Phenotypic and genotypic investigation of metallo-β-lactamases in Pseudomonas aeruginosa clinical isolates in Bushehr, Iran

Mahboubeh Seyedi 1, 2, Forough Yousefi 1, 3, Behrouz Naeimi 1, 3, Saeed Tajbakhsh 1, 3*

1 Department of Microbiology and Parasitology, School of Medicine, Bushehr University of Medical Sciences, Bushehr, Iran
2 Student Research Committee, Bushehr University of Medical Sciences, Bushehr, Iran
3 The Persian Gulf Tropical Medicine Research Center, The Persian Gulf Biomedical Sciences Research Institute, Bushehr University of Medical Sciences, Bushehr, Iran

OBJECTIVE: Production of metallo-β-lactamases (MBLs) is an important mechanism of resistance to carbapenems. This study aimed to detect the MBL-producing Pseudomonas aeruginosa clinical isolates and to investigate the presence of blaVIM, blaIMP, blaSIM, blaNDM, blaOXA, and blaGIM genes in these isolates in Bushehr, Iran.

MATERIALS AND METHODS: A total of 169 P. aeruginosa clinical isolates were collected from three hospitals in Bushehr. The modified carbapenem inactivation method (mCIM) was used for the phenotypic detection of carbapenemase production. A combination disk test (CDT) was performed for the phenotypic detection of MBL production. To investigate the presence of blaVIM, blaIMP, and blaGIM genes, PCR and sequencing was carried out.

RESULTS: Based on the results of mCIM, 40 (23.7%) of 169 isolates were carbapenemase producers. CDT revealed that 26 (15.4%) isolates were MBL producers. blaVIM and blaIMP genes were found in 18 (69.2%), 8 (30.8%), and 1 (3.8%) of the MBL-producing isolates, respectively. Coexistence of blaVIM and blaIMP was observed in 2 (7.7%) MBL-producing isolates. Among all 169 P. aeruginosa isolates, 23 (13.6%) harbored blaSIM 18 (10.6%) carried blaNDM, and 1 (0.6%) carried the blaGIM gene. blaVIM, blaIMP, and blaGIM genes were not found in the present study.

CONCLUSION: blaVIM, blaIMP, and blaGIM genes were detected in this study, which could be a warning sign about the prevalence of these genes among P. aeruginosa clinical isolates in our region. Proper monitoring and detection of MBL-producing isolates are essential steps to prevent the spread of these isolates.

© 2023 Iranian Journal of Basic Medical Sciences. All rights reserved.
Materials and Methods

P. aeruginosa clinical isolates

This study was approved by the Ethical Committee of Bushehr University of Medical Sciences, Iran, with reference number IR.BPU.MSC.REC.1398.082. A total of 169 P. aeruginosa clinical isolates recovered from clinical specimens including urine, endotracheal tube (ETT) secretions, blood, wound, throat, eye, urerter secretions, ear, and catheter (catheter culture to aid in the diagnosis of catheter-related infection), were collected from three hospitals in Bushehr, Iran, between May 2017 and August 2019. Identification of the isolates was carried out by conventional bacteriological methods and confirmed by PCR using specific primers to target 16S rDNA of P. aeruginosa (10) (Table 1).

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing for carbapenems including imipenem, meropenem, and doripenem was performed by the disk diffusion method and interpreted according to the Clinical and Laboratory Standards Institute (CLSI 2018) (11). Imipenem disk (10 µg), meropenem disk (10 µg), and doripenem disk (10 µg) (Mast Ltd, Merseyside, UK) were used. Pseudomonas aeruginosa ATCC 27853 and Escherichia coli ATCC 25922 were used for quality control. The minimum inhibitory concentration (MIC) of imipenem was determined for the isolates that were resistant to this antibiotic in the disk diffusion method. MIC Test Strip (Liofilchem, Italy) was used to determine the MIC of imipenem.

All tested isolates that were not susceptible to one or more carbapenems, were chosen for the phenotypic detection of carbapenemase production (11).

Modified carbapenem inactivation method (mCIM)

Isolates that were intermediate or resistant to one or more carbapenems, were selected for phenotypic detection of carbapenemase production. mCIM can detect serine carbapenemases and metallo-β-lactamases (11). The mCIM test was performed for each isolate according to the CLSI 2018 procedure as follows: briefly, a 10-µl loopful of bacteria from an overnight blood agar plate was emulsified in 2 ml tryptic soy broth (TSB). A 10-µg meropenem disk was added to the tube so that the entire disk was immersed in the suspension. The tube was incubated at 35 °C for 4 hr±15 min. Just before completion of the TSB-meropenem disk suspension incubation, a 0.5 McFarland suspension of E. coli ATCC 25922 (that is a meropenem-susceptible strain) was prepared and inoculated on a Mueller Hinton agar plate as for the routine disk diffusion procedure. Afterward, the meropenem disk was removed from TSB-meropenem disk suspension and placed on the Mueller Hinton agar plate previously inoculated with E. coli ATCC 25922. The Mueller Hinton agar plate was then incubated at 35 °C for 18-24 hr. Following incubation, the zone of inhibition was measured and the results were interpreted according to CLSI. A zone diameter of 6-15 mm or the presence of pinpoint colonies within a 16-18 mm zone was considered carbapenemase positive (mCIM positive). A zone diameter of ≥19 mm was considered carbapenemase negative (mCIM negative) (11).

A carbapenemase-producing strain of P. aeruginosa was used as positive control, and P. aeruginosa ATCC 27853 was used as negative control.

Combination disk test (CDT)

P. aeruginosa isolates that were intermediate or resistant to one or more carbapenems (5, 11), were also tested by CDT for the phenotypic detection of MBL production. CDT with imipenem (CDT-IMI) was carried out for each isolate as follows: a bacterial suspension with turbidity equal to 0.5 McFarland was prepared and inoculated on Mueller Hinton agar II (Biolife, Milano, Italy) plates as for the disk diffusion procedure. Two imipenem (10 µg) disks were placed on the Mueller Hinton agar II plate, then, 5 µl of 0.5 M EDTA (930 µg EDTA) was added to one of the imipenem disks. Since EDTA has some bactericidal activity, a blank disk without antibiotics was also inoculated with 5 µl of 0.5 M EDTA (5). The plates were incubated overnight at 35 °C. After incubation, an increase of ≥7 mm in zone diameter around imipenem plus EDTA disk in comparison with imipenem disk demonstrated the production of MBL by the bacterium (1, 5, 12). Furthermore, CDT-IMI with 750 µg EDTA was used.

Table 1. Primers used in this study for the identification of Pseudomonas aeruginosa and detection of metallo-β-lactamase genes

| Primer | Sequence (5'-3') | Target gene | Product size (bp) | Reference |
|--------|-----------------|-------------|------------------|-----------|
| PA-SS-F | GGGGATCTTGGGACCTCA | 16S rDNA | 956 | 10 |
| PA-SS-R | TCCTTAGGTCGCCACCCG | | | |
| VIM-F | GATGTTGTATTGTGCGATA | blaOXA | 390 | 14 |
| VIM-R | CGAATGGCCAGCACCAG | | | |
| IMP-F | GGAATTAGGTTGCTATTACC | blakIM | 233 | 14 |
| IMP-R | GTGTTGGAATCCGATTCC | | | |
| ND-MF | GGTGTTGGAATCCGATTCC | blakIM | 521 | 14 |
| ND-MR | GCGAATGGCCACATCCGGATC | | | |
| SPM-F | AAAATCTGGTAGCAAAGG | blakOXA | 271 | 14 |
| SPM-R | ACATATTCCGCTGGAGAGCG | | | |
| GIM-F | TGCAGACACCTGTGCTGAA | blakIM | 477 | 14 |
| GIM-R | AACGTGGCAATTGCCCATGC | | | |
| AIM-F | CTGAAAGGTGTCAGAAAGCAG | blakIM | 322 | 14 |
| AIM-R | GTCCGGCAACCTGCTAAGT | | | |
| SIM-F | TACAGGGATTCGGCATGC | blakIM | 571 | 14 |
| SIM-R | GATGGCGCTGTCGCCCATTG | | | |

*16S rDNA of Pseudomonas aeruginosa*
also performed and interpreted similarly (13).

Moreover, at the same time, CDT with 10 µg meropenem disks (CDT-MEM) was done by using 930 µg EDTA and 750 µg EDTA. The procedure and interpretation of CDT MEM results were similar to that described above (5).

A metallo-β-lactamase-producing strain of *P. aeruginosa* was used as positive control, whereas *P. aeruginosa* ATCC 27853 was applied as negative control (5).

**Detection of MBL genes by PCR assay and sequencing**

The total DNA from *P. aeruginosa* isolates was extracted using an extraction kit (GeneAll Biotechnology Co., Ltd, Seoul, South Korea) according to the manufacturer’s directions. Specific primers for the detection of MBL genes (bla<sub>TEM</sub>, bla<sub>IMP</sub>, bla<sub>NDM</sub>, bla<sub>VIM</sub>, bla<sub>AM</sub>, and bla<sub>DM</sub>) were used (14) (Table 1). Amplification of targeted DNA was carried out in 25 µl reaction volumes, each containing 12.5 µl Taq DNA polymerase 2X Master Mix (Ampliqon, Denmark), 1 µl of each oligonucleotide primer, 9.5 µl nuclease-free water, and 1 µl DNA template. PCR products were analyzed by electrophoresis on 2% agarose gel and finally visualized with a gel documentation system (UV, BioDoc-It Imaging System, USA). Positive PCR products were purified and sequenced by Kawsar Biotech Company (Tehran, Iran). The nucleotides and deduced protein sequence alignment were also performed online using the basic local alignment search tool (BLAST) program of the National Center for Biotechnology Information.

**Results**

In this project, 169 *P. aeruginosa* isolates were collected from clinical specimens including urine (70 isolates; 41.4%), ETT secretions (51 isolates; 30.2%), blood (19 isolates; 11.2%), wound (18 isolates; 10.6%), throat swab (4 isolates; 2.4%), eye (3 isolates; 1.8%), ureter secretions (2 isolates; 1.2%), ear secretions (1 isolate; 0.6%), and catheter (1 isolate; 0.6%). Eighty-eight (52.1%) isolates were from male and 81 (47.9%) from female patients.

**Table 2. Results of mCIM and CDT in 67 Pseudomonas aeruginosa isolates that were intermediate or resistant to one or more carbapenems**

| No. Isolates | mCIM | CDT-IMI 930 µg EDTA | CDT-IMI 750 µg EDTA | CDT-MEM 930 µg EDTA | CDT-MEM 750 µg EDTA |
|--------------|------|---------------------|---------------------|---------------------|---------------------|
| 21           | +    | +                   | +                   | –                   | –                   |
| 3            | +    | +                   | +                   | +                   | +                   |
| 2            | +    | +                   | +                   | +                   | +                   |
| 14           | +    | –                   | –                   | –                   | –                   |
| 27           | –    | –                   | –                   | –                   | –                   |

mCIM: modified carbapenem inactivation method; CDT: combination disk test; CDT-IMI: CDT with imipenem; CDT-MEM: CDT with meropenem

Antimicrobial susceptibility testing demonstrated that 67 isolates were not susceptible to at least one of the carbapenems including imipenem, meropenem, and doripenem, which were therefore selected to be tested by mCIM and CDT. The MIC of imipenem was determined for the 54 imipenem-resistant isolates. The MIC values of imipenem for 53 isolates were ≥32 µg/ml and for one isolate was 8 µg/ml. Out of the above-mentioned 67 isolates, 40 were mCIM positive. Therefore, in the present study, 40 (23.7%) of 169 isolates were carbapenemase producers. Out of 40 carbapenemase-producing isolates, 26 were MBL-positive using CDT with imipenem (CDT-IMI). Thus, based on the phenotypic test, 26 (15.4%) of 169 isolates were MBL producers. The results of CDT-IMI with 930 µg EDTA and CDT-MEM with 750 µg EDTA were the same (Table 2).

Also, CDT with meropenem (CDT-MEM) was performed in this project. Out of 26 MBL-producing isolates, 5 were positive using CDT-MEM with 930 µg EDTA and 3 were positive using CDT-MEM with 750 µg EDTA (Table 2).

It should be noted that 930 µg and 750 µg EDTA did not show an antibacterial effect around the blank disks.

All 169 isolates were tested by PCR for the detection of MBL genes. The presence of bla<sub>IMP</sub>, bla<sub>NDM</sub>, and bla<sub>VIM</sub> genes was confirmed via sequencing the PCR products and checking them using the BLAST program of the National Center for Biotechnology Information. Out of 26 isolates that were identified as MBL producers by the phenotypic method, 25 isolates carried the genes studied in this project. Coexistence of bla<sub>IMP</sub> and bla<sub>NDM</sub> was observed in 2 MBL-producing isolates. bla<sub>NDM</sub> was detected in 7 carbapenemase-producing isolates that were MBL negative by phenotypic test (CDT negative). bla<sub>NDM</sub> was also detected in 3 isolates that were carbapenemase negative and MBL negative by phenotypic methods (mCIM negative and CDT negative). In addition, bla<sub>NDM</sub> was found in 5 isolates that were susceptible to imipenem, meropenem, and doripenem (Table 3). Among

**Table 3. Frequency of MBL genes among Pseudomonas aeruginosa isolates**

| Gene          | mCIM (+), CDT (+) | mCIM (+), CDT (-) | mCIM (-), CDT (+) | mCIM (-), CDT (-) | NT No. Isolates (%) | Total isolates (%) |
|---------------|-------------------|-------------------|-------------------|-------------------|---------------------|---------------------|
| bl<sub>IMP</sub> | 16 (9.5)          | 0 (0)             | 0 (0)             | 0 (0)             | 16 (9.5)            |
| bl<sub>NDM</sub> | 6 (3.5)           | 7 (4.1)           | 3 (1.8)           | 5 (2.9)           | 21 (12.4)           |
| bl<sub>IMP</sub>, bl<sub>NDM</sub> | 2 (1.2)          | 0 (0)             | 0 (0)             | 0 (0)             | 2 (1.2)             |
| bl<sub>NDM</sub> | 1 (0.6)           | 0 (0)             | 0 (0)             | 0 (0)             | 1 (0.6)             |

MBL: metallo-β-lactamase; mCIM: modified carbapenem inactivation method; CDT: combination disk test; mCIM (+): mCIM positive; mCIM (-): mCIM negative; CDT (+): CDT positive; CDT (-): CDT negative; NT: isolates that were susceptible to imipenem, meropenem, and doripenem; thus, were not tested by mCIM and CDT
all 169 P. aeruginosa isolates, 23 (13.6%) harbored bla_{GIM}, 18 (10.6%) carried bla_{IMP}, and 1 (0.6%) carried bla_{VIM} gene. bla_{GIM}, bla_{IMP}, and bla_{SIM} were not found in the present study.

**Discussion**

The emergence and spreading of MBL-producing P. aeruginosa is a universal concern threatening not only immunosuppressed patients but also healthy members of the community. MBL-producing P. aeruginosa is an important organism due to its antibiotic resistance characteristics as well as its pathogenicity. It carries various antimicrobial resistance genes and is able to transfer these to other strains. Detection of MBL-producing isolates and studies on the frequency of these isolates are recommended in order to develop strategies that control and limit the transmission of MBL-producing P. aeruginosa (3).

In this study, CDT-IMI and CDT-MEM were used for phenotypic detection of MBL production; both methods were performed using 930 µg EDTA and 750 µg EDTA. The results of the CDT-IMI with 930 µg EDTA and CDT-MEM with 750 µg EDTA were the same, so that 26 MBL-producing isolates were detected. However, CDT-MEM with 930 µg EDTA and with 750 µg EDTA detected 5 and 3 isolates of 26 MBL-positive isolates, respectively. Of the 26 isolates identified by the phenotypic method as MBL producers, 25 isolates harbored MBL genes. Therefore, in our study, the results of CDT-IMI were much better than the results of CDT-MEM. Also, in the study performed by Heinrichs et al., CDT with imipenem showed the best results for the detection of MBL-producing P. aeruginosa (12). In contrast to our results, in the study conducted by Pitout and colleagues, the results of CDT with meropenem were better than the results of CDT with imipenem (3).

As mentioned above, of the 26 isolates identified as MBL producers by phenotypic methods, MBL genes were detected in 25 isolates but not in one. This isolate probably harbored a rare MBL gene such as bla_{SIM} (14, 15) that has not been investigated in our study.

In our work, out of 40 carbapenemase-producing P. aeruginosa, 26 isolates were MBL producers by phenotypic test (CDT positive), whereas 14 isolates were CDT negative. It is noteworthy that the bla_{SIM} gene was detected in 7 of these 14 isolates by PCR. Thus, it is probable that these 7 isolates were also MBL producers, but CDT was not able to detect MBL (false negative results).

Based on the results of the phenotypic test, out of 67 isolates that were not susceptible to one or more carbapenems, 26 (38.8%) were MBL producers. The frequency of MBL producers among all 169 isolates using CDT was 15.4% (26/169). In the study performed by Bagheri Bejestani et al. in Tehran, Iran, 3.3% of P. aeruginosa isolates were MBL producers based on CDT results, which was a lower frequency than the frequency obtained from our study (16). In a study conducted by Mirbagheri and colleagues in Mashhad, northeast of Iran, according to CDT results, 88.8% of imipenem-resistant isolates were MBL producers (17). In another study in Ahvaz, southwest of Iran, 90% of imipenem-resistant isolates were phenotypically MBL producers; these researchers also used CDT (1). Therefore, the frequency of MBL-producing isolates in these two studies was higher than the frequency in our study. In an investigation in India carried out by Arunagiri et al., 70.1% of multidrug-resistant P. aeruginosa were MBL producers based on the CDT results (18). In a study in Canada on 241 isolates that were not susceptible to imipenem, 110 (46%) isolates were detected as MBL positive by CDT (5). In Belgium, Heinrichs and colleagues studied 162 multidrug-resistant P. aeruginosa that were not susceptible to imipenem; 52 (32%) isolates were MBL positive in their investigation. Heinrichs et al. used CDT and disk synergy test (DST), but CDT with imipenem showed the best results (12). The reasons for these different results are difficult to explain but may be due to differences in the distribution of MBL genes across various geographical areas, studied isolates, and methods for the phenotypic detection of MBL producers.

In this study, the MIC of imipenem was determined for the 54 imipenem-resistant isolates. The MIC values of imipenem for 53 isolates were ≥32 µg/ml, which indicates a high-level resistance of these 53 isolates to imipenem. Imipenem showed a MIC ≥32 µg/ml for all 26 isolates that were MBL positive by the phenotypic method. In addition, the MIC values of imipenem were ≥32 µg/ml for 9 imipenem-resistant isolates that were MBL negative by phenotypic method but harbored the bla_{NDM} gene.

In this study, bla_{IMP}, bla_{NDM}, and bla_{VIM} were detected in 18 (69.2%), 8 (30.8%), and 1 (3.8%) of the MBL-producing isolates, respectively. Among all 169 P. aeruginosa isolates, 23 (13.6%) harbored bla_{GIM}, 18 (10.6%) carried bla_{IMP}, and 1 (0.6%) carried the bla_{VIM} gene. bla_{IMP}, bla_{GIM}, and bla_{SIM} were not found in our work. In a study in Ahvaz, Iran, bla_{IMP} and bla_{VIM} were detected in 1.6% and 55% of imipenem-resistant isolates, respectively, but, no bla_{SIM} gene was found in the isolates (1). In a study done by Haghi and colleagues in Zanjan, northwest of Iran, the frequency of bla_{IMP}, bla_{NDM}, and bla_{VIM} among the MBL-producing isolates was 80%, 17.1%, 57.1%, and 14.1%, respectively. Therefore in contrast to our results, bla_{SIM} and bla_{VIM} were detected in their study. However, similar to our results, no bla_{IMP} harboring isolate was detected. Also, these authors reported that 2 isolates contained bla_{NDM} and bla_{IMP}, and bla_{SIM} was also observed in 2 (7.7%) MBL-producing isolates in our work.

In an investigation conducted by Bagheri Bejestani et al. in Tehran, Iran, 3.3% of isolates carried the IMP gene (16) which was lower in frequency compared with our study. In the present study, no bla_{IMP} gene was identified in any of the isolates which was in accordance with the study performed by Yousefi et al. in Tabriz, northwest Iran (20). In the study conducted in Brazil, among the MBL-producing isolates, 55.6% were positive for bla_{SPM} (21).

In an investigation in Iraq, bla_{SPM} was detected in 4 of 22 P. aeruginosa isolates (4). In the study done in India, 17.3% of carbapenem-resistant P. aeruginosa isolates carried bla_{SPM} (22). In our work, among all 169 P. aeruginosa isolates, bla_{NDM} was the most frequently detected MBL gene which emphasizes the need for surveillance and precautions.

It should be noted that in our study bla_{NDM} was identified in 5 isolates that were susceptible to imipenem, meropenem, and doripenem (Table 3). The reason for susceptibility of these isolates to carbapenems despite the presence of the bla_{NDM} is unclear. These isolates may be silent reservoirs of the bla_{NDM}. A similar report on the bla_{NDM} gene has been published by Pellegrino and colleagues. These authors reported a carbapenem-susceptible P. aeruginosa strain that...
carried bla<sub>NDM</sub>; they stated that such isolates may be silent reservoirs of the bla<sub>NDM</sub> gene (23).

**Conclusion**

bla<sub>NDM</sub>, bla<sub>IMP</sub>, and bla<sub>SHV</sub> genes were detected in this study which could be a warning sign about the prevalence of these genes among P. aeruginosa clinical isolates in our region. Proper monitoring and detection of MBL-producing isolates are the essential steps to prevent the spread of these isolates.

**Acknowledgment**

This article was from the postgraduate MSc thesis of Mahboubeh Seyedi and was supported by the Vice-Chancellor of Research of Bushehr University of Medical Sciences, Bushehr, Iran (grant no. 5185). We thank Clinical Research Development Center, The Persian Gulf Martyrs Hospital, Bushehr University of Medical Sciences, Bushehr, Iran for facilitating the process of sampling.

**Authors’ Contributions**

FY, BN, and ST designed the experiments; MS, FY, and ST performed experiments and collected data; MS, FY, BN, and ST discussed the results and strategy; FY and ST Supervised, directed, and managed the study; MS, FY, BN, and ST approved the final version.

**Conflicts of Interest**

The authors declare that there are no conflicts of interest.

**References**

1. Moossavian M, Rahimzadeh M. Molecular detection of metallo-β-lactamase genes, bla<sub>NDM</sub>, bla<sub>IMP</sub>, and bla<sub>SHV</sub>, in imipenem resistant Pseudomonas aeruginosa isolated from clinical specimens in teaching hospitals of Ahvaz, Iran. Iran J Microbiol 2015; 7: 2-6.

2. Vanegas JM, Cienfuegos AV, Ocampo AM, Lopez L, del Corral H, Roncancio G, et al. Similar frequencies of Pseudomonas aeruginosa isolates producing KPC and VIM carbapenemases in diverse genetic clones at tertiary-care hospitals in Medellin, Colombia. J Clin Microbiol 2014; 52: 3978-3986.

3. Hong DJ, Bae IK, Jang I-H, Jeong SH, Kang H-K, Lee K. Epidemiology and characteristics of metallo-β-lactamase-producing Pseudomonas aeruginosa. Infect Chemother 2015; 47: 81-97.

4. Ismail SJ, Mahmoud SS. First detection of New Delhi metallo-β-lactamases variants (NDM-1, NDM-2) among Pseudomonas aeruginosa isolated from Iraqi hospitals. Iran J Microbiol 2018; 10: 98-103.

5. Pitout JD, Gregson DB, Poirel L, McClure J-A, Le P, Church DL. Detection of Pseudomonas aeruginosa producing metallo-β-lactamases in a large centralized laboratory. J Clin Microbiol 2005; 43: 3129-3135.

6. Fournier D, Garnier P, Jeannot K, Mille A, Gomez A-S, Plésiat P. A convenient method to screen for carbapenem-producing Pseudomonas aeruginosa. J Clin Microbiol 2013; 51: 3846-3848.

7. Jabalameli F, Taki E, Emaneini M, Beigverdi R. Prevalence of metallo-β-lactamases-encoding genes among carbapenem-resistant Pseudomonas aeruginosa strains isolated from burn patients in Iran. Rev Soc Bras Med Trop 2018; 51: 270-276.

8. Ghamgosha M, Shahrekizadehani S, Kafizadeh F, Bamerti Z, Taheri RA, Farnoosh G. metallo-β-lactamases VIM-1, SPM-1, and IMP-1 genes among clinical Pseudomonas aeruginosa species isolated in Zahedan, Iran. Jundishapur J Microbiol 2015; 8: e17489.

9. Farhan SM, Ibrahim RA, Mahran KM, Hetta HE, Abd El-Baky RM. Antimicrobial resistance pattern and molecular genetic distribution of metallo-β-lactamases producing Pseudomonas aeruginosa isolated from hospitals in Minia, Egypt. Infect Drug Resist 2019; 12: 2115-2133.

10. Spilker T, Coene Y, Vandamme P, LiPuma JJ. PCR-based assay for differentiation of Pseudomonas aeruginosa from other Pseudomonas species recovered from cystic fibrosis patients. J Clin Microbiol 2004; 42: 2074-2079.

11. Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility testing. 28<sup>th</sup> ed. CLSI 2018.

12. Heinrichs A, Huang TD, Berhin C, Bogarets P, Glupczynski Y. Evaluation of several phenotypic methods for the detection of carbapenemase-producing Pseudomonas aeruginosa. Eur J Clin Microbiol Infect Dis 2015;34:1467-1474.

13. Yong D, Lee K, Yum JH, Shin HB, Rossolini GM, Chong Y. Imipenem-EDTA disk method for differentiation of metallo-β-lactamases-producing clinical isolates of Pseudomonas spp. and Acinetobacter spp. J Clin Microbiol 2002; 40: 3798-3801.

14. Poirel L, Walsh TR, Cuvillier V, Nordmann P. Multiplex PCR for detection of acquired carbapenemase genes. Diagn Microbiol Infect Dis 2011; 70: 119-123.

15. Janice J, Agycopong N, Owusu-Ofori A, Govinden U, Essack SY, Samuelsen Ø, et al. Carbapenem resistance determinants acquired through novel chromosomal integrations in extensively drug-resistant Pseudomonas aeruginosa. Antimicrob Agents Chemother 2021; 65: e0028921.

16. Bagheri Bejestani F, Hakemi-Vala M, Momtaheni R, Bagheri Bejestani O, Gholami M. The frequency of imp and vim genes among Pseudomonas aeruginosa isolates from children’s medical center of Tehran. Arch Clin Infect Dis 2015; 10: e02991.

17. Mirbagheri SZ, Meshkat Z, Naderinasab M, Rostami S, Nabavinia MS, Rahmati M. Study on imipenem resistance and prevalence of blaVIM1 and blaVIM2 metallo-beta-lactamases among clinical isolates of Pseudomonas aeruginosa from Mashhad, Northeast of Iran. Iran J Microbiol 2015; 7: 72-78.

18. Arunagiri K, Sekar B, Sangeetha G, John J. Detection and characterization of metallo-β-lactamases in Pseudomonas aeruginosa by phenotypic and molecular methods from clinical samples in a tertiary care hospital. West Indian Med J 2012; 61: 778-783.

19. Haghi F, Keramati N, Hemmati F, Zeighami H. Distribution of integrons and gene cassettes among metallo-β-lactamase producing Pseudomonas aeruginosa clinical isolates. Infect Epidemiol Med 2017; 3: 36-40.

20. Yousefi S, Farajnia S, Naheai MR, Akhi MT, Ghotaslou R, Soroush MH, et al. Detection of metallo-β-lactamase-encoding genes among clinical isolates of Pseudomonas aeruginosa in Northwest of Iran. J Glob Antimicrob Resist 2010; 68: 322-325.

21. Sader HS, Reis AO, Silbert S, Gales AC. IMPs, VIMs and SPMs: the diversity of metallo-β-lactamases produced by carbapenem-resistant Pseudomonas aeruginosa in a Brazilian hospital. Clin Microbiol Infect 2005; 11: 73-76.

22. Ellapan K, Narasimha HB, Kumar S. Coexistence of multidrug resistance mechanisms and virulence genes in carbapenem-resistant Pseudomonas aeruginosa strains from a tertiary care hospital in south India. J Glob Antimicrob Resist 2018; 12: 37-43.

23. Pellegrino FLPC, Casali N, Noueir SA, Riley LW, Moreira BM. A carbapenem-susceptible Pseudomonas aeruginosa strain carrying the bla<sub>NDM</sub> gene. Diagn Microbiol Infect Dis 2008; 61: 214-216.