Multiple Mechanisms Synergistically Induce *Pseudomonas Aeruginosa* Multiple Drug Resistance

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Abstract

Background: This study was designed to detect the molecular epidemiological characteristics and resistant mechanism of carbapenem resistant *Pseudomonas aeruginosa* (CRPA) which provide reference for the prevention and treatment of hospital CRPA infection.

Methods: 34 strains of CRPA from 2018 to 2019 were isolated and their resistance to 13 commonly used antibiotics was detected using TDR-300B Plus VitEK-2 compact automatic bacterial identification instrument. Then carbapenemase production was detected using Carbe NP test. The efflux pumps MexA and outer membrane protein OprD proteins were detected using RT-PCR and class integron carried with drug-resistant genes were detected using PCR and sequences analysis.

Results: Among 34 strains of CRPA, 22 strains were multiple drug resistance (MDR) and 5 strains were extensively drug-resistant (XDR). The results of class integron carried drug-resistant gene sequencing analysis showed the class integron mainly carried aminoglycoside or quinolone antibacterial drug resistant genes.

Conclusion: Multiple mechanisms play an important role in the formation and development of MDR or XDR resistance.

Background

P. aeruginosa is the common opportunistic pathogenic bacteria in hospitals, which often get adhered to the surface of medical machinery equipment. Once the immunity of the human host is compromised, it can lead to number of infections including, respiratory tract infection, skin infection, urinary tract infection and burn infection. *P. aeruginosa* can also be spread in blood, inducing disseminated bacteremia, sepsis and even death[1]. In the recent years, a clinical concern aroused due to *P. aeruginosa* resistant to multiple drug resistance (MDR) and extensively drug-resistant (XDR), causing difficulties to the clinical treatment[2]. Carbapenem are commonly used drugs in clinical treatment of MDR- *P. aeruginosa* (MDR-PA) and XDR- *P. aeruginosa* (XDR-PA). Although new-lactam antibacterial drugs with broad antibacterial spectrum, strong antibacterial activity and inhibiting almost all gram-negative bacteria constantly are developed, *P. aeruginosa* often develops resistance against carbapenems [3].This results in the decrease of treatment efficacy and the increase of the dosage[4]. Based on the emerging studies, the drug resistances to carbapenems of *P. aeruginosa are* usually associated with carbapenemase production, excessive expression of active efflux system; outer membrane protein expression and integron carried drug-resistant genes. Besides, bacterial biofilm to a great extent, prevents antimicrobial drugs from entering[5, 6]. Therefore, we investigated the drug resistance in *P. aeruginosa* isolated from the clinic, detected multiple mechanisms and analyzed the possible mechanisms of MDR and XDR.

Methods

Bacterial strains

A total of 34 CRPA clinical bacterial isolates were collected from various clinical laboratories in Hunan Brain Hospital, China, during the period from October 2018 to March 2019. Reference strains *P. aeruginosa* ATCC 27853 and *P. aeruginosa* ATCC 15692 (PAO1) were purchased from the Clinical Laboratory Center of the Ministry of Health.

Antimicrobial susceptibility testing

All strains were identified and tested with TDR-300B Plus VitEK-2 compact automatic bacterial identification instrument. 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 drugs class were defined as “MDR-PA or XDM-PA” [7]. *P. aeruginosa* ATCC 27853 was used as the control for antibiotic resistance.

Detection of carbapenemase production

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

Quantification of *Mex A* and *OprD*

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

The cDNAs were subjected to semi-quantitative PCR using primers (Table 1), relative gene expressions were evaluated using *RpsL* representing housekeeping gene. *P. aeruginosa*-PAO1 was used as a reference for normalization of relative mRNA levels. The *MexA* were considered over expressed when their transcriptional levels were at least diploid higher than those of PAO1, and the expression of *OprD* decreased when their transcriptional levels were equal to or less than 30% those 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
MexA	F R	GGCCGTGAGCAAGCAGCAGT CGACGGAAACCTCGGAGAA		377
IntI1	F R	GGCATCCAAGCAGCAAG	AAGCAGACTTGACCTGA	Variable

PCR amplification and sequencing of the class I integron

Class I Integron variable region primer was designed as previously described[10]. Total DNA was extracted by TIANamp Bacteria DNA Kit (TIANGEN®, 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, ddH_2O 10.2 μl. PCR amplification conditions were pre-denaturation at 9℃5 for 5 mins, 35 cycles of denaturation at 95 ℃ for 30s, annealing at 55 ℃ for 30s, extension at 72 ℃ for 1min, and last extension at 72 ℃ for 10 mins. The products were sequenced at Sunnybio (China).The nucleotide sequences of variable regionwere analyzed with BLAST tool of NCBI (https://www.ncbi.nlm.nih.gov/) by comparison with sequences of the reference strain and PAO1 retrieved from the data bank.

Statistical analysis

All experimental data were analyzed by WHONET 5.6 and SPSS 22.0 software.

Results

Antibiotic sensitivity

The drug resistance rates of the 34 *P. aeruginosa* strains to meropenem and imipenem were 100% and 85.29% respectively. Antibiotic resistances were as follows, there were 22 strains with antibiotics resistant to 3 and more, defined as multi-drug resistant bacteria (MDPA). Five strains of bacteria were resistant to 6 types and more of antibiotics, defined as extensively resistant strains (XDPA).

Carbapenemase production

Among the 34 CRPA strains detected by Carbe NP, 6 strains (n = 34) were positive for Carbe NP test (Table 3). Among them, 5 strains showed XDR and 1 strain showed MDR.

Gene expression analysis

The relative expression levels of *MexA* and *OprD* genes were determined by semi-quantitative (Table 3). The results revealed that 23.53% (n = 8) of isolates displayed increased *MexA* mRNA, and 47.06% (n = 16) of isolates displayed decreased transcription of OprD mRNA. According to the drug sensitivity test, 34 CRPA strains were divided into three groups: MDPA, XDPA and CRPA (Figure 1a-c). Compared with the control group PAO1, OprD expression was significantly down-regulated, although in CRPA, XDPA or MDPA, there were varying degrees of down-regulation. The expression of MexA was slightly up-regulated in CRPA, but a significant up-regulation in the XDPA and MDPA groups, which may help increase the resistance of *P.aeruginosa.*

PCR amplification and sequencing of the class I integron

Class 1 integrons were detected in 13 isolates (Fig.2). The positive strains were sequenced, and the sequence comparison results showed that they carried 3 kinds of drug-resistant gene cassettes (Table 2). *Acc(6')-Ib*, *catB3*, *aadB* and *clmA6* cause *P.aeruginosa* to be insensitive to aminoglycoside drugs, while qnrvc1 leads to resistance to quinolones. See Supplementary Figure 1 for sequencing results.

Table 2. Antimicrobial Resistance Phenotypes and Molecular Characterization of Genes in Class 1 Integrons

Strains	Drug resistance classification	Size	Resistance genes
34, 37,42	MDR	1.1k-3.8k	*Acc(6′)-Ib□catB3, aadB*
13, 27,55	XDR		
78, 84	XDR	1.4k	*Acc(6′)-Ib□clmA6*
3,7,21,77,79	MDR	1.1k	*qnrvc1*

Table 3. CRPA resistance characteristics

Strains	Carbapenemase	class I integron	Relative expression[a]		IMP	MEM	CAZ	Drug resistance
			OprD	Mex A	MIC (μg/ml)			
3	-	+	0.911	1.243	≥32	≥32	32	MDR-PA
4	-	-	**0.215 ↓**	1.365	≥32	4	0.5	CRPA
7	-	+	0.865	1.447	≥32	≥32	1	MDR-PA
9	-	-	**0.283 ↓**	0.988	≥32	4	1	CRPA
11	-	-	0.931	**2.337 ↑**	≥32	≥32	0.5	MDR-PA
13	+	+	0.415	1.933	≥32	≥32	16	XDR-PA
16	-	-	**0.176 ↓**	1.057	≥32	≥32	0.5	CRPA
21	-	+	0.655	1.324	≥32	≥32	1	MDR-PA
27	+	+	0.512	**4.476 ↑**	≥32	≥32	256	XDR-PA
29	-	-	0.709	1.666	8	≥32	0.5	CRPA
30	-	-	**0.242 ↓**	1.245	≥32	≥32	12	CRPA
32	-	-	**0.017 ↓**	1.496	≥32	4	0.5	CRPA
34	+	+	0.589	**4.134 ↑**	≥32	≥32	0.75	MDR-PA
37	-	+	**0.019 ↓**	1.907	≥32	≥32	1	MDR-PA
42	-	+	**0.143 ↓**	1.222	≥32	≥32	1	MDR-PA
46	-	-	0.775	0.988	≥32	4	0.5	CRPA
55	+	+	0.611	1.747	≥32	≥32	0.5	XDR-PA
57	-	-	**0.128 ↓**	1.932	≥32	≥32	0.5	MDR-PA
61	-	-	0.386	1.978	≥32	≥32	32	MDR-PA
66	-	-	**0.211 ↓**	**2.133 ↑**	≥32	≥32	1	MDR-PA
70	-	-	0.692	**4.023**	≥32	≥32	32	MDR-PA

				↑				
72	-	-	0.408	1.759	≥32	≥32	256	MDR-PA
74	-	-	**0.293 ↓**	1.634	≥32	4	1	MDR-PA
76	-	-	**0.217 ↓**	1.326	≥32	≥32	16	MDR-PA
77	-	+	0.534	0.976	≥32	≥32	16	MDR-PA
78	+	+	**0.045 ↓**	**4.165 ↑**	≥32	≥32	256	XDR-PA
79	-	+	0.427	1.034	≥32	≥32	1	MDR-PA
80	-	-	**0.068 ↓**	0.953	≥32	≥32	0.5	MDR-PA
81	-	-	0.523	**4.447 ↑**	≥32	≥32	0.5	MDR-PA
82	-	-	**0.102 ↓**	1.561	≥32	≥32	1	MDR-PA
84	+	+	0.598	0.956	≥32	≥32	256	XDR-PA
85	-	-	0.477	**4.052 ↑**	≥32	≥32	1	MDR-PA
87	-	-	**0.143 ↓**	1.043	≥32	≥32	1	MDR-PA
90	-	-	**0.176 ↓**	1.555	≥32	12	0.5	MDR-PA

+ and – represent carbapenemase and non-carbapenemase producers, Class 1 integron and non-Class 1 integron producers, respectively.

[a] Relative to expression level in the reference strain PAO-1, assigned with a value of 1.0

↑and↓mRNA expression was up-regulated or down-regulated compared with PAO1

CRPA resistance characteristics

All strains with down-regulated *OprD* had high imipenem MIC (≥ 32 μg/ml) resistance. All the *MexA* over expressed strains showed high resistance to imipenem (≥ 32μg/ml) and meropenem (≥ 32μg/ml). The bacterial strains with multiple drug resistance mechanisms at the same time were very low, among which, it carried class I integron, up-regulated *MexA* and down-regulated *OprD* 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 down-regulated *OprD* were 5.88% (n = 2), which was manifested as MDR. The strains with down-regulated *OprD* and over expressed efflux pump *MexA* were

2.94% (n = 1), presenting as MDR. This suggests that multiple mechanisms play an important role in the development of MDR or XDR(Table 3).

Discussion

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

Different kinds 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 strains of CRPA in hospital were taken as the research objects, and their drug resistances were analyzed. The results showed that the drug resistance rates of CRPA to meropenem and imipenem were 100% (n = 34) and 85.29% (n = 29), respectively, indicating that carbapenem antibiotics should be used more carefully in clinic. The multi-drug resistance rate was 52.94% (n = 18), and the pan-drug resistance rate was 14.71% (n = 5). As it 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[12-14]. In addition, *P. aeruginosa* can also capture external drug-resistant genes through gene horizontal transfer elements integrons. The common integrons in *P. aeruginosa* is class integron, which has many kinds of drug-resistant gene boxes and a wide range of hosts, making bacteria multi-drug resistance [15-17]. In this study, class I integron detection rate was 38.24% (n = 13) among 34 strains, mediated to quinolone and aminoglycoside resistance. Moreover, class I integron genes are more integrated on the bacterial chromosome, making the genetic stability and maintaining the resistance[18]. However, we did not detect carbapenemase in the 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.

Conclusion

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 the formation and development of CRPA. Moreover, the 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. We will further explore the formation mechanism of multi-drug resistance of *P. aeruginosa* by inducing the synergy of multiple mechanisms *in vitro.*

Declarations

Ethics approval and consent to participate: This article does not contain any studies with human participants or animals performed by any of the authors.

Consent for publication: Not applicable

Availability of data and material: the data are available from the corresponding author on reasonable request.

Competing interests: The authors declare that they have no competing interests

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Authors' contributions: YT and FJ designed the experiment; FJ, LY and OB performed the experiment; KA analysed the data; LY wrote original draft; YT and GW performed writing - review & editing.

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Figures

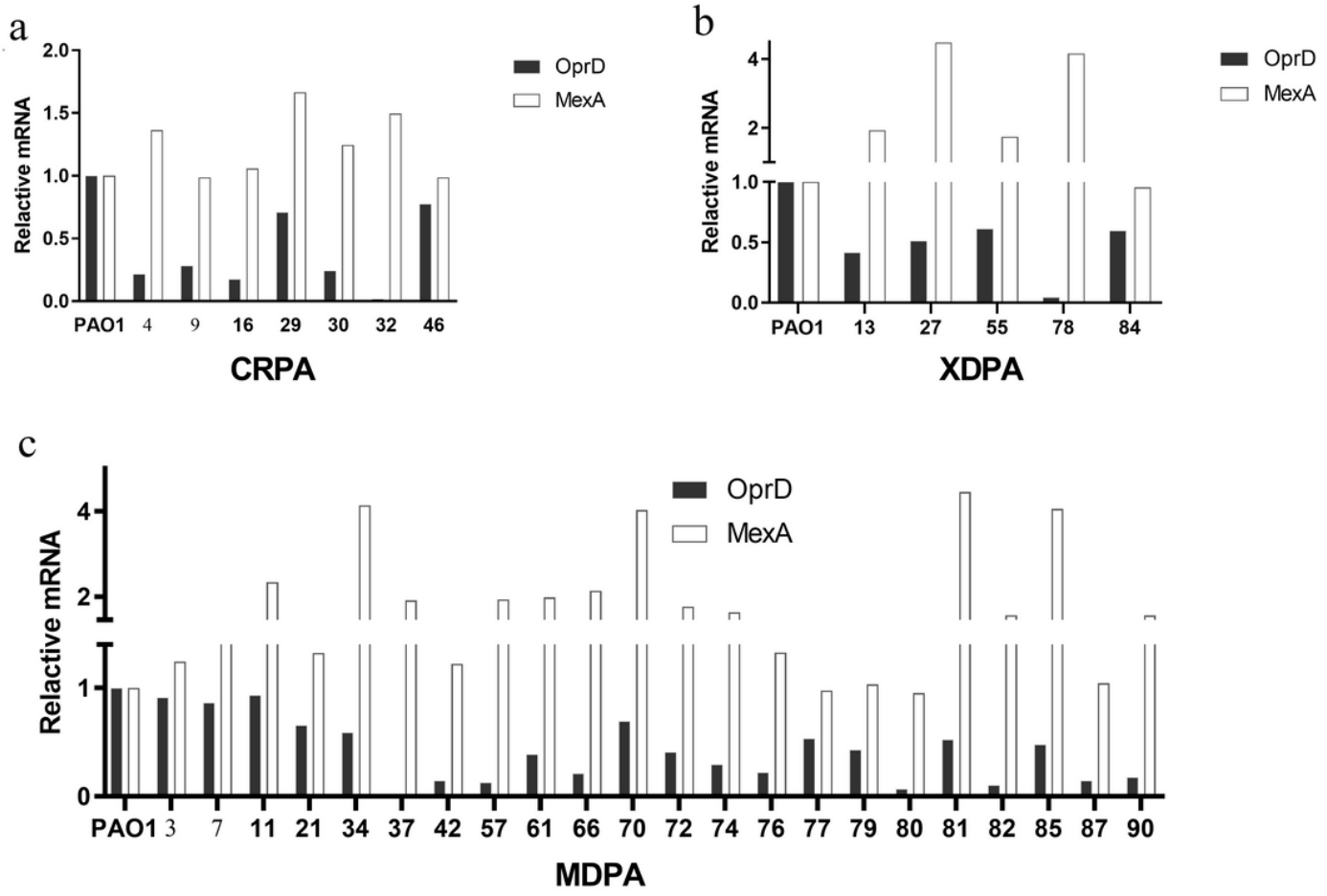

Figure 1

The expression of OprD and MexA mRNA increased in clinical isolated P. aeruginosa. (a) Carbapenem drug resistance group. (b) Extensively drug resistant group.(c) Multidurg resistance group. The MexA were considered over expressed when their transcriptional levels were at least diploid higher than those of PAO1, and the expression of OprD were decreased when their transcriptional levels were equal to or less than 30% those of PAO1

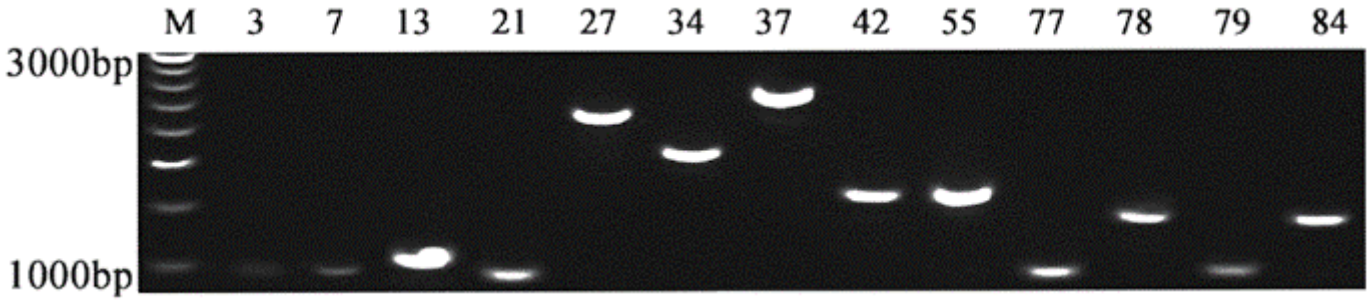

Figure 2

The variable regions of Class I integron were assayed using PCR. M: DNA ladder. Other lanes showed P. aeruginosa clinical isolates. Expression class I integrons in 3, 7, 13, 21, 27, 34, 37,42,55,77,78,79 and 84.

Supplementary Files

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- SupplementaryMaterial.tif