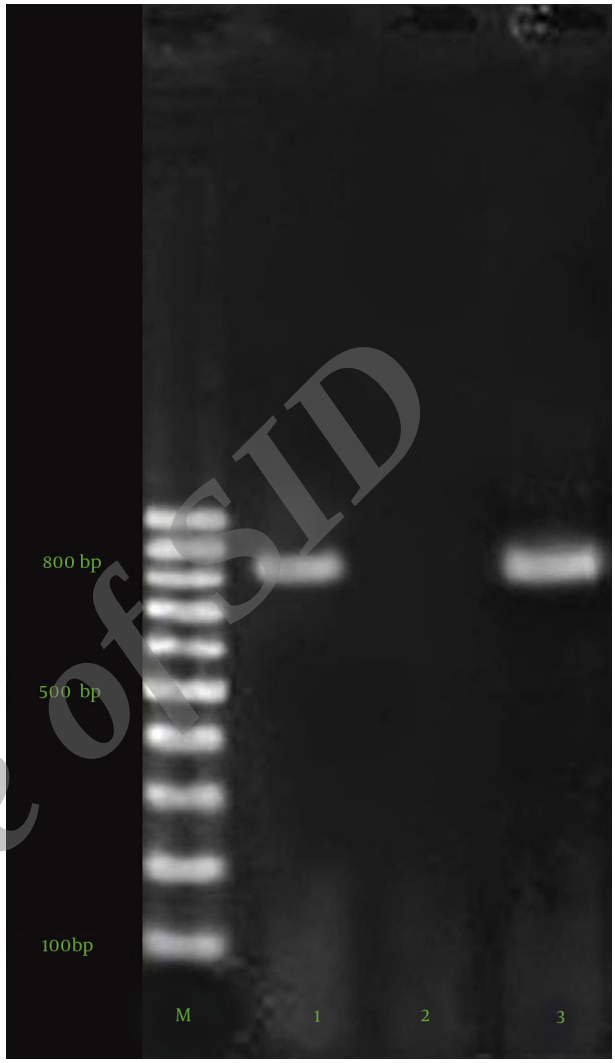
Target	Oligonucleotide Sequence (5' - 3)	Polymerase Chain Reaction Condition	No. of Cycles	Fragment Size, bp	Reference
***bla*IMP-1**		94°C, 30 s	25	448	(23)
	CATGGTTTGGTGGTTCTTGT	57°C, 45 s			
	ATAATTTGGCGGACTTTGGC	72°C, 60 s			
***bla*VIM-2**		94°C, 30 s	25	801	(24)
	ATGTTCAAACTTTTGAGTAAG	57°C, 45 s			
	CTACTCAACGACTGAGCG	72°C, 60 s			

Figure 1. Electrophoresis Results of the bla_{IMP-1} Gene Polymerase Chain Reaction



Lane 1: Positive control (clinical *P. aeruginosa* with the sequenced bla_{IMP-1} gene). Lane M: 100 bp DNA ladder. Lanes 2, 3, and 4: Isolates with the bla_{IMP-1} gene. Lane 5: Negative control (distilled water).

Figure 2. Electrophoresis Results of the bla_{VIM-2} Gene Polymerase Chain Reaction



Lane M: 100 bp DNA ladder. Lane 1: Isolate with the bla_{VIM-2} gene. Lane 2: Negative control (distilled water). Lane 3: Positive control (clinical *P. aeruginosa* with the sequenced bla_{VIM-2} gene).

4. Results

Totally, 225 confirmed isolates of *P. aeruginosa* recovered from different clinical specimens were tested for antibiogram. The findings showed that 137 (61%) isolates were resistant to ceftazidime, 131 (58.5%) to gentamicin, 130 (57.7%) to piperacillin/tazobactam, 101 (44.7%) to ciprofloxacin, 96 (42.8%) to cefepime, 92 (40.9%) to aztreonam, 76 (33.7%) to imipenem, and 41 (18.1%) to meropenem. According to these percentages, imipenem and meropenem were the most effective drugs. The imipenem-EDTA double-disk synergy test showed that 45 out of the 76 (59.2%) imipenem-resistant strains were MBL positive. The isolates were collected from different wards, including ICU (n = 22), urology (n = 2), emergency (n = 4), burn (n = 11), respiratory (n = 3), and surgery (n = 3).

Furthermore, our molecular studies showed that only the MBL-positive strains were positive in PCR. Moreover, 75% (n = 34) of the MBL-positive strains were detected to have bla_{IMP-1} and 2.2% bla_{VIM-2} (n = 1). bla_{IMP-1} was taken from burned tissue (n = 10, 29.4 %), respiratory tract (n = 10, 29.4 %), urine (n = 8, 23.5%), blood (n = 2, 5.8%), eye (n = 3, 8.8%), and vagina (n = 1, 2.9%). bla_{VIM-2} (n = 1) was recovered from burned tissue. Antibiogram showed that the strains resistant to imipenem possessed high-level resistance to gentamicin (100%), cefepime (94.7%), and ceftazidime (94.7%). Among the imipenem-resistant strains, 30.3% (n = 23) were resistant to meropenem and the rest (n = 53) were sensitive to it. On the other hand, there were 18 meropenem-resistant strains sensitive to both imipenem and cefepime.

5. Discussion

Microbial resistance has increased drastically in recent years in both developed and developing countries and it has rapidly become a leading public health concern. The prevalence of antimicrobial resistance varies greatly between and within countries and between different pathogens. Resistance to antibiotics among organisms is increased either by mutations or by acquiring new genetic material via horizontal gene transfer. Several factors

favor the development of bacterial resistance to antibiotics in developing countries such as the less potent activity of the drugs, i.e. some of the antibiotics provided in developing countries have decreased potency due to the degradation or adulteration of the drug or because of the presence of a lower concentration of active substances, lack of well-equipped diagnostic laboratories, and excessive use of antimicrobials in domestic animals (8-10).

Antimicrobial agents suitable for use in pediatric clinics are mainly limited to β-lactam antibiotics, especially carbapenems. However, the problem of drug resistance to carbapenems is gradually worsening owing to their increasing clinical use. The present study analyzed the patterns of resistance to several antibiotics such as carbapenems. The results showed that 33.7% of the isolates were resistant to imipenem. In a previous study in the Iranian city of Kermanshah in 2004, Mohajeri et al. (25) reported a lower rate of resistance (10%). However, another investigation conducted in the Iranian city of Shiraz in 2012 reported that 127 (59.16%) of the 240 isolates were resistant to this antibiotic (18). The reported rates of resistance to imipenem in Japan, Russia, France, Canada, and Spain were 8.3%, 13.4%, 18.5%, 12%, and 14%, respectively (26-30). As was mentioned, numerous factors contribute to these variations. In addition, the increased rate of resistance to imipenem in Kermanshah is in line with the increase throughout the world. Resistance to carbapenems in *P. aeruginosa* may be due to MBLs, which hydrolyze all carbapenems (5-7). Since MBLs are placed in movable elements, they are horizontally transmitted between organisms easily (8-10, 31). Large outbreaks by MBL-producing *P. aeruginosa* strains have been described in hospitals in Italy, Greece, and Korea (32-37). *IMP* and *VIM*-producing *P. aeruginosa* strains have been reported worldwide (10, 38-42).

In our study, the majority of the MBL producers carried the *blaIMP* gene (75%); however, the presence of this gene has been previously proved to be variable in different regions (17, 18). In a previous report from the Iranian province of Markazi, 37% (40 out of 108) of the *P. aeruginosa* isolates obtained from the hospitalized patients were demonstrated to be resistant to imipenem. The EDTA/imipenem test showed that 50% of the imipenem-resistant strains were MBL positive, and PCR revealed that 50% of the imipenem-resistant strains had bla_{VIM-1}, 56.6% bla_{VIM-2}, and 6.6% bla_{IMP-1} (17). In another study in Iran, from 240 *P. aeruginosa* isolates mainly recovered from wound, urine, and sputum, 82 (34.16%) isolates were imipenem-resistant (minimum inhibitory concentration [MIC] ≥ 4 μg/mL). Among these imipenem-resistant isolates, 19 (23.3%) MBL-producing *P. aeruginosa* isolates were screened using the double-disk synergy test. A specific PCR test confirmed the presence of 8 (9.75%) and 10 (12.19%) *IMP-1* and *VIM-2* types, respectively (18). In contrast, Khosravi and Mihani (2008) reported that none of the 8 MBL-producing *P. aeruginosa* strains in their study were positive for the bla_{IMP} gene (40).

The most and also the sole frequent reported MBL-producing *P. aeruginosa* in Iran is *VIM-1*, previously cited by several researchers in different cities of Iran (4, 17, 22, 40, 43, 44). Nonetheless, there are only a few investigations reporting the presence of the *VIM-2* type of *P. aeruginosa* in Iran. For the first time in 2012, Sadeghi et al. (17) detected this genotype in the Iranian city of Arak. Subsequently, in 2013, among the 82 imipenem-resistant isolates tested for MBLs, 10 isolates were positive for this gene (18). According to our findings, it seems that the MBL-producing isolates of *P. aeruginosa* are the main cause of IPM resistance among this species. We suggest that molecular typing via standard methods such as pulsed-field gel electrophoresis be conducted to define the clonality of the isolates.

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Authors' Contributions

Ramin Abiri: study concept and design, interpretation of data and final revision of the manuscript; Pantea Mohammadi: research and technical advisor, drafting of the manuscript; Navid Shavani: contribution in all laboratory experiments; Mansour Rezaei: Statistical analysis.

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