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Original article

Distribution of Integrons and Gene Cassettes among Metallo-β-Lactamase Producing *Pseudomonas aeruginosa* Clinical Isolates

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Abstract

Background: Integrons are considered as to play a significant role in the evolution and spread of antimicrobial resistance genes.
Materials and Methods: A total of 120 clinical isolates of *Pseudomonas aeruginosa* (collected from Zanjan hospitals between March 2015 and February 2016) were investigated for molecular characterization of MBLs and Class I and II integrons. Antimicrobial susceptibility testing was also performed based on the CLSI guidelines. The frequency of MBL producing isolates and the susceptibility to various antimicrobial agents were investigated.
Results: Based on the obtained results, *Bla*$_{IMP}$ was the most frequently detected metallo-β-lactamase. The frequency of *bla*$_{VIM}$, *bla*SPM, and *bla*$_{SIM}$, in MBL producing isolates was 17.1, 57.1, and 14.1%, respectively. No *bla*$_{GIM}$ harboring isolate was detected in our study. We detected two (5.7%) multidrug resistant *P. aeruginosa* strains isolated from the urine and sputum samples, which harbored *bla*$_{NDM-1}$. These isolates also contained *bla*$_{IMP}$ and *bla*$_{SPM}$. Class I integron was detected in 94.3% of the MBL positive isolates while 8.5% of the isolates contained Class II integrons. Of five different gene cassettes identified in Class I and II integrons, cassette encoding resistance to trimethoprim (*dfr*) was found to be predominant.
Conclusion: These results indicate that Class I integrons are widespread among the MBL producing *P. aeruginosa* isolates. Therefore, appropriate surveillance and control measures are essential to prevent the further spread of MBL and integron producing *P. aeruginosa* in hospitals.

Keywords: Antibiotic resistance, Integron, Metallo-β-lactamase, *Pseudomonas aeruginosa*

1. Background

The emergence of multidrug resistant (MDR) *Pseudomonas aeruginosa* has become a serious problem for healthcare settings in developing countries (1). The dissemination of antibiotic resistance genes by horizontal transfer has currently been thought to play a major role in development of MDR strains. Carbapenems as imipenem and meropenem are the most potent antibiotics for treatment of *P. aeruginosa* infections (2). However, carbapenem resistance have also been reported in some studies to be due to metallo-β-lactamase (MBL) production in *P. aeruginosa* (3), decreased outer membrane permeability via loss of the OprD (D_2) porins (4-5), over expression of an efflux pump system (6), and hyper production of the chromosomally encoded cephalosporinase AmpC (7-8). According to Bush grouping, class B β-lactamases or MBLs are zinc-dependent enzymes that hydrolyze all β-lactams (except aztreonam) (9). To date, several families of MBLs have been identified in *P. aeruginosa*, including IMP, VIM, GIM, SPM, SIM, AIM, KHM, NDM, and DIM (10). Among the acquired MBLs, the IMP and VIM types have been observed frequently worldwide (11). A novel MBL named NDM-1 (New Delhi metallo-β-lactamase) was identified from *Klebsiella pneumoniae* and *Escherichia coli* isolates recovered from a Swedish patient transferring from India. Amino acid sequence of NDM-1 has only 32% identity with the most closely related enzymes VIM-1 and VIM-2. Sporadic cases of NDM-1 producing isolates have been reported from different countries, suggesting its widespread dissemination (12-14). Recently, the presence of NDM-1 in *K. pneumoniae* has been reported from Iran (15). The multidruge resistance nature of these isolates limits their treatment options (15). The MBL encoding genes are usually carried by plasmids that associated with genetic elements, mostly class I integrons. Integrons, as mobile genetic elements, are primary source of gene cassettes and disseminate antibiotic resistance genes within the microbial populations. Mobile integrons capture gene cassettes from the environment and incorporate them in the variable region of integron by a site-specific integrase. More than 130 different cassettes have been characterized within integrons. There are more than nine classes of integrons, in which class I integrons are mostly and commonly found in nosocomial and community environments, followed by class II and III (2, 16-17).

2. Objective

We investigated the antibiotic susceptibility patterns and frequency of MBLs (*bla*$_{IMP}$, *bla*$_{VIM}$, *bla*$_{SPM}$, *bla*$_{SIM}$, *bla*$_{GIM}$ and *bla*$_{NDM-1}$) in clinical isolates of *P. aeruginosa*. Furthermore, we determined the frequency of class I and II integrons and their gene cassette assortments in MBL producing isolates in Zanjan, Iran.

3. Materials and Methods

3.1. Bacterial isolates

During March 2015to February 2016, one hundred and twenty *P. aeruginosa* clinical isolates were collected randomly from four major university hospitals in Zanjan, Iran. These strains were isolated from different clinical specimens such as urine, blood, sputum, and stool. The identification of the intended isolates was performed by routine biochemical tests. Confirmed isolates of *P. aeruginosa* were placed at –70°C in Tripticase Soy Broth (Merck, Germany) containing 20% (v/v) glycerol for further analysis.

3.2. Antimicrobial susceptibility testing and phenotypic characterization

Isolates susceptibility to the following antibiotics was examined using the disk diffusion method according to the

Clinical and Laboratory Standards Institute (CLSI, 2015) guidelines (18), including: Aztreonam (30µg), Amikacin (30µg), Cefotaxime (30µg), Ceftazidime (30µg), Cefepime (30µg), Ciprofloxacin (5µg), Gentamicin (10µg), Imipenem (10µg), PolymyxinB (300 unit), Piperacillin (100µg), (MAST, Merseyside, U.K). *P. aeruginosa* ATCC27853 was used as the control strain for susceptibility testing. All the strains resistant to imipenem were tested for the production of carbapenemases. MBL E-test strips (AB Biodisk, Solna, Sweden) using imipenem and imipenem-ethylenediamine-tetra acetic acid (EDTA) were used according to the protocol recommended by the manufacturer (18).

3.3. PCR amplification of metallo- β-lactamase genes

MBL producing isolates were tested for *bla* genes including bla_{IMP}, bla_{VIM}, bla_{SPM}, bla_{SIM}, bla_{GIM}, and bla_{NDM-1} using the primers listed in Table 1. Extraction of DNA was performed according to the protocol provided with the Qiagen Mini Amp kit. The PCR mixture with a final volume of 25 µL contained 2 µL template DNA, 0.2 mM of each deoxy nucleoside triphosphate, 10 pmol of each primer, 10 mM Tris-HCl, 1.5 mM MgCl2, 50 mM KCl, 1.5 U of Taq DNA polymerase. PCR was performed with the Gene Atlas 322 system (ASTEC, Japan). Amplification involved an initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 50 s, annealing at 52°C for 40 s for bla_{IMP}, bla_{VIM}, bla_{SPM}, bla_{SIM}, bla_{GIM} and at 58°C for 60 s for bla_{NDM-1}, and an extension at 72°C for 1 min, with a final extension step at 72°C, for 8 min. The expected amplicons were ascertained by electrophoresis on a 1.5% agarose gel with appropriate molecular size markers (100bp DNA ladder; MBI Fermentas).

3.4. Characterization of integrons and sequencing of gene cassettes

Metallo-β-lactamase producing isolates were tested for characterization of Class I and II integrons and their gene cassettes. The primer sequences used in this study are shown in Table 1 The PCR was performed in a reaction mixture with a total volume of 25 µL, containing 2 µL template DNA, 0.2 mM of each deoxynucleoside triphosphate, 10 pmol of each primer, 10 mM Tris-HCl, 1.5 mM MgCl2, 50 mM KCl, 1.5 U of Taq DNA polymerase. Amplification was done as follows: an initial denaturation step at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 62°C for 1 min for *intI1* and *intI2*and at60°C for 1 min for *in* and *hep*, and an extension at 72°C for 2 min, followed by a final extension step at 72°C for 10 min. Amplified products were purified using QIAquick Gel Extraction Kit (Qiagen), and direct sequencing of internal variable regions (gene cassettes) of Class I and II integron was done using ABI 3730X capillary sequencer (Genfanavaran, Macrogen, Seoul, Korea). Nucleotide sequences were analyzed and compared using BLAST software (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov).

4. Results

4.1. Frequency of MBL producing isolates

A total of 120 clinical *P. aeruginosa* isolates were collected from the clinical specimens of four hospitals in Zanjan. A majority of the isolates were recovered from urine specimens (56 isolates, 46.7%), followed by blood (33 isolates, 27.5%), sputum (21 isolates, 17.5%), and stool (10 isolates, 8.3%). Among which, 35 isolates (29.2%) were MBL positive.

4.2. *Susceptibility to antimicrobial agents*

Antimicrobial susceptibility of *P. aeruginosa* isolates are presented in Tables 2The highest resistance rate among the isolates was shown against to Cefotaxime (43.3%), followed by Piperacillin (41.6%), Aztreonam and Gentamicin (37.5%). Only 68.3% of the isolates were imipenem susceptible whilst Polymixin B showed the highest activity against all the isolates (95.8% of the isolates were susceptible). A total of 50 (41.6%) isolates of *P. aeruginosa* were multidrug resistant (MDR). Moreover, all of the MBL producing isolates (35 isolates) were MDR. The most prevalent MDR pattern was resistance to β-lactams, Gentamicin, and Ciprofloxacin.

Table 1. Primer Sequences for Detection of metallo-β-lactamase, integrons and gene cassettes genes in *Pseudomonas aeruginosa*.

Target gene	Primer sequence (5' → 3')	Ampliconsize (bp)	Reference
bla_{IMP-F}	GGAATAGAGTGGCTTACATCTC	188	29
bla_{IMP-R}	CCAAACCACTACGTTATCT		
bla_{SPM-F}	AAAATCTGGGTACGCAAACG	271	29
bla_{SPM-R}	ACATTATCCGCTGGAACAGG		
bla_{SIM-F}	TACAAGGGATTCGGCATCG	570	29
bla_{SIM-R}	TAATGGCCTGTTCCCATGTG		
bla_{VIM-F}	GATGGTGTTTGGTCGCATA	390	29
bla_{VIM-R}	CGAATGCGCAGCACCAG		
bla_{GIM-F}	TCGACACACCTTGGTCTGAA	477	29
bla_{GIM-R}	AACTTCCAACTTTGCCATGC		
bla_{NDM1-F}	CTTCCAACGGTTTGATCGTC	263	15
bla_{NDM1-R}	ATTGGCATAAGTCGCAATCC		
Int1-F	CAGTGGACATAAGCCTGTTC	160	17
Int1-R	CCCGAGGCATAGACTGTA		
Int2-F	CACGGATATGCGACAAAAAGGT	788	17
Int2-R	GATGACAACGAGTGACGAAATG		
in-F (5'CS)	GGCATCCAAGCAGCAAGC	Variable	30
in-R (3'CS)	AAGCAGACTTGACCTGAT		
hep-F	CGGGATCCCGGACGGCATGCACGATTTGT	Variable	30
hep-R	GATGCCATCGCAAGTACGAG		

Table 2. Antimicrobial susceptibility of *P. aeruginosa* clinical isolates collected from Zanjan hospitls.

Intermediate [n (%)]	Susceptible [n (%)]	Resistant [n (%)]	Antimicrobial agent
22(18.3)	53(44.2)	45(37.5)	Aztreonam
8(6.6)	86(71.7)	26(21.7)	Amikacin
10(8.3)	58(48.4)	52(43.3)	Cefotaxime
5(4.1)	80(66.7)	35(29.2)	Ceftazidime
7(5.8)	84(70)	29(24.2)	Cefepime
14(11.3)	77(64.2)	39(32.5)	Ciprofloxacin
2(1.1)	73(60.4)	45(37.5)	Gentamicin
3(2.5)	82(68.3)	35(29.2)	Imipenem
2(1.6)	68(56.6)	50(41.6)	Piperacillin
2(1.7)	115(95.8)	3(2.5)	PolymixinB

4.3. Characterization of MBL genes

Metallo-β-lactamase genes were detected in all MBL producing isolates. *bla*$_{IMP}$ was the most frequently isolated metallo- β-lactamase, which was detected in 80% (28/35) of MBL producing *P. aeruginosa* isolates. The frequency of *bla*$_{VIM}$, *bla*$_{SPM,}$ and *bla*$_{SIM,}$ among the MBL producing isolates was 17.1% (6 isolates), 57.1% (20 isolates), and 14.1% (5 isolates), respectively. No *bla*$_{GIM}$ harboring isolate was detected in our study. Two (5.7%) multidrug resistant strains of *P. aeruginosa* isolated from the urine and sputum samples harbored *bla*$_{NDM-1.}$These isolates contained *bla*$_{IMP}$ and *bla*$_{SPM}$ simultaneously.

4.4. Analysis of integrons

The presence of integrons was confirmed in 33 (94.3%) cases of MBL producing isolates, of which 33 (94.3%) and 3 (8.5%) cases were identified as Class I (*intI1*) and Class II (*intI2*) integrons, respectively. Three (8.5%) isolates harboread both *intI1* and *intI2*; moreover, *intI1*was more frequent in comparison with *intI2* (*P*<.001). All of the MBL positive isolates harboring *intI1* or *intI2* were MDR. We amplified cassette regions of Class I and II integrons by primers 5'CS/3'CS and *hep*F/*hep*R, respectively. Four different amplicons were identified in Class I integrons with the following size, including 480 bp (14 isolates), 707 bp (2 isolates), 750 bp (7 isolates), and 990 bp (3 isolates). No product was obtained for seven (21.2%) of the*intI1* positive isolates. Six strains (3.8%) had two amplicons. A 1400 bp amplicon was obtained from a single isolate of *P. aeruginosa* harboring *intI2*. The analysis of cassettes sequence indicated the presence of dihydrofolate reductase Type A17 (*dfrA17*), dihydrofolate reductase Type I (*dfrA7*), aminoglycoside-2'-adenylyltransferase (*aadB*), aminoglycoside 3'-adenyltransferase (*aadA1*), and dihydrofolatereductase 1-streptothricin acetyltransferase 2 (*dhfr1-sat2*) resistance gene cassettes among the isolates, corresponding to 480, 750, 707, 990, and 1400 bp PCR products, respectively.

5. Discussion

The emergence of metallo-β-lactamase and carbapenemase producing *P. aeruginosa* has become a serious problem in healthcare settings in developing countries. These multidrug resistant isolates are associated with higher mortality and morbidity and as reported in previous studies, the treatment of these infections has been further complicated in Asian countries (2, 19). Furthermore, the increase in carbapenem-resistant Enterobacteriaceae and non-fermenting Gram-negative bacilli is a major concern worldwide (15). In our study, 41.6% of the *P. aeruginosa* isolates were resistant to at least three different classes of antimicrobial agents and determined as multidrug resistant. Only 68.3% of the isolates were imipenem susceptible whilst polymixin B (95.8%) showed the highest activity against all the isolates, followed by Amikacin (71.7%) and Cefepime (70%). On average, resistance to third and fourth generation of cephalosporins was 32.2%: 43.3% to cefotaxime, 29.2% to ceftazidime, and 24.2% to cefepime.

During the last decade, several metallo-β-lactamases have been identified in *P. aeruginosa*, beginning with IMP-1 and its derivatives, which are widespread in Japan and China (20). Previous investigations indicated that IMP and VIM types of MBLs are also widespread in Asian countries such as Japan, Korea, China, Taiwan, and Iran (21-23). According to the results, 29.1% (35 isolates) of the *P. aeruginosa* isolates were MBL positive. Similar to a study conducted in China, *bla*$_{IMP}$ was the most commonly detected MBL in *P. aeruginosa* isolates (21). The frequency of *bla*$_{VIM}$, *bla*$_{SPM,}$ and *bla*$_{SIM}$ among the MBL producing isolates was 17.1, 57.1, and 14.1%, respectively. No *bla*$_{GIM}$ harboring isolate was detected in our study. The frequency of *bla*$_{IMP}$ was significantly higher than *bla*$_{VIM}$ (*P*<.001). In contrast, Saderi et al. (2010) (25) and Shahcheraghi et al. (2010) (21) indicated higher frequency of *bla*$_{VIM}$ in comparison with *bla*$_{IMP}$ (20-24).

According to our knowledge, this is the first report of NDM-1 producing *P. aeruginosa* isolates in Iran. Sporadic cases of NDM-1 producing isolates have been reported from different countries, suggesting its widespread dissemination (12-14). NDM-1 producing *P. aeruginosa* isolates are capable to destroy carbapenem antibiotics. Therefore, the spread of *P. aeruginosa* carrying NDM-1 gene is a serious global health threat (25). According to the results, two *P. aeruginosa* isolates harboring NDM-1 were resistant to all evaluated antibiotics, including third-generation cephalosporins, imipenem, ciprofloxacin, amikacin, gentamicin, aztreonam, piperacillin, and polymixin B. Although aztreonam is not hydrolyzed by MBLs, azteronam resistance observed in our study could be due to the presence of other beta-lactamase genes. In this study, NDM-1 positive isolates also harbored *bla*$_{IMP}$ and *bla*$_{SPM.}$ As reported in previous studies, patients with NDM-1-positive bacterial infection usually have a travel to India or Pakistan (15), but in the present study, the patients had traveled to Iraq before hospitalization. The ability of NDM-1 to spread among Enterobacteriaceae and Pseudomonaceae implies the possibility of numerous new NDM-1 cases to be detected in the near future.

Integrons are primary source of resistance gene cassettes and serve as reservoirs of antimicrobial resistance genes within microbial populations (26). The present study

characterized Class I and II integrons in 91.4 and 8.5% of the MBL producing *P. aeruginosa* isolates. Class II integrons are most frequently associated with members of the family Enterobacteriaceae (2). The association between integron production and nosocomial outbreaks was demonstrated previously (27). We found high frequency of Class I integrons among MBL producing isolates. Previous reports also have indicated the higher frequency of Class I integrons among *P. aeruginosa* in comparison to Class 2 (2, 28-29). Also, two NDM-1 positive isolates harbored Class I integrons. Most *P. aeruginosa* isolates carried Class I integrons with identical cassette array, suggesting that these isolates may have the same mechanisms for resistance acquisition. Gene cassettes encoding resistance to trimethoprim (*dfr*) were found to be predominant in the Class I integrons (63.6% of the isolates harboring *intI1*). Also, two NDM-1 positive isolates harbored *dfrA17* gene cassette. The *aad* cassettes conferring resistance to aminoglycosides were also detected in 15.1% of the isolates harboring *intI1*. *dhfr1-sat2* gene cassette detected in one isolate of Class II integrons may reflect co-transfer of resistance genes due to the genetic linkage between *dhfr* and *sat* cassettes. Our results are also consistent with the previous reports worldwide on the predominance of *dfrA* and *aad* gene cassettes among Enterobacteriaceae (19, 30).

6. Conclusion

According to our results, high frequency of metallo-β-lactamases and integrons in P. aeruginosa is serious threat in healthcare setting. Therefore, suitable surveillance and control methods are necessary to reduce the spread of metallo-β-lactamases and integron producing P. *aeruginosa* in hospitals.

Conflict of interests

Authors have no conflict of interest to declare.

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Authors' Contribution

Dr Zeighami and Dr Haghi designed the study and wrote the manuscript, Keramati and Hemati performed the experiments and analyzed data.

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