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Multiple Mechanisms Synergistically Induce Pseudomonas Aeruginosa Multiple Drug Resistance

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Abstract: The aim of this study was to understand the molecular epidemiological characteristics and drug resistance mechanism of carbapenem-resistant *Pseudomonas aeruginosa* (CRPA) and to provide a theoretical basis for the prevention and treatment of CRPA infection in hospitals. A total of 34 CRPA strains were isolated, and resistance to 13 commonly used antibiotics was detected using the TDR-300B Plus VitEK-2 compact automatic bacterial identification instrument. Then, carbapenemase production was detected using the Carbe NP test. RT-qPCR was used to detect the expression of efflux pump *MexA* and outer membrane protein *OprD*, and PCR amplification and sequence analysis were used to detect class I integrons carried by drug resistance genes. Our results showed that of the 34 CRPAs, 22 were multi-drug resistant (MDR), and five were extensively drug-resistant (XDR). Sequencing analysis showed that class I integron mainly carried aminoglycosides or quinolones resistance genes. Multiple mechanisms play important roles in the formation and development of MDR or XDR.

Keywords: *Pseudomonas aeruginosa*; carbapenem; drug resistance mechanisms; MDR; XDR

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1. Introduction

Pseudomonas aeruginosa (*P. aeruginosa)* is a common opportunistic pathogenic bacteria in hospitals, which often adheres to the surface of medical equipment. Once the immunity of the human host is compromised, it can lead to a number of infections, including respiratory tract infections, skin infections, urinary tract infections, and burn infections. *P. aeruginosa* can also be spread in the blood, inducing disseminated bacteremia, sepsis, and even death [1]. In recent years, a clinical concern has been raised due to *P. aeruginosa*'s multiple drug resistance (MDR) and extensive drug resistance (XDR), causing difficulties in clinical treatment [2]. Carbapenem is a commonly used drug in the clinical treatment of MDR-*P. aeruginosa* (MDR-PA) and XDR-*P. aeruginosa* (XDR-PA). Although novel Lactam antibacterial drugs with a broad antibacterial spectrum, strong antibacterial activity, and inhibition of almost all gram-negative bacteria are constantly being developed, *P. aeruginosa* often develops resistance against carbapenems [3]. This results in a decrease in treatment efficacy and an increase in dosage [4]. Based on emerging studies, resistance to carbapenems of *P. aeruginosa* is usually associated with carbapenemase production, excessive expression of active efflux system, expression of outer membrane protein, and integron-carrying drug-resistant genes. In addition, bacterial biofilm largely

prevents antimicrobial drugs from entering [5,6]. Therefore, we investigated drug resistance in *P. aeruginosa* isolated from the clinic, detected multiple mechanisms, and analyzed possible mechanisms of MDR and XDR.

2. Materials and Methods

2.1. Bacterial Strains

A total of 34 CRPA clinical bacterial isolates were collected from various clinical laboratories in Hunan Brain Hospital, China. Reference strains *P. aeruginosa* ATCC 27853 and *P. aeruginosa* ATCC 15692 (PAO1) were purchased from the Clinical Laboratory Center of the Ministry of Health.

2.2. Antimicrobial Susceptibility Testing

All strains were identified and tested with the TDR-300B Plus VitEK-2 compact automatic bacterial identification instrument (bioMérieux, Marceletouille, France). The minimum inhibitory concentration (MIC) method recommended by CLSI standards 2016–2018 was used for the determination. The antibiotics chosen were amikacin (AK), ceftazidime (CAZ), ciprofloxacin (CIP), levofloxacin (LEV), cefepime (CFPM), gentamicin (GM), tobramycin (TOB), imipenem (IPM), aztreonam (ATM), Polymyxin B (PB), piperacillin (PRL), piperacillin/tazobactam (TZP), and meropenem (MEM). Isolates shown to be resistant to IPM or MEM were defined as "CRPA", and those resistant to three or more drug classes were defined as "MDR-PA or XDR-PA" [7]. P. aeruginosa ATCC 27853 was used as a control for antibiotic resistance. Drug resistance rates were calculated according to the ratio of the number of drug-resistant bacteria in 34 CRPA clinical bacterial isolates.

2.3. Detection of Carbapenemase Production

Carbapenemase production was detected using the Carbe NP test as described by Bouslah [8]. This carbapenemase in bacteria was completely released through lysate-free tissue and hydrolyzed imipenem to produce acid. This changed the pH and caused the phenolic red color to change from red to yellow or orange, indicating carbapenemase as positive.

2.4. RT-qPCR Was Used to Detect the Expression Levels of Mex A and OprD

Total RNA was extracted from the exponential growth of bacteria in the Luria Bertani medium using TRT-101 (TOYOBO, Shanghai, China), and residual DNA was removed by DNase I. A cDNA was then synthesized using a reverse transcription kit (TOYOBO, China) with some modifications. The PCR reaction system was as follows: total volume was 25 µL, including 1 µL of reverse transcription product, 0.25 µL of upstream and downstream primers, 2× PCR buffer mix 12.5 µL, and 11.25 µL ddH_2O. The reaction conditions were pre-denaturation at 94 °C for 2 min, 35 cycles of denaturation at 94 °C for 30 s, annealing at 54 °C for 30 s, extension at 72 °C for 30 s, and final extension at 72 °C for 10 min.

cDNAs were subjected to semi-quantitative PCR using primers (Table 1), and relative gene expression was evaluated using *RpsL* representing the housekeeping gene. *P. aeruginosa*-PAO1 was used as a reference for the normalization of relative mRNA levels. *MexA* were considered overexpressed when their transcriptional levels were at least two times higher than those of PAO1, and *OprD* expression decreased when their transcriptional levels were equal to or less than 30% of PAO1 [9].

Table 1. Primers used in the experiment.

Genes	Primers	Primers Sequences(5′-3′)	Amplicon Size (bp)
rpsL	F R	CGCAACGTCGTGGCGTAT ACCCGAGGTGTCCAGCGAAC	226
OprD	F R	TTTCAACATCTACCGCACAAA CGTAGCCGTAGTTCTTATAGCC	389

Table 1. *Cont.*

Genes	Primers	Primers Sequences(5′-3′)	Amplicon Size (bp)
MexA	F R	GGCCGTGAGCAAGCAGCAGT CGACGGAAACCTCGGAGAA	377
IntI1	F R	GGCATCCAAGCAGCAAG AAGCAGACTTGACCTGA	Variable

2.5. PCR Amplification and Sequencing of Class I Integron

A Class I Integron variable region primer was designed as previously described [10]. Total DNA was extracted by TIANamp Bacteria DNA Kit (TIANGEN@, Beijing, China), PCR system was 25 µL, including Premix Taq 12.5 µL, template DNA 0.7 µL, upstream and downstream primers 0.8 µL each, and ddH_2O 10.2 µL. PCR amplification conditions were pre-denaturation at 95 °C for 5 min, 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 1 min, and final extension at 72 °C for 10 min. The products were sequenced at Sunnybio (Shanghai, China). Nucleotide sequences from variable regions were analyzed with the BLAST tool of NCBI (https://www.ncbi.nlm.nih.gov/, accessed on 1 January 2020) by comparison with sequences of reference strain sequences and PAO1 retrieved from the data bank.

2.6. Statistical Analysis

GraphPad Prism ware version 7.0 (GraphPad Software, SanDiego, CA, USA) was used for statistical analysis, and the data were expressed as mean ± standard deviation. A T-test was used to compare the two groups; $p < 0.05$ was considered significant.

3. Results

3.1. Antibiotic Sensitivity

The drug resistance rates of 34 strains of *P. aeruginosa* to meropenem and imipenem were 100% and 85.29%, respectively. Antibiotic resistance was as follows. There were 22 strains with antibiotic resistance of three or more, defined as MDR-PA. Five strains of bacteria were resistant to six types and more antibiotics, defined as XDR-PA (Table 2).

Table 2. Antimicrobial Resistance Phenotypes of Class I Integrons.

Strains	Drug Resistance Classification	Size	Resistance Genes
34, 37, 42	MDR	1.1–3.8 k	*Acc(6′)-lb, catB3, aadB3*
13, 27, 55	XDR		
78, 84	XDR	1.4 k	*Acc(6′)-lb, clmA6*
3, 7, 21, 77, 79	MDR	1.1 k	*qnrvc1*

3.2. Carbapenemase Production

Of the 34 CRPA strains detected by the Carbe NP test, six strains (n = 34, 17.6%) tested positive for the Carbe NP test (Table 3). Of these, five strains showed XDR and one strain showed MDR.

Table 3. CRPA resistance characteristics.

Strains	Carbe NP Test	Class I Integron	Relative mRNA		IMP	MEM	CAZ	Drug Resistance
			OprD	Mex A	MIC (µg/mL)			
3	−	+	−	−	≥32	≥32	32	MDR-PA
4	−	−	↓	−	≥32	4	0.5	CRPA
7	−	+	−	−	≥32	≥32	1	MDR-PA
9	−	−	↓	−	≥32	4	1	CRPA
11	−	−	−	↑	≥32	≥32	0.5	MDR-PA
13	+	+	−	−	≥32	≥32	16	XDR-PA

Table 3. *Cont.*

Strains	Carbe NP Test	Class I Integron	Relative mRNA		IMP	MEM	CAZ	Drug Resistance
			OprD	Mex A	MIC (μg/mL)			
16	−	−	↓	−	≥32	≥32	0.5	CRPA
21	−	+	−	−	≥32	≥32	1	MDR-PA
27	+	+	−	↑	≥32	≥32	256	XDR-PA
29	−	−	−	−	8	≥32	0.5	CRPA
30	−	−	↓	−	≥32	≥32	12	CRPA
32	−	−	↓	−	≥32	4	0.5	CRPA
34	+	+	−	↑	≥32	≥32	0.75	MDR-PA
37	−	+	↓	−	≥32	≥32	1	MDR-PA
42	−	+	↓	−	≥32	≥32	1	MDR-PA
46	−	−	−	−	≥32	4	0.5	CRPA
55	+	+	−	−	≥32	≥32	0.5	XDR-PA
57	−	−	↓	−	≥32	≥32	0.5	MDR-PA
61	−	−	−	−	≥32	≥32	32	MDR-PA
66	−	−	↓	↑	≥32	≥32	1	MDR-PA
70	−	−	−	↑	≥32	≥32	32	MDR-PA
72	−	−	−	−	≥32	≥32	256	MDR-PA
74	−	−	↓	−	≥32	4	1	MDR-PA
76	−	−	↓	−	≥32	≥32	16	MDR-PA
77	−	+	−	−	≥32	≥32	16	MDR-PA
78	+	+	↓	↑	≥32	≥32	256	XDR-PA
79	−	+	−	−	≥32	≥32	1	MDR-PA
80	−	−	↓	−	≥32	≥32	0.5	MDR-PA
81	−	−	−	↑	≥32	≥32	0.5	MDR-PA
82	−	−	↓	−	≥32	≥32	1	MDR-PA
84	+	+	−	−	≥32	≥32	256	XDR-PA
85	−	−	−	↑	≥32	≥32	1	MDR-PA
87	−	−	↓	−	≥32	≥32	1	MDR-PA
90	−	−	↓	−	≥32	12	0.5	MDR-PA

+ and − represent carbapenemase and non-carbapenemase producers, Class I integron and non-Class I integron producers, respectively. Relative to the expression level in reference strain PAO-1, assigned a value of 1.0. ↑ and ↓ mRNA expression was up-regulated or down-regulated compared to PAO-1.

3.3. Gene Expression Analysis

MexA, a membrane fusion protein attached to the inner membrane with a lipid anchor, is found in the periplasmic space, which is essential for in vivo transport and pump assembly. Additionally, *OprD*, is a channel from the outer membrane factor family located in the outer membrane that allows the extrusion of antibiotics. *MexA* and *OprD* are the two main genes that lead to *P. aeruginosa* resistance. The relative expression levels of *MexA* and *OprD* genes were determined by semi-quantitative PCR (Table 3). Results showed that 23.53% (n = 8) of isolates showed increased *MexA* mRNA, and 47.06% (n = 16) of isolates showed decreased transcription of *OprD* mRNA. According to the drug sensitivity test, 34 CRPA strains were divided into three groups: MDR-PA, XDR-PA, and CRPA (Figure 1A–C). Compared to the control group *PAO1*, *OprD* expression was significantly downregulated, although, in CRPA, XDR-PA, or MDR-PA, there were varying degrees of downregulation. *MexA* expression was slightly up-regulated in CRPA but significantly up-regulated in XDR-PA and MDR-PA groups, which may help increase *P. aeruginosa* resistance.

3.4. PCR Amplification and Sequencing of Class I Integron

Class I integrons were detected in 13 isolates (Figure 2A), of which eight were MDR-PA and five were XDR-PA (Figure 2B). The positive strains were sequenced, and the sequence comparison results showed that they carried three types of drug-resistant gene cassettes, Acc(6′)-lb, catB3, aadB, and Acc(6′)-lb, clmA6 cause *P.aeruginosa* to be insensitive to aminoglycoside drugs, while the resistance gene box qnrvc1 leads to resistance to quinolones. Both MDPA and XDPA strains carry aminoglycoside resistance genes (Figure 2C), which leads to a decrease in *P.aeruginosa*'s clinical sensitivity to aminoglycoside drugs. See Supplementary Figure S1 for sequencing results.

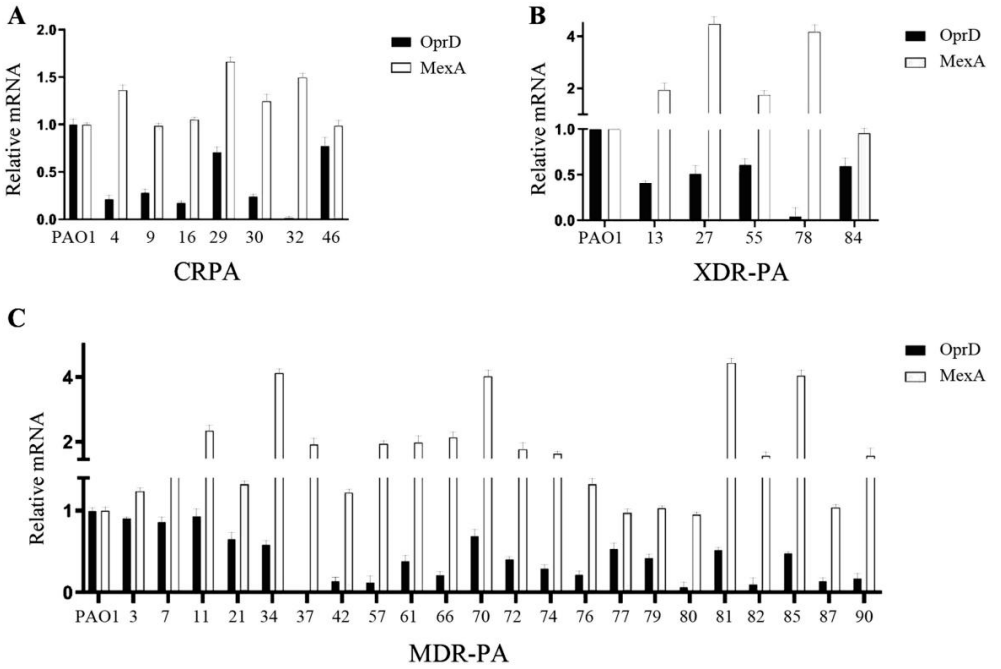

Figure 1. Expression of *OprD* and *MexA* mRNA increased in clinically isolated *P. aeruginosa*. (**A**) Carbapenem drug resistance group. (**B**) Extensively drug-resistant group. (**C**) Multi-drug resistance group. *MexA* were considered overexpressed when their transcriptional levels were at least two times higher than PAO1 levels, and *OprD* expression was decreased when their transcriptional levels were equal to or less than 30% PAO1 levels.

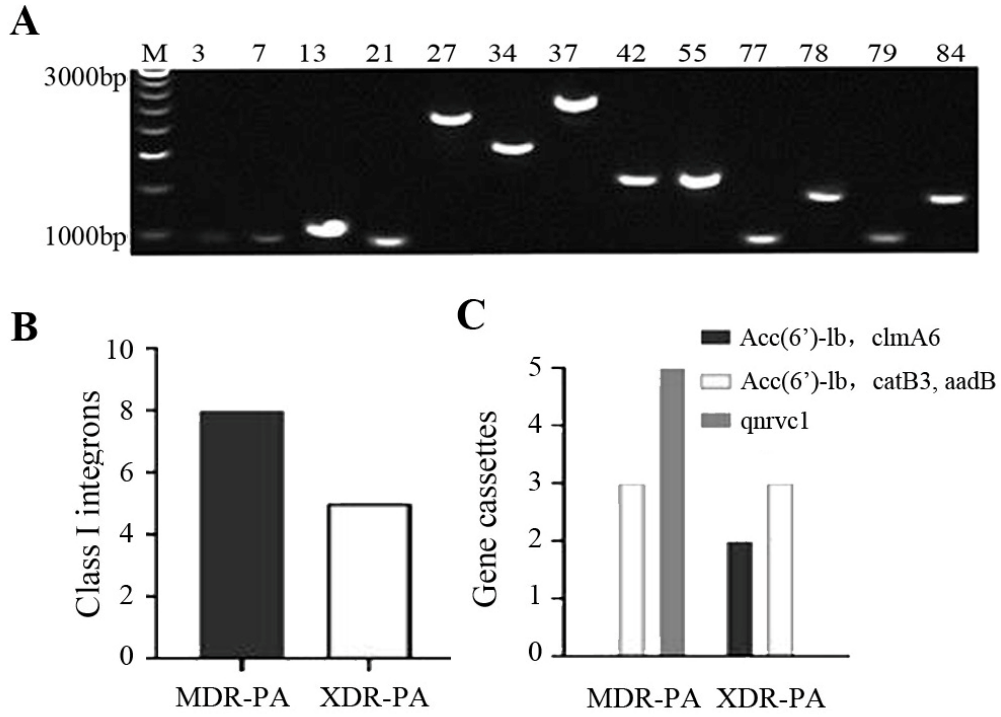

Figure 2. Resistance genes carried by Class I integrons. (**A**) Identification of gene cassettes by PCR. (**B**) Number of Class I integrons in MDPA and XDPA strains. (**C**) Gene cassettes carried by Class I integrons in MDPA and XDPA strains.

3.5. CRPA Resistance Characteristics

In carbapenem-resistant strains, only, low expression of the outer membrane protein *OprD* promoted bacterial drug resistance (Table 3). Among the MDR-PA and XDR-PA strains, carbapenemase, *MexA* efflux pump, outer membrane protein *OprD,* and Class I integrons all promoted the resistance of *P. aeruginosa* to varying degrees. More significantly, *OprD* expression was downregulated, and Class I integrons had a higher detection rate in MDR-PA and XDR-PA strains (Table 3). Analyzing the resistance mechanism of *P. aeruginosa*, we can see the joint action of multiple resistance mechanisms; six strains have two resistance mechanisms, and two strains have three resistance mechanisms. Meanwhile, there is one strain with these four resistance mechanisms, the XDR-PA strain, indicating that multiple resistance mechanisms are synergistic, leading to the high resistance of *P.aeruginosa* (Table 3).

All strains with down-regulated *OprD* had high imipenem MIC (≥32 μg/mL) resistance. All *MexA* over-expressed strains showed high resistance to imipenem (≥32 μg/mL) and meropenem (≥32 μg/mL). The bacterial strains with multiple drug resistance mechanisms at the same time were very low, including Class I integron, up-regulated *MexA,* and downregulated *OprD,* which was 2.94% (n = 1), manifested as XDR. The strains with Class I integron and increased *MexA* were 5.88% (n = 2), manifested as MDR or XDR. The proportion of Class I integron and downregulated *OprD* were 5.88% (n = 2), which was manifested as MDR. The strains with downregulated *OprD* and overexpressed efflux pump *MexA* were 2.94% (n = 1), presenting MDR. This suggests that multiple mechanisms play an important role in the development of MDR or XDR.

4. Discussion

Invasive hospital operation is an important cause of opportunistic infection of *P. aeruginosa*. In general, patients undergoing invasive surgery need to use antibiotics to control infection. However, such patients are usually in poor health and have compromised immunity. Especially in elderly patients, *P. aeruginosa* has become the main pathogen of infection in middle-aged and elderly patients, accounting for pathogens in the first place of pathogens [11].

Different types of antibacterial drugs are often used alone or in combination for the treatment of patients with *P. aeruginosa* infection, and β -lactamase antibiotics are the most common, especially carbapenem. In this study, 34 CRPA strains were taken as research subjects, and their drug resistance was analyzed. Results showed that the drug resistance rates of CRPA to meropenem and imipenem were 100% and 85.29%, respectively, indicating that carbapenem antibiotics should be used more carefully in clinics. The multi-drug resistance rate was 52.94%, and the pan-drug resistance rate was 14.71%. As can be seen from this situation, the drug resistance of *P. aeruginosa* has been severe, and more attention should be paid to the detection of *P. aeruginosa* resistance in clinical treatment, rational use of antibacterial drugs, and reduction of the spread of multiple drug-resistant *P. aeruginosa* in hospitals.

The mechanism of *P. aeruginosa* resistance to carbapenems is complex, including metal enzyme (MBLs), active outer membrane efflux system, and decreased outer membrane permeability [6,12]. In addition, *P. aeruginosa* can also capture external drug-resistant genes through horizontal transfer element integrons. Common integrons in *P. aeruginosa* are Class I integrons, which have many types of drug-resistant gene boxes and a wide range of hosts, making bacteria multi-drug resistant [13–15]. In this study, the Class I integron detection rate was 38.24% (n = 13) among 34 strains, mediated by quinolone and aminoglycoside resistance. In addition, Class I integron genes are more integrated on the bacterial chromosome, creating genetic stability and maintaining resistance [16]. However, we did not detect carbapenemase in integron I. The possible reasons are that the carbapenemase exists in other types of integrons, exists in the genome of living bacteria, or there are other mechanisms of drug resistance to β -lactamase antibiotics in bacteria, which will be further explored in the following research.

In 34 strains, the ratio of strains with multiple drug resistance mechanisms was very low. The bacterial strain with Class I integron, up-regulated *MexA*, and down-regulated *oprD* was 2.94% (1/34). Strains with two of the three mechanisms at the same time were 14.7% (5/34). The above results suggest that multiple mechanisms play an important role in CRPA formation and development. In addition, drug resistance genes carried by Class I integrons and the synergistic effect of multiple mechanisms play a synergistic role in the formation of MDR and XDR.

However, the study is only an epidemiological investigation. We will further investigate the whole-box resistance mechanism in bacterial multi-drug resistance and the coordination of multiple resistance mechanisms induced in vitro. Attempts will also be made to develop drugs to address resistance.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/microbiolres14020044/s1, Figure S1: Sequencing analysis of *aadB3* and *clmA6*.

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