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Original Article

The correlation between the biofilm formation ability and the drug resistance of *Acinetobacter baumannii*

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Abstract: This study investigated the correlation between the biofilm formation ability and the drug resistance of *Acinetobacter baumannii* from different sources and examined the differences in the expressions of the biofilm formation related genes in *A. baumannii* strains. All the *A. baumannii* strains were collected from the Affiliated Hospital of Jiangsu University, and the bacterial biofilm formation ability was determined using the crystal violet staining method. The expressions of *csuA*, *csuE*, *bfs*, *ompA,* and *bfmS* were determined using RT-qPCR. We found that there was no significant difference in the biofilm formation ability in *A. baumannii* from the different sources ($P>0.05$), and the biofilm formation ability of the non-multi-drug resistant strains was stronger than that of the multi-drug resistant strains ($P<0.05$). The biofilm formation ability of the drug-sensitive strains was stronger than that of the drug-resistant strains in every drug in our experiment ($P<0.05$). The results of a RT-qPCR showed that the expressions of *ompA*, *bfmS,* and *csuE* in the strong biofilm formation ability strains were significantly higher than they were in those with the weak biofilm formation ability strains, and the expressions of *csuA* and *bfs* had no significant differences between the strong biofilm formation ability strains and the weak biofilm formation ability strains ($P>0.05$). In clinical treatment, it is necessary to attach great importance to the biofilm formation ability of strains with weak drug resistance. The analysis of the biofilm formation related genes *ompA*, *bfmS* and *csuE* of *A. baumannii* can provide a new method for the rapid evaluation of bacterial biofilm formation ability.

Keywords: *Acinetobacter baumannii*, biofilm formation, drug resistance, biofilm formation related genes

Introduction

Acinetobacter baumannii, a conditional pathogen with strong adhesion, is widely found in the natural environment, hospitals, and even organisms [1]. The results of CHITE China bacterial resistance monitoring in 2017 showed that the clinical isolation rate of *A. baumannii* in China exceeded that of *Pseudomonas aeruginosa* , which is a type of non-fermentative bacteria resistant to carbapenems [2]. It exceeds 65%, and the resistance rate to other antibiotics (such as cefoperazone/sulbactam, minocycline, etc.) also exceeds 40%. In recent years, strains resistant to polymyxin have also appeared [3]. The infection and spread of *A. baumannii* has become a public health problem and caused huge losses to the national economy, a problem which needs to be solved urgently [4].

Bacterial biofilm is an important bacterial virulence factor [5]. The biofilm is filled with various water channels, so the bacterial gene expression is active and can more easily escape the immune clearance of the host [6]. The relationship between the bacterial biofilm formation ability and drug resistance is more complicated [7]. It is known that bacterial biofilms have an osmotic restriction that reduces the sensitivity of bacteria to antibiotics [8]. However, there are still dozens of research conclusions indicating that the resistance of bacteria with strong biofilm formation abilities is weak [9, 10]. In this study, samples of *A. baumannii* derived from sputum specimens, blood specimens, and urine specimens were examined to analyze the

Table 1. Criteria of the antimicrobial susceptibility test using the K-B method

Drugs	diameter of the inhibition zone (mm)		
	S	I	R
tigecycline	≥16	13~15	≤12
minocycline	≥16	13~15	≤12
cefoperazone/sulbactam	≥21	16~20	≤15

biofilm formation ability and drug resistance of *A. baumannii*.

Materials and methods

Bacterial strains

A total of 107 strains of *A. baumannii,* including 33 strains taken from blood, 14 strains taken from urine, and 60 strains taken from sputum were isolated from patients at the Affiliated Hospital of Jiangsu University from July 2017 to June 2019. All the strains were identified as *A. baumannii* using a VITEK 2-Compact system (BioMrieux, France) and were then confirmed as *A. baumannii* using bacterial 16S rRNA gene sequencing. Sample sources including from the blood, urine, and sputum, and duplicate strains of the same source from the same patient were excluded. The quality control strains antimicrobial susceptibility tests were *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853).

Antimicrobial susceptibility test

The tigecycline, minocycline, and cefoperazone/sulbactam sensitivity tests were determined using the Kirby-Bauer method (K-B method). We confirmed that the diameters of the inhibition zones of the quality control strains were within the allowable range. The sensitivity of the drug sensitivity results (S), intermediate (I), and drug resistance (R) are shown in **Table 1**. The minocycline refers to the Clinical and Laboratory Standards Institute (CLSI) standard established in 2016, cefoperazone/sulbactam identifies the breakpoint with the CLSI 2016 cefoperazone breakpoint, and the tigecycline drug sensitivity judges the breakpoint established by the US Food and Drug Administration (FDA).

Other drugs were tested according to the Standard Microbiological Laboratory Standard Operating Procedure (SOP) using the VITEK 2-Compact system. The drug sensitivity tests of the experimental strain and the quality control strain *E. coli* (ATCC 25922) and *P. aeruginosa* (ATCC 27853) were used at the same time. A total of 12 commonly used drugs were tested, including levofloxacin (LVX), amikacin (AMK), compound sulfamethoxazole (SXT), piperacillin/tazobactam (TZP), gentamicin (GEN), tobramycin (TOB), ceftriaxone (CRO), imipenem (IPM), ceftazidime (CAZ), ampicillin/sulbactam (SAM), ciprofloxacin (CIP) and cefepime (FEP).

Biofilm formation ability determination

The fresh colonies of *A. baumannii* were used for this assay. The bacterial colonies were adjusted to 1.0 McF with sterile saline. We added 200 μL of LB medium and 1.0 μL of 1.0 McF bacterial suspension to each well in a 96-well bacterial culture plate. The negative control wells contained only 200 μL of the LB medium. All the tests were repeated 6 times. The *A. baumannii* in 96-well bacterial culture plate was incubated at 35°C for 48 h to form a biofilm. In the dying process, the culture medium in the well was aspirated, and the free bacteria were removed by washing with normal saline. Then 200 μL of methanol was added into each well, and 20 min later, the wells were washed twice with saline. Then we added 200 μL of 1% crystal violet dye solution to each well and let them stand for 10 min. After the dying was completed, the wells were washed twice with distilled water. Finally, 200 μL of ethanol was added to each well to dissolve the crystal violet, and the absorbance at 570 nm of each well was determined using an ELISA microplate reader (A_{570}).

Determination of the gene expression of the A. baumannii biofilm formation

We determined the bacterial biofilm formation abilities in all the bacterial strains and calculated the mean and standard deviation (SD). Twenty strains of *A. baumannii* were selected randomly from among all the strains, and the 20 strains with significant differences in their biofilm formation abilities were divided into a biofilm formation high ability group (BF-H group) and a biofilm formation low ability group (BF-L group). The value of the bacterial biofilm formation ability in the BF-H group was above (Mean + 2×SD), and the value of the bacterial biofilm

Table 2. Primer sequences of the real-time PCR

Gene name	Primers sequence (5'→3')
ompA	F: CAATTGTTATCTCTGGAG
	R: ACCTTGAGTAGACAAACGA
bfs	F: GCGCATATGAAAAATGATGCAAATATC
	R: GCGCTCGAGTCATTTCAAATCATATCGAG
bfmS	F: CGTATGCATCAGGTCGAC
	R: ACAGACAAAAGCCTGCC
csuA	F: ATGAGTAGACCTGTTTTAAA
	R: TTAAAACTCAATCGTAATTG
csuE	F: GTTCTCTGGACTGATGTTGACG
	R: GGAAGCCGTATGTAGAAAGGTA
16S rRNA	F: CAGCTCGTGTCGTGAGATGT
	R: CGTAAGGGCCATGATGACTT

Table 3. Departmental sources of the clinical isolates of *Acinetobacter baumannii*

department	Strains	Proportion
ICU	45	42.06%
Respiratory medicine	15	14.02%
Thoracic surgery	8	7.48%
Old cadre	6	5.61%
Urology and breasts	5	4.67%
Neurosurgery	5	4.67%
Neurology	4	3.74%
Cardiovascular Medicine	4	3.74%
Burns	3	2.81%
Rheumatology	2	1.87%
Emergency Department	2	1.87%
Radiotherapy	2	1.87%
Orthopedics	2	1.87%
General surgery	1	0.93%
Chemotherapy	1	0.93%
Gastroenterology	1	0.93%
Hematology	1	0.93%
Total	107	100.00%

formation ability in the BF-L group was above (Mean - 2×SD). Each group had 10 strains. All the above strains were inoculated into an LB medium and cultured for 12 h. The total RNA of *A. baumannii* was extracted using a Bacterial Total RNA Extraction Kit (Sangon Biotech., Shanghai, China). The quality of the purified RNA was tested, and 20 μg-30 μg total RNA were reverse transcribed using M-MLV reverse transcriptase (Vazyme Biotech., Nanjing, China) according to product's instructions. The expressions of the biofilm formation genes with *csuA*, *csuE*, *OmpA*, *bfs* and *bfmS* were determined using Real-time PCR (Perkin-Elmer 9600 PCR system, Applied Biosystems, Foster City, CA). The reaction tubes were incubated for 1 min at 95°C, followed by 35 cycles of the cycling conditions with denaturation at 95°C for 10 sec and annealing at 55°C for 20 sec. The real-time PCR primers are listed in **Table 2**, and the *16S rRNA* gene of *A. baumannii* was used as the internal reference gene.

Statistical analysis

The statistical analysis was performed using SPSS 23.0 software. Chi-square tests or Fisher's exact probability method was used to analyze the relationship among biofilm-related genes and drug resistance, and biofilm-related genes and biofilm formation ability. Because the real-time PCR data were skewed, the relative expressions of the mRNA of the biofilm-related genes in the BF-H group and the BF-L group were expressed as the median and interquartile range [M (P_{25}, P_{75})], using a Mann-Whitney U test. The differences in mRNA expression levels of the biofilm-related genes between the BF-H group and the BF-L group were analyzed. $P<0.05$ was considered statistically significant.

Results

Sample description

There were 107 strains of *A. baumannii* in this study, including 33 strains taken from blood (30.84%), 14 strains taken from urine (13.09%), and 60 strains taken from sputum (56.07%). The bacteria originated from various departments of our hospital, with the highest proportion from the ICU and respiratory medicine, which were 42.06% (45/107) and 14.02% (15/107) respectively (**Table 3**). The majority of the patients were males, accounting for 78.50% (84/107); the age distribution was 31 to 93 years old, with a majority of elderly patients, and with a median age of 69 years.

Antimicrobial susceptibility test

One hundred and seven strains of *A. baumannii* had the highest resistance rate to ciprofloxacin and cefepime, and both were 71.03% (76/107). The resistance rate to imipenem was 63.55% (68/107). The relatively low resistance rates were to tigecycline (14.02%), cefo-

Table 4. Antimicrobial susceptibility test results of *A. baumannii* strains

Antibacterial agents	Antimicrobial susceptibility [*n* (%)]		
	R	I	S
Cefoperazone-Sulbactam	26 (24.30)	32 (29.91)	49 (45.79)
Ampicillin-Sulbactam	66 (61.68)	3 (2.80)	38 (35.51)
Piperacillin-Tazobactam	63 (58.88)	5 (4.67)	39 (36.45)
Levofloxacin	54 (50.47)	18 (16.82)	35 (32.71)
Ciprofloxacin	72 (67.29)	0 (0.00)	35 (32.71)
Amikacin	58 (54.21)	3 (2.80)	46 (42.99)
Gentamicin	69 (64.49)	0 (0.00)	38 (35.51)
Tobramycin	68 (63.55)	0 (0.00)	39 (36.45)
Ceftazidime	69 (64.49)	5 (4.67)	33 (30.84)
Ceftriaxone	70 (65.42)	28 (26.17)	9 (8.41)
Cefepime	72 (67.29)	0 (0.00)	35 (32.71)
Imipenem	68 (63.55)	0 (0.00)	39 (36.45)
Sulfamethoxazole-Trimethoprim	64 (59.81)	0 (0.00)	43 (40.19)
Minocycline	27 (25.23)	30 (28.04)	50 (46.73)
Tigecycline	15 (14.02)	30 (28.04)	62 (57.94)

Table 5. The biofilm formation abilities of *A. baumannii* in the MDRAB and non-MDRAB groups

group (*n*)	biofilm formation ability [M (P_{25}, P_{75})]	*Z* value	*P* value
MDRAB (69)	0.84 (0.69, 1.01)	-6.321	<0.001
non-MDRAB (38)	1.71 (1.23, 1.98)		

perazone/sulbactam (24.30%), and minocycline (25.23%). Among all the strains, the MDRAB strains accounted for 64.49% (69/107), and the non-MDRAB strains accounted for 35.51% (38/107). The proportions of MDRAB originating from blood and urine were 87.88% (29/33) and 71.43% (10/14) respectively. (**Table 4**).

The correlation between biofilm formation ability and the drug resistance of A. baumannii

All the strains in our study had some biofilm formation ability. The MDRAB biofilm formation ability was significantly lower than the non-MDRAB (P<0.001) (**Table 5**). Among all the tested drugs in our study, for each antibiotic the biofilm formation ability of the MDRAB group was significantly lower than that of the non-MDRAB group (P<0.005) (**Table 6**).

The differences in biofilm formation abilities from the different sources of A. baumannii

In the MDRAB group, there was no significant difference in the biofilm formation abilities among the blood, urine, and sputum-derived strains (P>0.05), and in the non-MDRAB group, there was also no significant difference in biofilm formation abilities (P> 0.05) (**Table 7**).

The gene expressions in the different biofilm formation ability strains

We used the RT-qPCR to determine the expressions of the biofilm formation related genes. We found the expressions of *ompA*, *bfmS* and *csuE* were higher in the BF-H group than they were in the BF-L group (P<0.05), but the *csuA* and *bfs* expressions were not significantly different in the two groups (P>0.05) (**Table 8**).

Discussion

The concept of bacterial biofilm was first proposed in 1978 by Professor William J. Costerton. Bacterial biofilms are bacterial colonies formed by large molecules such as bacteria extracellular mucopolysaccharides, which are related to planktonic bacteria [11]. The formation of biofilm helps bacteria survive under harsh conditions [12]. In addition to bacteria, other microorganisms such as fungi and protozoa can also form biofilm [13]. A large number of studies have shown that the tolerance of antibiotics to bacteria after biofilm formation is significantly improved [14], and the tolerance can be increased by 10 to 1000 times, so the formation of bacterial biofilm plays an important role in bacterial resistance [15]. The formation of biofilm can help bacteria adhere to the surface of various plastic catheters (such as catheters, venous catheters, etc.) implanted in the human body [16], causing various invasive infections [17]. At the same time, because biofilm can limit the infiltration of antibiotics, and the metabolism of bacteria in biofilm different from its metabolism in the planktonic state [18], some special genes are expressed by the activation of the quorum sensing (QS) system, which leads to bacterial resistance and the enhancement of virulence [19].

We found that the biofilm formation ability of multi-drug resistant strains in this study was

Table 6. Comparisons of the bacterial biofilm formation abilities in the resistant and sensitive groups for 15 kind of antibiotics

biofilm formation ability [M (P_{25}, P_{75})]		biofilm formation ability [M (P_{25}, P_{75})]	Z value	P value
Cefoperazone-Sulbactam	resistant (26)	0.78 (0.65, 0.89)	-3.726	<0.001
	sensitive (81)	1.03 (0.84, 1.66)		
Ampicillin-Sulbactam	resistant (66)	0.83 (0.68, 1.00)	-6.325	<0.001
	sensitive (41)	1.61 (1.12, 1.96)		
Piperacillin-Tazobactam	resistant (63)	0.84 (0.68, 1.02)	-5.654	<0.001
	sensitive (44)	1.50 (1.00, 1.95)		
Levofloxacin	resistant (54)	0.84 (0.68, 1.00)	-5.035	<0.001
	sensitive (53)	1.39 (0.91, 1.91)		
Ciprofloxacin	resistant (72)	0.87 (0.70, 1.02)	-6.122	<0.001
	sensitive (35)	1.80 (1.26, 2.04)		
Amikacin	resistant (58)	0.82 (0.69, 1.00)	-5.296	<0.001
	sensitive (49)	1.41 (0.99, 1.93)		
Gentamicin	resistant (69)	0.88 (0.71, 1.01)	-5.598	<0.001
	sensitive (38)	1.71 (1.13, 1.98)		
Tobramycin	resistant (68)	0.87 (0.71, 1.01)	-5.754	<0.001
	sensitive (39)	1.71 (1.15, 1.96)		
Ceftazidime	resistant (69)	0.84 (0.69, 1.01)	-6.321	<0.001
	sensitive (38)	1.71 (1.23, 1.98)		
Ceftriaxone	resistant (70)	0.85 (0.69, 1.01)	-6.281	<0.001
	sensitive (37)	1.72 (1.32, 2.00)		
Cefepime	resistant (72)	0.86 (0.70, 1.02)	-5.970	<0.001
	sensitive (35)	1.80 (1.26, 2.04)		
Imipenem	resistant (68)	0.84 (0.69, 1.01)	-6.324	<0.001
	sensitive (39)	1.71 (1.15, 1.96)		
Sulfamethoxazole-Trimethoprim	resistant (64)	0.83 (0.68, 1.00)	-6.094	<0.001
	sensitive (43)	1.56 (1.05, 1.95)		
Minocycline	resistant (27)	0.79 (0.70, 1.05)	-2.686	0.007
	sensitive (80)	1.01 (0.82, 1.69)		
Tigecycline	resistant (15)	0.77 (0.63, 1.11)	-2.270	0.023
	sensitive (92)	1.00 (0.80, 1.42)		

Table 7. The biofilm formation abilities of *A. baumannii* in different samples

group (n)		biofilm formation ability [M (P_{25}, P_{75})]	H value	P value
MDRAB group	blood (29)	0.84 (0.70, 0.97)	2.683	0.261
	urine (10)	0.69 (0.56, 0.96)		
	sputum (30)	0.90 (0.72, 1.06)		
non-MDRAB group	blood (4)	1.59 (1.45, 1.87)	1.450	0.480
	urine (4)	1.27 (0.95, 1.78)		
	sputum (30)	1.80 (1.22, 2.14)		

significantly weaker than it was for non-multi-drug resistant strains. Among the multi-drug resistant strains, there was no significant difference in the biofilm formation ability between the strains derived from sputum, blood, or urine, and the biofilm formation ability of the strains from different sources in non-multi-drug resistant strains also lacked any significant difference. Further analysis of the biofilm formation abilities of the resistant strains and sensi-

Table 8. The expressions of the biofilm-related genes in the different strains

gene	Gene expression [M (P_{25}, P_{75})]		Z value	P value
	BF-H	BF-L		
csuA	1.012 (0.425, 2.605)	1.350 (0.641, 2.560)	-0.378	0.705
ompA	0.942 (0.519, 1.425)	0.483 (0.262, 0.605)	-2.495	0.013
bfmS	1.119 (0.963, 3.779)	0.511 (0.338, 1.123)	-2.721	0.007
bfs	1.287 (0.984, 3.144)	1.103 (0.446, 1.643)	-1.285	0.199
csuE	1.603 (0.685, 4.005)	0.343 (0.142, 1.091)	-2.268	0.023

tive strains of an antibiotic (a total of 15 kinds of antibiotics) revealed that the biofilm formation ability of all the sensitive strains of the drug was stronger than the biofilm formation ability of the resistant strain. Our results are consistent with Espinal's research conclusion that the strains with low drug resistance can form stronger biofilms. Our results seem to explain to some extent why strains with weaker drug resistance in *in vitro* antimicrobial susceptibility tests were difficult to remove in clinical treatment [20]. In the current antimicrobial susceptibility test, the bacterial culture time was only 12 h to 16 h, and it took at least 48 h to form a mature biofilm. At this time, the bacteria had not yet formed a mature biofilm, and the results of antimicrobial susceptibility tests were the resistance of the bacteria to the non-biofilm state (i.e., the state of the floating bacteria), and does not truly reflect the resistance of the bacteria in the human body [21]. We believe that a bacteria's biofilm formation ability is an indispensable factor in the evaluation of its drug resistance, and the current routine antimicrobial susceptibility tests cannot extend the bacterial culture time to 48 h but can be quickly done by detecting the presence of bacterial biofilm formation related genes to assess the bacterial biofilm formation ability.

In our study, we found a significant difference in the biofilm formation abilities in the two groups using RT-qPCR. We found the expressions of the biofilm formation ability related genes such as *OmpA*, *bfmS* and *csuE* were significantly higher in the strong biofilm formation ability strains than in the weak biofilm formation ability strains, but the expressions of *csuA* and *bfs* were not significantly different in the two groups. We believe that *A. baumannii OmpA*, *bfmS,* and *csuE* can be used as potential molecular markers for assessing bacterial biofilm formation, but their exact assessment efficacy remains to be further studied.

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Disclosure of conflict of interest

None.

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