Analysis of Metallo-β-lactamases, oprD Mutation, and Multidrug Resistance of β-lactam Antibiotic-Resistant Strains of *Pseudomonas aeruginosa* Isolated from Southern China

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Abstract

The purpose of this study was to analyze the metallo-β-lactamases (MBLs) genotype and oprD mutations of the β-lactam antibiotic-resistant *Pseudomonas aeruginosa* (PA) strains isolated from southern China. We collected 110 strains of β-lactam antibiotic-resistant PA from 2 hospitals during January 2016–December 2017 from Dongguan, South China. MBLs were detected, amplified, and typed using EDTA disc synergy test, PCR, and Sanger gene sequencing. The mutations and expression levels of oprD were detected using Sanger gene sequencing and qPCR. A total of 16.36% (18/110) β-lactam antibiotic-resistant PA strains produced MBLs, and the main genotypes of MBLs were IMP-25, VIM-2, and SIM-2. Sanger gene sequencing results showed that 107 of the 110 strains harbored mutations in oprD sequence, while 3 strains were negative for oprD amplification (2.73%). Among the 107 strains with positive amplification (97.27%), the rate of intentional mutations (including deletions, insertions, and premature stop codons) was 93.46% (100/107) and that of no disrupted mutation was 6.54% (7/107). qPCR analysis confirmed that the expression level of the OprD protein in the 7 strains of no disrupted mutation was significantly reduced. Among the β-lactam antibiotic-resistant PA strains in southern China, 16.36% were positive for MBLs. The loss rate of oprD was 2.73%, and almost all PA strains showed oprD amplification variation or transcription downregulation. Thus, impaired oprD expression and MBLs production may be some of the mechanisms of β-lactam antibiotic-resistance of PA strains in southern China.

Introduction

*Pseudomonas aeruginosa* (PA) is a common opportunistic pathogen that can cause infections in immunocompromised patients or in patients with burns. Since the 1990s, the incidence of clinical isolates of PA has increased [1]. Clinically, β-lactam antibiotics are one of the most commonly used antibiotics for treatment of PA infections. However, the use of antibiotics for a long time may increase the drug resistance rate and multidrug resistance to β-lactam antibiotics in PA, thereby leading to difficulties in the treatment of PA infections [2, 3]. China’s monitoring report suggests that PA resistance to various antimicrobial agents is quite high in 2016 [4]. Therefore, the study of β-lactam antibiotic resistance and multidrug resistance mechanisms in PA is conducive to the prevention and treatment of PA infection.

Metallo-β-lactamases (MBLs), also known as class B β-lactamases, are carbapenemases that are usually identified in PA. MBLs can effectively hydrolyze β-lactams, including carbapenems, and aminoglycosides, and other antibiotics such as quinolones [5]. Various types of MBLs have been identified in PA, including IMP, VIM, SPM-1, GIM-1, SIM-1, NDM-1, and FIM-1. The production of MBLs is considered to be one of the important mechanisms for PA resistance [6, 7]. In addition, outer membrane porins, like OprD, that uptake amino acids, peptides, and carbapenems undergo insertion, frameshift, and mutation-mediated gene inactivation or expression suppression, which can increase carbapenem resistance in PA [8–11].

Electronic supplementary material

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Although the production of MBLs and the inactivation of \textit{oprD} in PA resistant to carbapenems have been reported in specific countries and regions such as Korea or in specific populations such as patients with fibrosis in South Korea [1, 9], the drug resistance mechanisms of \(\beta\)-lactam antibiotic-resistant strains of PA in China have not been still elucidated. The aim of this study was to analyze the drug susceptibility profiles of 110 isolates of \(\beta\)-lactam antibiotic-resistant PA from Dongguan, South China, and to determine the expression of MBLs and \textit{oprD} for revealing the drug resistance mechanisms in \(\beta\)-lactam antibiotic-resistant PA. This study is expected to provide valuable information on the drug resistance in PA for the diagnosis and treatment of infections caused by PA.

Materials and Methods

Bacterial Isolates and Susceptibility Testing

\textit{Pseudomonas aeruginosa} samples isolated from the patients who visited Dongguan People’s Hospital and Dongguan Donghua Hospital from 2016 to 2017 were stored in skimmed milk at \(-70\,^\circ\text{C}\). The strains were reconstituted from skimmed milk to a blood plate, and a single colony was picked from the blood plate. Antibiotic susceptibility and drug resistance of each strain was measured using the VITEK 2 Compact system (BioMérieux, Lyon, France). Drugs that were used for antibiotic susceptibility determination were ampicillin, ampicillin/sulbactam, piperacillin/tazobactam, cefazolin, cefotetan, ceftazidime, ceftriaxone, cepfepime, imipenem, amikacin, gentamicin, tobramycin, ciprofloxacín, levofloxacín, nitrofurantoin, meropenem, and compound sulfamethoxazole. Multiple \(\beta\)-lactam antibiotic-resistant samples were selected for subsequent analyses.

Pulsed-Field Gel Electrophoresis (PFGE)

A single colony was picked, inoculated on a nutrient plate, and incubated overnight at \(37\,^\circ\text{C}\). The concentration of the bacterial suspension was adjusted with a DENSIMAT turbidimeter with a turbidity of 3.8–4.2. Next, 1% Seakem Gold Glue was prepared. Bacterial genomic DNA was isolated on the gel block. The enzyme was digested with 40 U SpeI (Takara Bio, Otsu, Japan) endonuclease and incubated at \(37\,^\circ\text{C}\) for 4 h. PFGE was performed in a CHEF-DRIII (Bio-Rad Laboratories, CA, USA) electrophoresis apparatus. The electrophoresis parameters were 5–15 s for 9 h and 15–50 s for 9 h. After the electrophoresis was completed, the gel was stained with a GelRed nucleic acid dye. Correlation analysis of PFGE patterns was performed using the BioNumerics software version 4.0 (Applied Maths, Sint-Martens-Latem, Belgium).

Ethylendiaminetetraacetic Acid (EDTA) Disc Synergy Test

Since divalent cations (usually zinc) act as cofactors for maintaining the MBL enzyme activity and are inhibited by EDTA, EDTA is often used to detect MBL activity in PA [12]. Each isolate was inoculated in the Mueller–Hinton broth at \(1 \times 10^6\,\text{CFU/mL}\) and then inoculated on the agar medium with a cotton swab. Two discs (antibiotic disc and antibiotic + EDTA disc) were placed on the agar surface using sterile forceps with a spacing of approximately 30 mm. The EDTA disc was prepared by adding 250 \(\mu\text{L}\) of neutral EDTA to imipenem. The bacteriostatic inhibition zone was observed by incubation at \(37\,^\circ\text{C}\) for 24 h. When the inhibition zone was > 4 mm in diameter, the strain was considered to be an MBL-positive phenotype. In addition, a control tray containing only EDTA was used to determine the activity of EDTA to ensure that the test isolate was inhibited by EDTA without causing false-positive results.

\textit{Mbls} and \textit{oprD} Gene Amplification

DNA of each bacterial isolate was extracted using the TaKaRa Ex Taq® Hot Start Version (RR006Q, TaKaRa, Japan). Fragments of \textit{mbls} and full-length \textit{oprD} were amplified with the primers presented in Supplementary Table 1. The PCR program was set as follows: initial denaturation at \(94\,^\circ\text{C}\) for 5 min; followed by 30 cycles of \(94\,^\circ\text{C}\) for 30 s, \(52\,^\circ\text{C}\) for 40 s, and \(72\,^\circ\text{C}\) for 50 s; and final extension at \(72\,^\circ\text{C}\) for 5 min. The PCR products were sequenced for gene typing using Sanger sequencing (Sangon Biotech, Shanghai, China).

\textit{OprD} Mutation and Expression

Total RNA was isolated from the \(\beta\)-lactam antibiotic-resistant strains using the Trizol reagent (Thermo Fisher Scientific, IL, USA) according to the standard protocol. cDNA was synthesized using M-MLV Reverse Transcriptase (Promega, WI, USA). \textit{OprD} was amplified using the Bestar qPCR RT Kit (DBI Bioscience, Ludwigshafen, Germany) according to the manufacturer’s instructions. The relative expression of \textit{oprD} was normalized to that of the PAO1 standard strain and calculated by the \(2^{-\Delta\Deltact}\) method. \textit{RpsL} was used as the internal control.
Results

Drug Sensitivity and Resistance of β-Lactam Antibiotic-Resistant PA

The 110 strains of PA isolated from Dongguan were resistant to β-lactams, fluoroquinolones, tetracyclines, and sulfonamides. Among them, 100%, 100%, 98.18%, 97.27%, and 96.36% of the isolates were resistant to imipenem, ampicillin, nitrofurantoin, compound sulfamethoxazole, and cefazolin, respectively. Approximately, 31.82%, 27.27%, and 26.36% of the strains were resistant to ceftriaxone, ciprofloxacin, and levofloxacin, respectively. The resistance rate to amikacin was 10.00%, and the resistance rates to tobramycin, gentamicin, cefepime, and piperacillin/tazobactam were 17.27%, 19.09%, 21.82%, and 21.82%, respectively (Table 1).

MBLs Production in β-Lactam Antibiotic-Resistant PA

The EDTA disc synergy test showed that there were 18 (16%) isolates that were MBL-positive PA. Further typing results showed that 9 isolates were IMP-25 (8.18%), 8 isolates were VIM-2 (7.27%), and 4 isolates were SIM-2 (3.64%); in addition, in 3 isolates, both VIM-2 and SIM-2 were identified. The sequence alignment of these isolates in the GenBank was found to be identical to IMP-25 (EU352796), VIM-2 (AF191564), and SIM-2 (KT013203).

OprD Variation, Phylogenetic Analysis, and Expression Analysis of β-Lactam Antibiotic-Resistant PA

The phylogenetic analysis of the 110 β-lactam antibiotic-resistant PA isolates according to the oprD sequence revealed that the gene could be divided into 4 classes, and each class had a sequence with high homology in GenBank. The standard strain (PAO1) reference sequence was in the third class (Fig. 1).

The oprD typing results showed that 107 isolates were positive for oprD, while 3 were negative. The rate of oprD deletion rate was 2.73%. Further sequencing of the oprD-positive isolates revealed that there were no disrupted mutations in 7 isolates, while there were frameshift mutations in 84 isolates and single-base single-point mutations in 16 isolates with premature stop codons (Table 2).

The mRNA expression levels of oprD were detected in the 7 non-disruptive mutant isolates (numbered: 5, 11, 18, 23, 43, 81, and 107). The qPCR results showed a significant decrease in the expression of oprD in all the isolates compared to that in the carbapenem-susceptible PAO1 strains (Fig. 2). In addition, we detected the expression of oprD in 10 imipenem-sensitive PA strains, however, did not find any strains with reduced oprD expression (data not shown).

Discussion

There are only few studies on the drug resistance mechanisms of β-lactam antibiotic-resistant PA in southern China. One study had shown a mutation in oprD in imipenem-resistant PA in southern China [9]. Compared to the isolates in the previous study, the isolates in this study were characterized by multiple β-lactam antibiotic-resistance, and we analyzed the production and typing of MBLs from these isolates. We found that 18 of the 110 isolates of the β-lactam antibiotic-resistant PA in Dongguan, China, produced MBL, and 107 isolates were positive for oprD amplification. Further phylogenetic analysis of β-lactam antibiotic-resistant PA based on the oprD sequence revealed that these resistant isolates were mainly divided into 4 groups. OprD sequence analysis revealed a variation in oprD sequence of most β-lactam antibiotic-resistant PA strains. Among them, 84 isolates showed frameshift mutations, 16 showed premature stop codons, and 7 showed non-disruptive mutations. In particular, the 7 non-disruptive mutants showed a significant decrease in the gene expression level of oprD.

Currently, acquired MBLs include IMP, VIM, SPM, GIM, SIM, AIM, KHM, DIM, NDM, with IMP and VIM

Table 1 Sensitivity of β-lactam antibiotic-resistant Pseudomonas aeruginosa (PA) to commonly used antibiotics

| Antibiotics                  | Sensitivity | Resistance |
|------------------------------|-------------|------------|
| Imipenem                    | 0 (0%)      | 110 (100%) |
| Ampicillin                   | 0 (0)       | 110 (100%) |
| Nitrofurantoin              | 2 (1.82%)   | 108 (98.18%)|
| Compound sulfamethoxazole   | 3 (2.73%)   | 107 (97.27%)|
| Cefazolin                    | 0 (0%)      | 106 (96.36%)|
| Cefotetan                    | 2 (1.81%)   | 104 (94.55%)|
| Ampicillin/sulbactam         | 7 (6.36%)   | 103 (93.64%)|
| Ceftriaxone                  | 6 (5.45%)   | 100 (90.91%)|
| Meropenem                    | 14 (12.73%) | 82 (74.54%) |
| Ceftazidime                  | 54 (49.09%) | 35 (31.82%) |
| Ciprofloxacin               | 64 (58.19%) | 30 (27.27%) |
| Levofloxacin                | 69 (62.73%) | 29 (26.36%) |
| Piperacillin/tazobactam      | 47 (42.73%) | 24 (21.82%) |
| Cefepime                     | 75 (68.18%) | 24 (21.82%) |
| Gentamicin                   | 78 (70.91%) | 21 (19.09%) |
| Tobramycin                   | 83 (75.45%) | 19 (17.27%) |
| Amikacin                     | 96 (87.27%) | 11 (10%)    |
being the most common ones [13]. IMP, VIM, SPM, SIM, GIM, and NDM are of epidemiological and clinical significance in antibiotic resistance [14–16]. A previous national PA multidrug resistance test across 27 hospitals—mostly from southeastern China and southern China—shows that 8.2% of the strains produce MBLs, with IMP-9 being the most common one, followed by VIM-2 [17]. Another study shows that approximately 13 (35.1%) of the 37 multidrug-resistant PA strains isolated from the Hunan Province, China, produced IMP-4 and VIM-2 [18]. In our study, 18 resistant isolates (16.36%) carried MBLs—mainly IMP-25, VIM-2, and SIM-2. This result suggested that IMP-25, VIM-2, and SIM-2 played important roles in β-lactam antibiotic-resistance. In terms of the major epidemic types, our research suggested that the dominant type of MBLs in the southern region differed from the dominant types in the Hunan Province and the rest of the country. Simultaneously, there was also a consensus that the IMP type was dominant, followed by the VIM-2 type. This result was consistent with worldwide trends, indicating a potential association of IMP and VIM with PA multidrug resistance.

The inactivation of oprD is considered to be one of the mechanisms of PA resistance. Both sequence insertion and mutation can affect the activity of oprD [10, 19]. Our study found that oprD sequence of 107 isolates was mutated, while 3 isolates showed gene loss. In particular, the PCR results verified that the expression of oprD was decreased in the isolates with non-disruptive mutations, suggesting that the function of the oprD of these isolates was impaired. In addition, 3 isolates showed negative oprD amplification, suggesting the absence of oprD. Thus, we hypothesized that oprD, including positive or negative oprD amplification, might show abnormality in expression and function. The expression of oprD has been reported to be downregulated in multidrug-resistant PA in China [9, 18]. Our study further provided evidence that oprD expression was reduced in the non-disruptive mutant isolates. Studies have suggested that more than 50% of isolates have consistent characteristics of MBL and oprD [20]. Our study also showed a similar trend of both impaired MBLs production and mutated oprD sequence, which further supported this opinion.

**Conclusion**

In summary, we analyzed the MBLs production of the β-lactam antibiotic-resistant PA strains in southern China and the variations in the oprD sequence, which were the 2 important mechanisms of PA resistance. Our results indicated that the drug resistance mechanism of
β-lactam antibiotic-resistant PA in southern China might be attributed to the impaired oprD expression and MBLs production. Our study further provided evidence of the MBLs production and the variation in the oprD sequence as the clue for the treatment of infections caused by β-lactam multidrug-resistant PA in southern China.

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Author Contributions The study concept and design, and the manuscript revision were performed by BZ. The experiments performance, data analysis and the manuscript draft were performed by FL and DC. The study design, study implementation and manuscript revision were performed by LL, DL and FW. All authors read and approved the final manuscript.

Data Availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Research Involving Human Participants and/or Animals This article does not contain any studies with human participants or animals performed by any of the authors.

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