Insertional inactivation of oprD in carbapenem-resistant \textit{Pseudomonas aeruginosa} strains isolated from burn patients in Tehran, Iran

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Abstract

In this study, we report the insertion sequence ISP\textit{Ppu}21 in the \textit{oprD} porin gene of carbapenem-resistant \textit{Pseudomonas aeruginosa} isolates from burn patients in Tehran, Iran. Antibiotic susceptibility tests for \textit{P. aeruginosa} isolates were determined. Production of metallo-\textit{β}-lactamas (MBLs) and carbapenemase was evaluated and the \textit{β}-lactamase-encoding and aminoglycoside-modifying enzyme genes were investigated by PCR and sequencing methods. The mRNA transcription level of \textit{oprD} and \textit{mex} \textit{efux} pump genes were evaluated by real-time PCR. The outer membrane protein profile was determined by SDS–PAGE. The genetic relationship between the \textit{P. aeruginosa} isolates was assessed by random amplified polymorphic DNA PCR. In all, 10.52\% (10/95) of clinical isolates of \textit{P. aeruginosa} harboured the ISP\textit{Ppu}21 insertion element in the \textit{oprD} gene. The extended-spectrum \textit{β}-lactamase-encoding gene in ISP\textit{Ppu}21-carrying isolates was \textit{bla}\textsubscript{TEM}. PCR assays targeting MBL and carbapenemase-encoding genes were also negative in all ten isolates. The \textit{rmt}A, \textit{aad}A, \textit{aad}B and \textit{arm}A genes were positive in all ISP\textit{Ppu}21 harbouring isolates. The relative expression levels of the \textit{mexX}, \textit{mexB}, \textit{mexT} and \textit{mexD} genes in ten isolates ranged from 0.1- to 1.4-fold, 1.1- to 3.68-fold, 0.3- to 8.22-fold and 1.7- to 35.17-fold, respectively. The relative expression levels of the \textit{oprD} in ten isolates ranged from 0.57- to 35.01-fold, which was much higher than those in the control strain \textit{P. aeruginosa} PA01. Evaluation of the outer membrane protein by SDS–PAGE suggested that \textit{oprD} was produced at very low levels by all isolates. Using random amplified polymorphic DNA PCR genotyping, eight of the ten isolates containing ISP\textit{Ppu}21 were shown to be clonally related. The present study describes a novel molecular mechanism, ISP\textit{Ppu}21 insertion of the \textit{oprD} gene, associated with carbapenem resistance in clinical \textit{P. aeruginosa} isolates.

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Introduction

Carbapenems, such as imipenem and meropenem are often used as last resort antibiotics for the treatment of multidrug-resistant (resistant to at least three different classes of antibiotics) \textit{Pseudomonas aeruginosa} infections \cite{1}. \textit{Pseudomonas aeruginosa} is an opportunistic bacterial pathogen causing a variety of infections such as endocarditis, pneumonia, urinary tract infection, septicaemia, and skin, eye and ear infections. This organism is also a leading cause of morbidity and mortality among hospitalized burn patients worldwide \cite{2}. Selecting the appropriate drug to initiate antibiotic therapy is important to control the clinical outcome. Unfortunately, treatment with the most appropriate antibiotics is complicated because of the possible resistance of this bacterium to multiple classes of antibacterial agents, such as carbapenems (imipenem and meropenem) \cite{3}. \textit{Pseudomonas aeruginosa} can use

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a combination of chromosomally encoded and/or plasmid-encoded mechanisms to evade carbapenem therapy. However, in non-carbapenemase-producing P. aeruginosa, resistance to doripenem and meropenem is associated with other mechanisms [4]. The main reported mechanism of resistance to carbapenems involves the loss of OprD porin from the outer membrane through deletions, mutations or insertions in the oprD gene. One important mechanism of carbapenem resistance is insertion inactivation of the oprD gene by insertion sequence (IS) elements of various sizes [5]. In this study we define a novel insertion sequence in the oprD porin gene of carbapenem-resistant P. aeruginosa isolates from burn patients in Tehran, Iran.

Materials and methods

Ethics statement
This study was approved by the Ethics Committee of Shahid Beheshti University of Medical Sciences “IR.SBMU.RETECH.REC.1396.85” and financially supported by the Infectious Diseases and Tropical Medicine Research Centre, Shahid Beheshti University of Medical Sciences, Tehran, Iran (grant No. 9369).

Clinical samples and bacterial identification
A total of 95 clinical isolates of P. aeruginosa were obtained from patients hospitalized in Shahid Motahari Burn Care Centre, Tehran, Iran from March 2014 to March 2015. Wound swabs were collected from patients and immediately transported to the microbiology laboratory of the Department of Microbiology of Shahid Beheshti University of Medical Sciences, Tehran, Iran. Blood culture was also carried out in patients with a suspicion of sepsis. Strains were identified as P. aeruginosa using the standard biochemical tests, including OF, SIM and MR-VP tests, and pigment production on Mueller–Hinton agar (Merck, Darmstadt, Germany). Isolates identified as P. aeruginosa were preserved at −70°C in trypticase soy broth (Merck) supplemented with 20% glycerol until further processing [6].

Susceptibility testing
Susceptibility of the P. aeruginosa isolates to imipenem (10 μg), cefazidime (30 μg), cefotaxime (30 μg), meropenem (10 μg), doripenem (10 μg), ticarcillin (75 μg), piperacillin (100 μg), pipercillin/tazobactam (100/10 μg), ciprofloxacin (5 μg), cefepime (30 μg), aztreonam (30 μg), amikacin (30 μg) and gentamicin (10 μg) were determined using a disc diffusion method according to the CLSI [7]. The MIC values of seven selected antibiotics—amikacin, gentamicin, meropenem, imipenem, cefazidime, ciprofloxacin and colistin—were also determined using the microbroth dilution method at a final concentration for each antimicrobial agent from 0.5 mg/L to 256 mg/L [7]. Pseudomonas aeruginosa ATCC 27853 was used as the quality control strain.

Carbapenemase and metallo-β-lactamase determination
Phenotypic determination of metallo-β-lactamase (MBL) producers and carbapenemase producers was performed by the combined disc diffusion test [8] and Modified Hodge test, respectively [1].

Conventional PCR and sequencing analysis of resistance determinants
Responsible resistance genes encoding extended spectrum β-lactamases, MBLs, carbapenemases and aminoglycoside-modifying enzymes are listed in Table 1. DNA was extracted using the DNA extraction kit (GeNet Bio Company, Daejeon, Korea; Cat. No, K-3000) according to the manufacturer’s guidelines. PCR was conducted in a final volume of 25 μL with 12.5 μL of 2× Master Mix (SinaClon, Tehran, Iran; Cat. no. PR901638), including 1× PCR buffer, 0.4 mmol/L dNTPs, 3 mmol/L MgCl2 and 0.08 IU Taq DNA polymerase, 1 μL of 10 pmol of each primer and 7.5 μL of sterile distilled water. Amplification reactions were performed on a thermal cycler (Eppendorf, Mastercycler Gradient; Eppendorf, Hamburg, Germany) followed by 36 cycles of denaturation at 94°C for 45 s, annealing at 50°C to 59°C, according to the primers for each gene, for 45 s and extension at 72°C for 45 s. PCR products were electrophoresed on a 1%–1.5% agarose gel, visualized by ethidium bromide staining and photographed under UV light. Amplicons representing each studied gene were confirmed by sequencing analysis (Macrogen Korea). The obtained sequence results were examined by the NCBI BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/). Insertion sequences were characterized by the online tool ISfinder (https://www.is.biotoul.fr/).

Real-time RT-PCR
The expression levels of oprD, mexB, mexD, mexT and mexX genes were assessed by RT-PCR. After an overnight culture of bacteria on Luria–Bertani broth [9], RNAs were extracted using the RNX-Plus kit (Cat. No., RN7713C; SinaClon) according to the manufacturer’s instructions. The contaminating DNA was removed by DNase I (Fermentas, Waltham, MA, USA). The total RNA concentration was determined using the Nanodrop (DS-11 Spectrophotometer, USA). DNase-treated RNA was reverse-transcribed into cDNA using the Takara kit (Shiga, Japan). The primers used for real-time RT-PCR are shown in Table 1. Real-time RT-PCR assay was performed using the Power SYBR Green PCR Master Mix (Bioneer, Daejeon, Korea).
Korea) on a Corbett Rotor-Gene 6000 Real-Time rotary analyser (Corbett Life Science, Sydney, Australia). Samples were run in triplicate and contained 2 µL of cDNA per reaction. Controls run without reverse transcriptase confirmed the absence of contaminating cDNA in any of the samples. The relative expression of the investigated genes was normalized against the rpsL housekeeping gene, and was calculated based on a 2^ΔΔCT method. Results were obtained as the relative expression of the mRNA compared with that of P. aeruginosa PAO1.

### Outer membrane protein analysis

Bacterial outer membrane proteins were examined using previously described methods [9]. Following sonication, membranes were collected by ultracentrifugation at 100 000 g for 45 min. Outer membrane proteins were separated by SDS–PAGE, and gels were stained with Coomassie blue.

### DNA fingerprinting analysis

Random amplified polymorphic DNA (RAPD)-PCR was performed as described previously [6]. The RAPD fingerprints were analysed and genotypes were assigned on the basis of weight and number of band differences.

### Nucleotide sequence accession numbers

The DNA sequences of ISPpu21/oprD and ISPpu21 have been deposited in GenBank under accession numbers KT736319 and KT728193, respectively.

### Results

In this study, 95 P. aeruginosa isolates were identified as imipenem-resistant with MICs ranging from 4 to 256 mg/L. Antibiotic susceptibility tests in this study showed that P. aeruginosa isolates were resistant to all currently used antibiotics, including ß-lactams, fluoroquinolones and aminoglycosides, but remained susceptible to colistin. In all, 10.52% (10/95) clinical isolates of P. aeruginosa harboured an insertion element in the oprD gene (Fig. 1).

Phenotypic and genotypic determination of carbapenemase and MBLs in 10.52% (10/95) of P. aeruginosa isolates revealed absence of any carbapenemase activity and production of MBL in isolates. In contrast, these isolates were positive for the ESBL gene (blaTEM). In this study, the rmtA, aadA, aadB and armA genes were positive in all ISPpu21-harbouring isolates. The relative expression levels of the mexX, mexB, mexT and mexD genes in ten isolates ranged from 0.1-fold to 1.4 fold, 1.1-fold to 3.68-fold, 0.3-fold to 8.22-fold and 1.7-fold to 35.17-fold, respectively and relative expression levels of the oprD in ten

**FIG. 1.** PCR amplification of oprD gene from carbapenem-resistant Pseudomonas aeruginosa. M, DNA Ladder 100 bp; N, negative control; P, P. aeruginosa PAO1 strain; lane 4, oprD gene; lanes 2.3.5–9, ISPpu21/oprD combination.

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**TABLE 1.** DNA sequences used in PCR and real-time RT-PCR

| Primer for PCR | Sequence (5’→3’) | Expected size of amplicon (bp) | Reference |
|---------------|------------------|-------------------------------|-----------|
| oprD          | ATGAAAGTGTGAGAAGTGGGAG | 1329 [13] |          |
| oprD for RT-PCR | CAG ACTGCAACAACCCAGTGTGTC  | 195 [14] |          |
| mexB          | GGGCCTGTTAGGATGGATGTGGCAG | 297 [15] |          |
| mexD          | CTGAGGTATACGTCGCTAACCAAGTG | 79 [16] |          |
| mexT          | GTCCTCCTCCTCCCTCCAGCG | 165 This study | |
| mexX          | ACACCTGACCGAGTTCGAGA | 64 [9] |          |
| oprL          | GGAGCGTGTGCTACACCAACGTGT | 92 [16] |          |
| TEM           | TCGGGGAAGATATGCGGCC | 972 [17] |          |
| SHV           | TCGTACATGATGAGGACCC | 861 [17] |          |
| CTX-M         | TTGGCGATGCTAGCTACAGGAATA | 544 [17] |          |
| VEB           | CGACCTCCATTTCCTCCATGCTG | 643 [18] |          |
| GES           | TTGCAATGTGTCACACAGTC | 351 This study | |
| KPC           | CCGTCACTGCCTGTTCGGTGGT | 798 [19] |          |
| IMP           | GAAACCGTTATTGTTACATCATA | 587 [19] |          |
| VIM           | TGCTTTCAGAAGAGGTAGTTC | 390 [20] |          |
| NDM           | GGGGCTGTCGATTCGCTGGTGGT | 621 [20] |          |
| GFP           | TGACACACATTGCTGCTGGAATT | 477 [20] |          |
| SPM           | AAAATCTTTGACCGAGCGA | 271 [20] |          |
| mscA          | GTAGACATGTGATGAGGACTG | 635 [21] |          |
| mscB          | ATCGATACATGATGAGGACTG | 320 [21] |          |
| armA          | CGGCTGTTGACACACGCGA | 212 [21] |          |

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isolates ranged from 0.57-fold to 35.01-fold, which was higher than those in the control strain *P. aeruginosa* PAO1 (Table 2).

Outer membrane profiles obtained by SDS–PAGE indicated that except for one isolate, nine remaining isolates had a corresponding band for oprD in comparison to that of PAO1. Using RAPD-PCR genotyping, eight of the ten isolates containing IS*Ppu*21 were shown to be clonally related (Fig. 2, Table 2).

**Discussion**

Carbapenems, such as imipenem and meropenem, have been widely used as active antimicrobials to treat infections associated with *P. aeruginosa*. Producing carbapenemases has been reported as a major mechanism of carbapenem resistance in Iran [22]. Loss of OprD has also contributed to carbapenem resistance, especially imipenem resistance, in Iran [10]. The present study describes isolates of *P. aeruginosa* that exhibit reduced susceptibility to carbapenems other than carbapenemase production [1]. Antibiotic susceptibility tests in this study showed that *P. aeruginosa* isolates were resistant to all antibiotics, but remained susceptible to colistin. PCR assays targeting MBL and carbapenemase-encoding genes were also negative in all ten isolates. It seems that imipenem resistance in such bacteria is not mediated by carbapenemase enzymes. It has been shown that one of the most important mechanisms for imipenem resistance in *P. aeruginosa* is the absence of OprD, which is the oprD expression levels in the control strain *P. aeruginosa* PAO1 (Table 2).

Using RAPD-PCR genotyping, eight of the ten isolates containing IS*Ppu*21 were shown to be clonally related (Fig. 2, Table 2).

**Table 2.** Antibiotic susceptibility, PCR results for genes and gene expression data for *IS*Ppu*21*-carrying *Pseudomonas aeruginosa* isolates

| Isolate | CO | GEN | AK | IMP | MBL | CAZ | CIM | Relative expression levels | Inactivated into oprD | IS*Ppu*21/OprD RAPD-PCR |
|---------|----|-----|----|-----|-----|-----|-----|----------------------------|----------------------|-------------------------|
| PA11    | 23 | 256 | 8  | 128 | 32  | 16  | 16  | aadA- aadB- rmtA-armA        | 1.2                  | + + + + + + + + +       |
| PA17    | 23 | 256 | 8  | 128 | 32  | 16  | 16  | aadA- aadB-rmtA             | 1.2                  | + + + + + + + + +       |
| PA21    | 23 | 256 | 8  | 128 | 32  | 16  | 16  | aadA- aadB-rmtA-armA        | 1.2                  | + + + + + + + + +       |
| PA42    | 23 | 256 | 8  | 128 | 32  | 16  | 16  | aadA- aadB- rmtA-armA       | 1.2                  | + + + + + + + + +       |
| PA49    | 23 | 256 | 8  | 128 | 32  | 16  | 16  | aadA- rmtA                  | 1.4                  | + + + + + + + + +       |
| Psa6    | 23 | 256 | 8  | 128 | 32  | 16  | 16  | aadA- rmtA- 1.10             | 3.68                 | + + + + + + + + +       |

Abbreviations: GEN, gentamicin; AK, amikacin; IMP, imipenem; MEM, meropenem; CAZ, cefotaxime; CIP, ciprofloxacin; ESBL, extended-spectrum β-lactamase; MBL, metallo-β-lactamase. RAPD-PCR, random amplified polymorphic DNA polymerase chain reaction.

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FIG. 2. Random amplified polymorphic DNA-PCR fingerprints of Pseudomonas aeruginosa isolates from burn patients using primer 272. M, DNA molecular weight marker (1 kb); N, negative control; 1–12, clinical samples.

(ISPa133), China (ISPa1328, ISPre2), the USA (ISPa8, ISPa1635 and ISPa1328) and France (ISPa46 and ISPa1328) [1]. Mobile elements, such as IS can help Gram-negative bacteria to promote their survival and adaptation to altered environmental niches through the interruption of genes and genomic modifications. This type of adaptation has been well reported in clinical isolates of P. aeruginosa that are resistant to β-lactams [4]. ISPpu21, identified in the present study, is a 1179-bp insertion sequence with a couple of 6-bp inverted repeats (TCTGAA) at its extremes. Interestingly, ISPpu21 elements introduced at insertion site 1 and insertion site 2 generate a pair of 7-bp and a pair of 4-bp target site duplication direct repeats, respectively. Just a single open reading frame was discovered in ISPpu21, which encodes a protein with 326 amino acids. The ISPpu21 nucleotide sequence is 100% identical to a region of O antigen biosynthesis gene cluster (GenBank accession no. AF498406) of P. aeruginosa serotype O15-ATCC with the open reading frame in this ISPpu21 orthologue transcribed in the opposite direction from the P. aeruginosa chromosomal gene cluster [24]. The putative product of the open reading frame in ISPpu21 has a homology (identities from 61% to 93%) to transposase proteins belonging to the IS5 family of transposases [24]. A previous study has demonstrated that resistance to carbapenems, such as meropenem and doripenem in P. aeruginosa isolates that do not produce metallo-β-lactamase, could be developed through both down-regulation and loss of OprD in the outer membrane and up-regulation of MexAB-OprM in these strains [4]. In this study, the relative expression levels of the mexX, mexB, mexT and, mexD genes in the isolates ranged from 0.1-fold to 1.4-fold, 1.1-fold to 3.68-fold, 0.3-fold to 8.22-fold, and 1.7-fold to 35.17-fold, respectively, and the oprD expression level in ISPpu21-carrying isolates ranged from 0.57-fold to 35.01-fold, which was much higher than in the control strain P. aeruginosa PAO1 [4]. However, the susceptibility analysis results for nine isolates were contrary to others, so imipenem resistance could not be attributed to the increased expression of the oprD gene. These results were similar to those by Sun and Dennis, in which the relative expression levels of the oprD gene in IS-positive isolates were higher than the control strain P. aeruginosa ATCC27853 [24]. In addition, we found one isolate that showed decreased expression of the oprD gene. These results were also similar to those of Wolter et al., who reported how an IS element with 97% similarity to ISPa16 inserted in the promoter of the oprD gene in two isolates, P. aeruginosa PA323 and P. aeruginosa PA415, reduced the oprD expression and increased imipenem MIC value [25]. Outer membrane proteins obtained by SDS–PAGE indicated that, with the exception of one isolate, the nine remaining isolates had a corresponding band for oprD in comparison to that of PAO1. Although these results signify the presence of a functional OprD in our clinical isolates, they strongly indicate that the OprD is absent or produced at very low levels. These results were similar to those of Shen et al., who reported a reduction in the size of OprD through the insertion of an IS element in the oprD gene of six isolates. In contrast, both imipenem-susceptible strains and the PAO1 control strain showed the expected protein band for OprD at 46 000 MW [26]. In the other study, Fournier et al., showed that an IS insertion in the oprD gene of one isolate may be associated with down-regulated OprD production [27]. RAPD-PCR was performed for typing all the isolates harbouring ISPpu21 in order to survey whether the IS-carrying strains collected in this study were associated with nosocomial infections. On observing DNA fingerprinting results, three patterns of these isolates were obtained. In the other word, eight of the ten isolates were clonally related. The results showed that the high prevalence of ISPpu21-carrying isolates was related to nosocomial infections.

Conclusion

Carbapenem resistance mediated by OprD deficiency in P. aeruginosa clinical isolates may be more frequent in Iranian hospitals, which could in turn be derived from the insertional inactivation. In this study, we report the emergence of ISPpu21 in carbapenem-resistant P. aeruginosa strains isolated from burn patients in Iran.

Transparency declaration

The authors declare that they have no competing interests.

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