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Interaction of biofilm and efflux pump in clinical isolates of carbapenem resistant *P. aeruginosa*

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Abstract. – OBJECTIVE: Carbapenem-resistant *P. aeruginosa* (CRPA) is particularly worrisome because of its resistance against multiple antimicrobial agents which reduces treatment options. The efflux pump decreases antibiotic abundance, and biofilm impairs the penetration of antibiotics. The aim of the present study was to evaluate the role and relationship of efflux pump and biofilm formation in CRPA isolates obtained from different clinical samples.

PATIENTS AND METHODS: A total of 110 different clinical samples were collected from three tertiary medical hospitals. The samples were subjected to isolation and identification by standard operating procedures. Species level were identified using Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry system. Antibiotic susceptibility testing was done by broth microdilution method. Crystal violet (CV) staining for observing the biofilm forming ability and amplification of efflux pump *mexA* gene were also performed on clinical CRPA isolates. Three efflux pump MexAB-OprM regulatory genes were analyzed using sequencing methods. The expression of *mexA* gene both in biofilm and planktonic bacteria was observed by Quantitative real-time PCR (qRT-PCR).

RESULTS: The results showed that 110 samples were CRPA and among them 83 (75.5%) were MDR isolates. The CV staining showed 105 (95.5%) isolates as biofilm producers while 78 (74.3%) MDR isolates showed biofilm formation. *mexA* hyperexpression was detected in 27 (24.5%) CRPA isolates while 26 (96.3%) in biofilm forming isolates and 96.3% (26/27) in MDR *P. aeruginosa*. Multiple mutations in *nalC*, *nalD*, and *mexR* genes were detected. The distinct difference confirmed that the expression of *mexA* gene in *P. aeruginosa* biofilm producer was significantly higher than that of planktonic bacteria *in vitro*, and the efflux pump inhibitor PAβN significantly inhibited biofilms in CRPA isolated from clinical samples.

CONCLUSIONS: The biofilm and efflux pumps might be two intertwined processes involved in CRPA isolates. Their synergistic effect magnified the drug resistance characteristics of *P. aeruginosa*.

Key Words:
P. aeruginosa, CRPA, MDR, Biofilms, Efflux pump.

Introduction

P. aeruginosa, an ultimate opportunistic gram-negative pathogen, caused life threatening infections in patients with the compromised immune system[1]. It is one of the main pathogenic bacteria causing nosocomial infections (including respiratory system, urinary system and skin wounds), and often lead to repeated and prolonged chronic infections in cystic fibrosis patients[2-4]. Carbapenem antibiotics, which have wide antibacterial spectrum and high antibacterial activity, are commonly used in the treatment of multidrug-resistant gram-negative bacterial infection. Due to the widely application and overuse of carbapenem antibiotics, the *P. aeruginosa* with multidrug resistance (MDR) and extended drug resistance (XDR) had clinically emerged, which brought huge burden and challenges to clinical anti-infection treatment[5]. In 2018, the World Health Organization (WHO) took the carbapenem resistant *P. aeruginosa* (CRPA) as one of the extremely important pathogens in urgent need of the development and application of new antibiotics[6]. *P. aeruginosa* belongs to the "ESKAPE" group of bacteria, which includes *Enterococcus faecalis*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter Baumanii*,

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P. aeruginosa, and *Escherichia coli*. These bacteria usually "escape" antibiotics and disinfectants, resulting in high morbidity and mortality and economic burden[7]. Exploring the mechanisms of drug resistance is of great significance for clinicians to reasonably use of antibiotics.

Multidrug resistance in *P. aeruginosa* can be attributed to various mechanisms, including the biofilm formation and efflux pumps activity[8]. Biofilm is a complex and it is composed of a large number of bacterial colonies, extracellular polysaccharides, proteins, alginate and DNA, and plays an important role in persistent infections[9]. Since the barrier formed by biofilm can effectively reduce the entry of antibiotics, the bacteria could not reach the effective inhibitory concentration. According to reports, biofilms were responsible for 65% of human infections, and they had a 10 to 1,000-fold stronger resistance than planktonic cells[10]. Furthermore, compared to planktonic bacteria, bacteria in biofilm had worse air and nutrient interaction. Under such environmental pressure, some bacteria had even evolved to greatly improve their tolerance. As a result, biofilm-related infections were more difficult to eradicate[11].

Efflux pump system widely exists in microorganisms, and it is another important cause of bacterial resistance. It can timely discharge the metabolism waste and can also actively excrete toxic substances as well as antibiotics. Since it has no selectivity for excreting antibiotics, its overexpression plays an important role in the mechanism of inherent drug resistance and multiple drug resistance[12]. Major facilitator super family (MFS), resistance-nodulation division family (RND), ATP binding cassette family (ABC), small multidrug resistance family (SMR), and multi-drug and toxic compound extrusion family (MATE) are the five types of bacteria efflux system[13]. The RND mainly includes MexAB-OprM, MexCD-OprJ, MexEF-OprN, MexXY-OprM and so on[14]. MexAB-OprM, the first to be found in the RND family, is most closely related to carbapenem resistance in *P. aeruginosa*[15]. The regulatory genes *mexR, nalD* and *nalC*[14,16] negatively regulate the expression of MexAB-OprM and any type of mutant may lead to the upregulation of MexAB-OprM, thereby increasing drug resistance in *P. aeruginosa*.

To sum up, the efflux pump decreases antibiotic abundance, and biofilm impairs the penetration of antibiotics. However, the quantitative relevance of efflux and biofilm formation in more relevant clinical isolates remained largely unknown. Here, we aimed to explore the relationship between them in order to provide new treatment strategies, so as to better prevent and treat *P. aeruginosa* related infection.

Patients and Methods

Bacterial Strains and Antimicrobial Susceptibility Testing (AST)

This study was approved by the First Affiliated Hospital of Kumming Medical University Ethics Research Committee (No. L-20/2021) and exempted the application for informed consent. 110 carbapenems-resistant *P. aeruginosa* (CRPA) strains used in this study were collected from January 2017 to May 2021 in three tertiary medical hospitals (both in the First and second Affiliated Hospital of Kumming Medical University, and The First People's Hospital of Yunnan Province). *P. aeruginosa*, the first clinically isolated, was defined as carbapenem-resistant with MIC≥4 μg/mL for imipenem or meropenem according to CLSI guidelines. Bacterial cultures were subcultured on blood agar plates overnight to obtain monoclonal colonies at 37°C. All the strains were unrepeated isolated from hospitalized patients. Identification and susceptibility testing were performed using VITEK 2 compact automatic bacterial identification system according to Clinical and Laboratory Standards Institute procedures (CLSI M100-S30). Species levels were identified using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry system. All were identified as CRPA. The *P. aeruginosa* ATCC27853 was used as the quality control strain.

Biofilm Formation Assay

Quantification of the biofilm-forming ability of *P. aeruginosa* was determined using crystal violet method[17]. 110 CRPA strains were transferred to Columbia blood plate and cultured in 37°C incubator for 24 hours. Then, we prepared 0.5 McFarland standard bacterial suspension (1.5×10^8 CFU/ml) with LB broth and added 200 μl of the prepared bacterial solution into a 96-well tissue culture plate. *P. aeruginosa* ATCC 27853 was used as control, while LB broth as negative control. We kept the plate in a 37°C incubator for 24 hours. Following that, the plates were gently washed three times with 1×phosphate-buffered

saline (pH 7.4) to eliminate the planktonic bacteria, and the adherent biofilms were stained for 15 min at room temperature with 200 µl of 0.1% crystal violet solution. After washing it with running water and allowing 200 µl of 95% ethanol to dissolve for 2 min, the sample was measured at 570 nm using a microplate reader and the process was done three times.

The optical density cut-off value (ODc) was established as three standard deviations (SD) above the mean of the optical density (OD) of the negative control: ODc=average OD of negative control+3x SD of negative control. The biofilm formation ability was classified into four categories as follows according to optical density (ODi): ODi≤ODc=negative; ODc<ODi<2x ODc=weakly positive; 2x ODc< ODi<4x ODc=moderate positive; 4x ODc<ODi=strongly positive[18,19].

Quantitative Real-Time PCR (qRT-PCR)

The culture was made into bacterial suspension with LB broth, and cultivated overnight at 37°C, 180 rpm. The extraction of total RNA from *P. aeruginosa* isolates was performed using the total RNA extraction kit (Omega, USA) according to the manufacturer's instructions. The concentration and purity were determined by NanoDrop spectrophotometer (ND-1000, Wilmington, DE, USA). Synthesis of cDNA was performed using the Prime Scrip RT reagent Kit (TaKaRa, Dalian, China) according to the manufacturer's instructions.

mexA mRNA level was determined using TB Green Premix Ex Taq II (TaKaRa, Dalian, China) and examined by Quantitative real-time PCR system (SLAN-96P, Shanghai, China).

The sequences were as follows: *mexA* forward 5'-AACCCGAACAACGAGCTG-3', reverse 5'-ATGGCCTTCTGCTTGACG-3'; *rpoD* forward 5'-GGGCTGTCTCGAATACGTTGA-3', reverse 5'- ACCTGCCGGAGGATATTTCC-3'.

The cycling conditions were as follows: initial denaturation at 95°C for 30 s, 40 cycles denaturation at 95°C for 5 s, and elongation at 60°C for 30 s. Relative expression was determined using the $2^{-\Delta\Delta Ct}$ method. The *P. aeruginosa* ATCC 27853 was used as control. The RNase-free water was as negative control. All gene expression levels were compared with housekeeping gene *rpoD*. The assay was performed three times for each sample, and the mean was considered as the expression level. When transcription level was at least two-fold higher compared with that of *P. aeruginosa* ATCC27853, it can be considered as overexpression of MexAB-OprM efflux pump.

Detection of MexAB-OprM Regulatory Genes mexR, nalC, nalD

Genomic DNAs were extracted using the extraction kit (TIANGEN biochemical technology Co., Ltd, Beijing) according to the manufacturer's instructions. The PCR assay was performed to detect the three regulatory genes *mexR*, *nalC* and *nalD*. PCR amplification was performed in a 25 µl reaction mixture, containing 12.5 µl 2×Taq Master Mix; 8 µl ddH_2O; 1 µl primers of *mexR*, *nalC* and *nalD* and 2 µl DNA template. The conditions were as follows: 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 55°C for 30 s, with a final extension at 72°C for 10 min. The PCR products were used to visualize in the 2% agarose gel. The *P. aeruginosa* ATCC27853 and ddH_2O were used as controls.

Gene mexR, nalC, nalD Sanger Sequencing

The positive PCR amplicons (regulatory gene *mexR*, *nalC* and *nalD*) were sent to Biomed biotech Co. Ltd (Beijing, China) for sequencing. The results were spliced with DNAMAN software and analyzed by MEGA-X. *P. aeruginosa* PA01877857, 880362 and 880183 were used as the reference sequences of *mexR, nalC*and *nalD*, respectively. The specific primers were as follows: *mexR* forward 5'-TGTTCTTAAATATCCTCAAGCGG-3', reverse 5'-GTTGCATAGCGTTGTCCTCA-3'; *nalC* forward 5'-TCAACCCTAACGAGAAACGCT-3', reverse 5'-TCCACCTCACCGAACTGC-3'; *nalD* forward 5'-GCGGCTAAAATCGGTACACT-3', reverse 5'- ACGTCCAGGTGGATCTTGG-3'.

Expression of MexAB-OprM both in Biofilm and planktonic bacteria

Random 20 positive strains that the biofilm formation ability was >2x ODc were selected to detect the expression of MexAB-OprM. *P. aeruginosa* strains incubated in 37°C for 18-24 h. A single colony was inoculated into fresh LB (overnight at 37°C, 180 rpm) to prepare bacterial suspension.

The bacterial suspension incubated overnight at 37°C, 180 rpm, and the bacteria grew as planktonic bacteria. At the same time, 4 ml bacterial suspension was added into 6-well plates with a total volume 24 ml to incubation at 37°C for 18-24 h. We carefully aspirated the superna-

tant from the 6-well plate and discarded it, then, gently washed it with saline solution to remove planktonic bacteria. Finally, 4 ml saline solution was added into each well, blew repeatedly to make the attached biofilm fall off, and collected biofilm bacterial solution into 50 ml centrifuge tube. The planktonic bacteria and biofilm bacteria were centrifuged at 4000 g for 5 min to collect bacteria precipitate. The process of total RNA extraction, cDNA synthesis and qRT-PCR were as above.

PAβN Inhibited Biofilm Formation

The efflux pump inhibitor phenylalanine-arginine-β-naphthylamide (PAβN) (Sigma, Aldrich, St. Lous, MO, USA) were diluted to three concentrations (8 μg/ml, 4 μg/ml, 0 μg/ml). The *P. aeruginosa* (ATCC27853) and the LB broth were used as control. The biofilm formation was mentioned above. After incubation for 24 h at 37°C, the OD570 was quantified by a microplate reader. All tests were repeated three times independently.

Statistical Analysis

Statistical analysis was conducted using the chi-square test using SPSS software version 25 (IBM, Armonk, NY, USA). The comparison among different groups was performed by Chi-square test. All statistical graphs were performed using GraphPad Prism 8.0. p-value <0.05 were considered as statistically significance.

Results

P. Aeruginosa Isolates from Diverse Sources Display a High Prevalence for Multidrug Resistance

110 strains of *P. aeruginosa* were isolated from diverse sources: the urine (46/110), secretion (17/110), sputum (16/110), blood (11/110), hydrothorax and ascites (11/110) and bronchoalveolar lavage fluid (BALF) (9/110). All the strains were identified as being carbapenem-resistant *P. aeruginosa* (CRPA). 109 isolates (99.1%) displayed resistance to imipenem and 90 (81.8%) strains showed resistance to meropenem. In addition, 105 (95.5%) isolates were susceptible to colistin. For fluoroquinolones, 75 (68.2%) isolates were resistant to ciprofloxacin and 76 (69.1%) were resistant to levofloxacin. For aminoglycosides, 50 (45.5%) were resistant to tobramycin. For beta-lactam antibiotics, 66 (60.0%) were resistant to ceftazidime and 60 (54.5%) to cefepime. Based on the Centers for Disease Control and Prevention's (CDC, Atlanta, GA, USA) definition of multi-drug resistance as "an isolate that is resistant to at least one antibiotic in three or more drug classes", 83 isolates (75.5%) were deemed to be multi-drug resistant (Figure 1).

Higher Biofilm-Forming Abilities Exist in Clinically Isolated CRPA

The switch toward the biofilm mode of growth was often considered to be a survival strategy

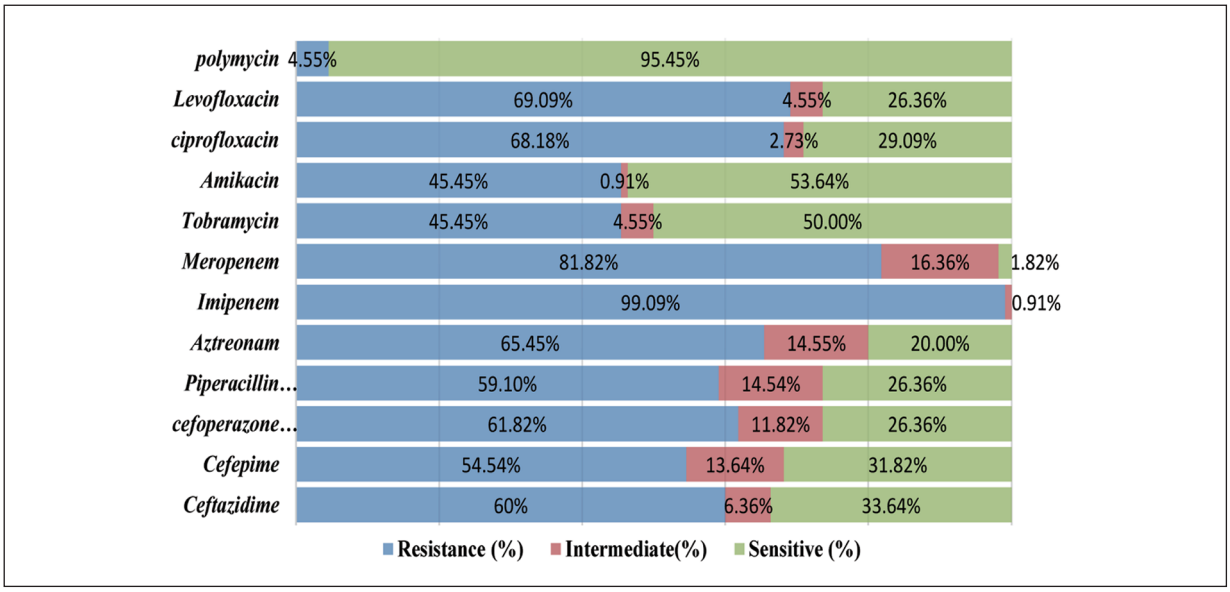

Figure 1. The resistance of *P. aeruginosa* isolates from different sources to antimicrobials.

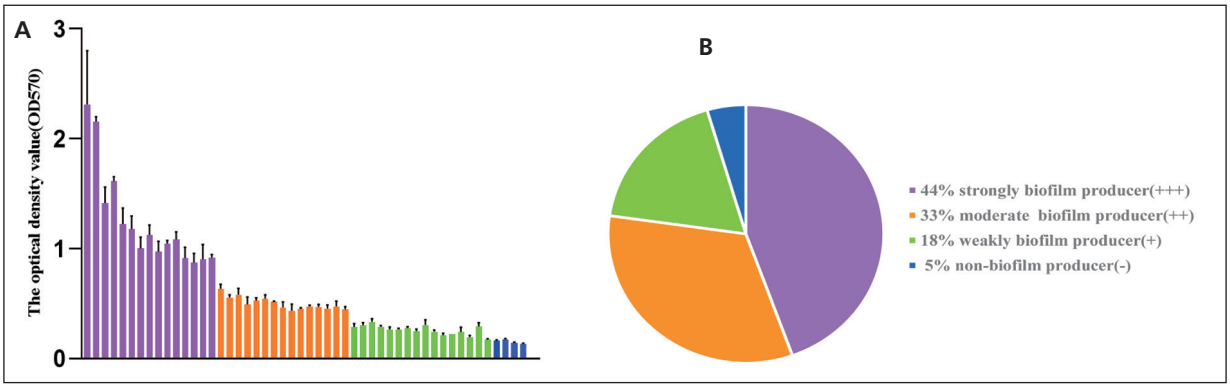

Figure 2. Biofilm-forming abilities of *P. aeruginosa* isolates analyzed by staining with crystal violet. Based on the OD values, the strains were classified in four categories: non-biofilm producer (ODi≤ODc), weakly biofilm producer (ODc<ODi≤2×ODc), moderate positive (2×ODc<ODi<4×ODc) and strongly biofilm producer(ODi>4×ODc). **A,** A representative diagram of biofilm formation strength in clinically isolated *Pseudomonas aeruginosa*. **B,** The percentage of different biofilm categories: strongly positive in purple, moderate positive in orange, weakly positive in green and the negative control in blue.

for bacteria[20], and we assessed whether all these CRPA isolates could form biofilms. Biofilm was classified into four groups according to crystal violet staining: strongly positive (+++), moderate positive (++), weakly positive (+) and negative (-). As shown in Figure 2, 105 strain (95.5%) produced significantly biofilm and only 5 (4.5%) isolates were negative (OD_{570}<0.18). Besides, strongly positive (+++), moderate positive (++), and weakly positive (+) biofilm producing strains accounted for 44.5% (49/110), 32.7% (36/110), and 18.2% (20/110) respectively.

As biofilms were shown to dramatically increase resistance to antimicrobial agents, we further analyzed resistance rates in the three biofilm-positive groups. Overall, 105 positive biofilm producing strains all showed high resistance with the exception of polymyxin. Interestingly, the resistance to tobramycin and amikacin was lower in the strongly positive group than in the moderate and weakly positive groups (p<0.05). (Table I).

Significant correlations between the Biofilm Formation and the Prevalence of Efflux pump MexAB-OprM Phenotype

Of the 110 CRPA strains, 27 strains overexpressed *mexA* gene, accounting for 24.5%. Only one strain was non-multidrug-resistant (no-MDR), and the rest were all multidrug-resistant (MDR). Among them, the relative expression of *mexA* gene were more than two times, even 4

Table I. The antibiotic resistance ratio in different biofilm formation ability groups.

Antibiotics	Strong positive (49)	Moderate Positive (36)	Weakly positive (20)	Total (105)	*p*-value
Ceftazidime	55.10%	61.11%	60.00%	58.10%	0.869
Cefepime	46.94%	61.11%	55.00%	53.33%	0.440
Piperacillin tazobactam	59.18%	50.00%	65.00%	57.10%	0.552
cefoperazone sulbactam	55.10%	63.89%	70.00%	60.95%	0.507
Aztreonam	59.18%	69.44%	70.00%	64.76%	0.554
Imipenem	100.00%	97.22%	100.00%	99.05%	0.533
Meropenem	81.63%	80.56%	80.00%	80.95%	1.000
Tobramycin	30.61%	50.00%	70.00%	44.76%	0.009*
Amikacin	30.61%	50.00%	70.00%	44.76%	0.009*
ciprofloxacin	61.22%	77.78%	70.00%	68.57%	0.259
Levofloxacin	67.35%	75.00%	65.00%	69.52%	0.698
polymycin	6.12%	2.78%	5.00%	4.76%	0.847

*p<0.05 indicates that there is a difference between strongly positive and weakly positive group.

isolates had exceeding five times. Interestingly, a high occurrence of biofilm phenotypes was found: 59.3% (16/27) of the isolates produced strong biofilm; 29.6% (8/27) produced moderate biofilm; 7.4% (2/27) produced weak biofilm, while 3.7% (1/27) of isolates were identified as non-biofilm producer (Table II).

Multiple Mutations Occurred in the Efflux Pump MexAB-OprM Regulatory Genes

In light of regulatory gene's role as a repressor of MexAB-oprM expression, the mutation reflected a loss of negative regulation. Three important regulators were sequenced. Regarding *mexR*, 40.7% (11/27) of the isolates showed point mutation or deletion mutations leading to amino acid changes, being V126E (8/11), the most frequent. One strain (PA041) had a deletion of the base fragment at position from 135th to 146th (sequence: TATCGACGAACA). In relation to *nalC*, 100% isolates showed point mutations, being G71E (27/27), S209R (24/27), Q182K (9/27), and L206V (10/27) the most frequent. Regarding *nalD*, 44.4% (12/27) of the isolates showed point mutations, being W49S the most frequent in 75% (9/12) of the isolates (Table III).

Evaluation of the Relationship Between Biofilm Forming Potential and Efflux Pump in P. Aeruginosa

As shown in Table II, 96.3% (26/27) of CRPA that upregulated efflux pump MexAB-OprM were characterized by biofilm producer strains, suggesting a significant relationship between the biofilm-forming ability and the MexAB-OprM overexpression phenotype. To identify this relationship, the expression levels of gene *mexA* in CRPA were compared under planktonic and biofilm conditions. The results showed that the expression level of *mexA* in biofilm bacteria was significantly higher than that in planktonic bacteria (p<0.001) (Figure 3A), with a difference of up to 177-fold in one strain. This result implied that strains with upregulated MexAB-OprM were more prone to form biofilms. Moreover, the efflux pump inhibitor phenylalanine-arginine-β-naphthamide (PAβN) significantly inhibited biofilms in 105 clinically isolated CRPA that produced biofilm (Figure 3B).

Discussion

P. aeruginosa is a bacterium known to produce robust biofilms. The unique biofilm properties further complicate the eradication of the biofilm infection, leading to limited treatment options[21]. The results of our study confirmed this observation. CV staining showed a high prevalence of biofilm-forming abilities (95.5%) in clinically isolated CRPA, and strongly positive (+++), moderate positive (++), and weakly positive (+) biofilm producing strains accounted for 44.5% (49/110), 32.7% (36/110), and 18.2% (20/110) respectively. Surprisingly, up to 75.5% of them were MDR isolates. The results suggested that multidrug resistant in *P. aeruginosa* might be heightened due to the production of biofilms. Consistent with our results, Karballaei et al[22] conformed that biofilm formation was higher in multidrug-resistant (MDR). This is justified, in part, because the growth of bacteria in the biofilm is about 64 times more resistant to antimicrobial[23].

Considering the high frequency of multidrug resistant strains, we speculated that other resistance mechanisms may coexist in these strains, such as efflux pumps. Studies[24,25] have shown that the efflux pump can actively pumped antibiotics out of the bacterial cells to reduce the drug concentration, resulting in drug resistance. Our study also showed that overexpression of *mexA* which characterized by the efflux pump MexAB-OprM (24.5%, n=27) was detected in CRPA, and only one was no-MDR, suggesting the efflux

Table II. Relationship between biofilm characteristic and overexpressed mexA gene among clinical isolates CRPA.

Phenotypic pattern of biofilm	MexAB-OprM overexpression phenotype no. (%)	Multiple drug resistance phenotype no. (%)
Strongly positive	16 (59.3%)	16 (61.5%)
Moderate positive	8 (29.6%)	7 (26.9%)
Weakly positive	2 (7.4%)	2 (7.7%)
Negative	1 (3.7%)	1 (3.9%)
Total	27 (100%)	26 (100%)

Table III. Results of MexAB OprM regulatory genes *mexR*, *nalC, nalD* mutations.

N.	MexR					NalC					NalD			
	V115E	L119K	V126E	L135H	R63H	G71E	A145V	Q182K	L206V	S209R	T11N	L17R	W49S	N130S
PA001	*	*	*	*	*	+	*	+	+	+	*	*	+	*
PA003	*	*	+	*	*	+	*	*	*	+	*	*		nt89-A
PA004	*	*	*	*	*	+	*	*	*	+	*	*	*	*
PA008	*	*	+	*	*	+	*	+	+	+	*	*	+	*
PA014	*	*	*	*	*	+	*	+	+	+	*	*	+	*
PA015	*	*	*	*	*	+	*	*	+	+	*	*	*	*
PA024	*	*	*	*	*	+	*	+	+	+	*	*	+	*
PA025	*	*	*	*	*	+	*	+	+	+	*	*	+	*
PA026	*	*	*	*	*	+	*	+	+	+	*	*	+	*
PA028	*	*	*	*	*	+	*	+	+	+	*	*	+	*
PA030	*	*	*	*	*	+	*	*	*	+	*	*	*	*
PA031	*	*	*	*	*	+	*	+	+	+	*	*	+	*
PA041	nt135-146 base deletion				+	+	+	+	*		*	*	+	*
PA042	*	*	*	*	*	+	*	*	*	+	*	*	*	+
PA055	+	+	+	+	*	+	*	*	*	+	*	*	*	*
PA061	*	*	*	*	*	+	*	*	*	+	*	*	*	*
PA062	+	+	+	+	*	+	*	*	*	+	*	*	*	*
PA075	*	*	*	*	*	+	*	*	*	+	*	*	*	*
PA076	*	*	*	*	*	+	*	*	*	+	*	*	*	*
PA087	*	*	+	*	*	+	*	*	*	+	*	*	*	*
PA088	*	*	*	*	*	+	*	*	*	*	+	*	*	*
PA089	*	*	*	*	+	+	*	*	*	+	*	*	*	*
PA095	*	*	*	*	*	+	*	*	*	*	*	+	*	*
PA098	*	*	*	*	+	+	*	*	*	+	*	*	*	*
PA102	*	*	+	*	*	+	*	*	*	+	*	*	*	*
PA104	*	*	+	*	*	+	*	*	*	+	*	*	*	*
PA108	*	*	+	*	*	+	*	*	*	+	*	*	*	*

+Denotes mutation, *Indicates no mutation.

pump to be an important factor for multidrug resistance. Mutations in multiple genes (*mexR*, *nalC*, and *nalD*) were reported to be responsible for *mexA* overexpression[15]. In the current study, multiple mutation sites in the *mexR*, *nalC* and *nalD,* which negatively regulated MexAB-OprM were found. Regarding the *mexR*, 40.7% (11/27) of the isolates showed point mutation or deletion

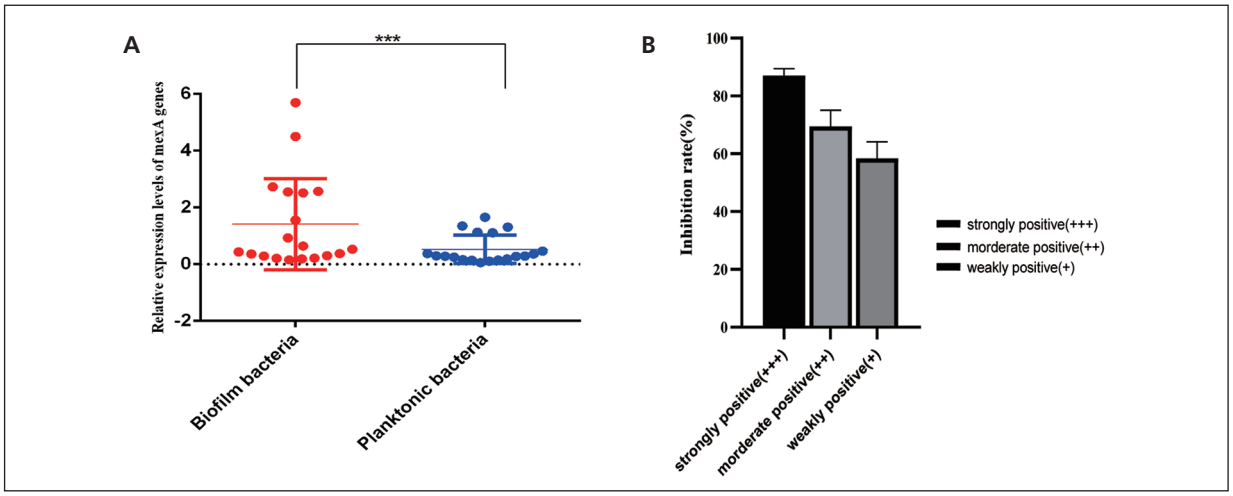

Figure 3. Conferring the relationship between biofilm forming potential and *mexA* in *P. aeruginosa*. **A,** The expression levels of gene *mexA* in *P. aeruginosa* were compared under planktonic and biofilm conditions. **B,** The efflux pump inhibitor phenylalanine-arginine-β-naphthylamide (PAβN) inhibited biofilm formation.

mutations leading to amino acid changes, being V126E (8/11) the most frequent. One strain (PA041) happened nt135-146 base deletion (sequence: TATCGACGAACA). In relation to the *nalC*, 100% isolates showed point mutations, being G71E (27/27), S209R (24/27), Q182K (9/27), and L206V (10/27) the most frequent. Regarding *nalD*, 44.4% (12/27) of the isolates showed point mutation, being W49S the most frequent in 75% (9/12) of the isolates.

Similar to our research, Pan et al[15] reported *nalC* mutation at position 71st and 209th, *nalD* mutations at 158th and *mexR* gene mutations sited at 3rd, 54th, 55th, 126th, 137th, and 138th amino acids. Quale et al[26] reported the same mutations sited at 71st and 209th amino acids in *nalC* gene. Our results also showed that the same mutation in *nalC* gene were detected in all isolates (n=27) overexpressing efflux pump, suggesting that *nalC* mutation was more common in CRPA. However, scholars[26] have pointed out that the amino acid mutations at position 126 of *mexR* and positions 71 and 209 of *nalC* were mostly nonsense mutations. Another study[27] confirmed mutations in *mexR*, *nalC* and *nalD* can yield increased MexAB-oprM expression and multidrug resistance. In our study, the point mutation found in the efflux pump MexAB-OprM should be responsible for carbapenem resistance. The deletion of base fragment of the regulatory gene *mexR* (sequence: TATCGACGAACA) caused a deletion mutation. Whether this mutation affects the function of the efflux pump MexAB-oprM remained to be further studied.

Multifactorial carbapenem resistance mechanisms were detected in the higher frequency than carbapenem resistance caused by one mechanism[28]. Considering the co-existance of efflux pump MexAB-OprM phenotype and biofilm production, 59.3% (16/27) of the strains were classified as strong producers. *In vitro*, we clearly observed that *mexA* was expressed up to 177-fold higher in biofilms than in planktonic state. The pathway by which the biofilm regulates it deserved further investigation.

Conclusions

Our study strengthened the understanding that biofilm and efflux pumps are two intertwined processes involved in Carbapenem-resistant *P. aeruginosa* phenotype. Their synergistic effect magnified the drug resistance characteristics of *P. aeruginosa*. By understanding the role and relationship between *P. aeruginosa* biofilm and efflux pump, new and effective anti-biofilm drugs can be developed to eradicate biofilm related infection.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Acknowledgements

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