ORIGINAL RESEARCH

Phenotypic and Genotypic Characteristics of Biofilm Formation in Clinical Isolates of *Acinetobacter baumannii*

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Background: *Acinetobacter baumannii* is an important pathogen in clinical infections, and biofilm formation is an effective way for *A. baumannii* to survive under external pressures. In this study, the aims were to examine the antimicrobial resistance, biofilm formation, and biofilm-specific resistance in clinical isolates of *A. baumannii*.

Materials and Methods: A total of 104 clinical *A. baumannii* isolates were collected from a large teaching hospital in Southwest China. The antibiotics susceptibilities were tested, and biofilm-forming ability was evaluated by crystal violet staining by confocal laser scanning microscopy (CLSM). Minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), minimum biofilm inhibitory concentration (MBIC), and minimum biofilm eradication concentration (MBEC) of ciprofloxacin, meropenem, and ceftazidime were tested on selected strains by broth microdilution method. Biofilm-associated genes were detected by polymerase chain reaction (PCR), and expression of genes at planktonic stage and biofilm stage were analyzed by real-time reverse transcription PCR (RT-PCR).

Results: Multidrug-resistant (MDR) isolates accounted for 65.4%, but no strain was resistant to tigecycline and polymyxin B. Moreover, non-MDR strains tended to form stronger biofilms than MDR strains, and a negative correlation between biofilm-forming ability and resistance profiles to each of tested antimicrobials were observed. The MBECs and MBICs of ciprofloxacin, ceftazidime, and meropenem were evidently increased compared with MICs and MBCs among all tested strains. Additionally, the biofilm formation ability of the *csuD*-positive strains was stronger than that of the *csuD*-negative strains. The strains in MDR group had higher carrying rate of *csuA* and *csuD* genes than non-MDR group, while non-MDR strains possessed more *ompA* gene than MDR group. Finally, *abaI* gene was significantly up-regulated after biofilm formation.

Conclusion: These results revealed valuable data for the negative correlation between antimicrobial resistance and biofilm formation, as well as phenotypic and genotypic characteristics of biofilm formation in *A. baumannii*.

Keywords: *Acinetobacter baumannii*, biofilm formation, antimicrobial resistance, gene expression

Introduction

Acinetobacter baumannii is one of the most common nosocomial pathogen that causes several severe infections in critically ill patients, and a great number of multidrug-resistant (MDR) strains attract the attention of the whole world.[1] The formation of biofilm contributes to *A. baumannii* adhere to various biological and non-biological surfaces, such as vascular catheters, cerebrospinal fluid shunts, and other medical devices.[2] Biofilm

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is defining composed of extracellular polymeric substances (EPS) that surrounding microorganisms, which mediates microbial adhesion and makes a lifestyle that is totally different from the planktonic state.[3] Indeed, biofilm formation presents a significantly higher antimicrobial resistance than planktonic cells, resulting in harder eradication and easier recurrence in biofilm-associated infections.[4] Biofilm formation is a complex process and regulated by a variety of factors. Taking *A. baumannii* for example, biofilm associated protein (Bap) is involved in the initial adhering of biofilm, promoting the maturation of biofilm and maintaining the structure of mature biofilm.[5,6] Moreover, synthesis and assembly of pili are encoded by *csuA/BABCDE* gene cluster to induce the initial adhesion process of biofilm.[7] The BfmR-BfmS regulatory system senses the changes of external signals then affects the synthesis and secretion of pili, and induces the biofilm formation by regulating the *csuA/BABCDE* gene cluster as well.[8,9] The *pgaABCD* locus encodes the production of poly-beta-1-6-N-acetylglucosamine (PNAG), which is not only an important virulence factor but also essential for biofilm formation.[10] The self-induced synthetase gene *abaI* is involved in the quorum sensing (QS) system and plays an important role in later stages of biofilm maturation.[11,12] Outer membrane protein A (OmpA) is a well-known virulence factor that contributes to adhesion, invasion, and biofilm formation of *A. baumannii*.[13] On the other hand, the latest research has reported that biofilm growth-associated repressor (BigR) directly or indirectly regulates the expression of genes related to epithelial cell adhesion and biofilm formation.[14] Several biofilm-associated genes affect the antimicrobial susceptibility, indicating a relationship between biofilm formation and antimicrobial resistance.[11]

However, it is still unclear whether there is a quantitative correlation between biofilm formation and antimicrobial resistance. The aims of this study were to analyze the antimicrobial susceptibility, biofilm formation, biofilm-specific resistance, and biofilm-associated gene carrying rate of 104 clinical isolates of *A. baumannii*. Furthermore, the phenotype-genotype correlation between planktonic and biofilm stage was also evaluated.

Materials and Methods
Identification and Antimicrobial Susceptibility Testing (AST)
A total of 104 non-repetitive *A. baumannii* strains were isolated from January 2020 to April 2021 in the Affiliated Hospital of Southwest Medical University, and ATCC19606 was used as the reference strain in this

study. Identification and antimicrobial resistance analysis were performed by the MicroScan Walk-Away 96 Plus system (Siemens, Germany), and results were interpreted according to the standards of the Clinical and Laboratory Standards Institute (CLSI) 2020-M100.[15] *Escherichia coli* ATCC25922 and *Pseudomonas aeruginosa* ATCC27853 (purchased from China National Health Inspection Center) were used as quality control. Based on the obtained antimicrobial resistance profiles, the tested strains were classified into multidrug-resistant (MDR) group if 'the isolates are resistant to three or more antimicrobial classes',[16] and non-MDR group which the isolates are resistant to 0–2 antimicrobial classes.

Biofilm Formation Assay
Biofilm formation ability was determined by crystal violet staining method as described previously,[17] with slight modifications. Briefly, the overnight culture of each strain was diluted to final optical density of 600 (OD_{600}) = 0.1, and then 180 μL of Luria-Bertani (LB) broth (Haibo, Qingdao, China) with 1% glucose (Sigma, USA) and 20 μL of bacterial suspensions were inoculated into 96-well polystyrene microtiter plates (Costar #3524, Corning, NY, USA) in triplicate. After 24 h incubation at 37 °C, the plates were gently washed with phosphate-buffered saline (PBS) for three times and stained with 200 μL of 0.1% crystal violet (Solarbio, Beijing, China) for 20 min. After washing with PBS for three times again, the plates were solubilized with 200 μL of 95% ethanol, followed by shaking slowly at room temperature for 20 min. The absorbance was measured at OD_{570}. The cut-off OD (ODc) was defined as three standard deviations (SD) above the mean OD of the negative control (LB broth only). Following were classification criteria: $OD \leq OD_C$, non-biofilm producers (-); $OD_C < OD \leq 2 \times OD_C$, weak biofilm producers (+); $2 \times OD_C < OD \leq 4 \times OD_C$, moderate biofilm producers (++); and $OD > 4 \times OD_C$, strong biofilm producers (+++).[18]

Determination of Biofilm Using Confocal Laser Scanning Microscopy (CLSM)
Four strains (two strong biofilm producers, one biofilm-negative producer, and ATCC19606) were selected for biofilm visualization by CLSM (Leica, Germany). In brief, strains were cultured in LB broth for 18 h and diluted to 0.5 McFarland standard. One hundred microlitre suspension and 2 mL fresh LB broth were added in the

petri dishes (FengRun, Beijing, China). After incubation at 37 °C for 24 h, the petri dishes were washed three times with PBS and fixed with 2 mL of 2.5% glutaraldehyde for 1.5 h. Then, the petri dishes were washed three times with PBS again and 200 μL fluorescein isothiocyanate-concanavalin A (FITC-ConA) (Sigma, Germany) were added and stored at 4 °C for 30 min. Two hundred microlitre propidium iodide (PI) (Sigma, Germany) were added and stored at 4 °C for 15 min after washing three times with PBS. Finally, the dishes were washed with PBS and sealed with 40% glycerol. FITC-conA binds to EPS of biofilm and reflects green fluorescence at the laser wavelength of 488 nm.[19] PI binds to bacterial DNA and emits red fluorescence at 543 nm.[20] If both lasers appear at the same time, an orange fluorescence will be shown.[21]

Growth Curve Assay

The growth of eight strong biofilm producers, eight weak biofilm producers, and ATCC19606 were evaluated. For each strain, 20 μL of diluted overnight culture (0.5 McFarland standard) and 180 μL LB broth were added in 96-well plates and incubated at 37 °C for 24 h, and the OD_{590} was detected by microplate reader every 2 h until 24 h. After biofilm modeling on plates, the OD_{570} was measured by microplate reader every 4 h until the number of biofilm biomass decreased. All assays were repeated in triplicate.

Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC), Minimum Biofilm Inhibitory Concentration (MBIC), and Minimum Biofilm Eradication Concentration (MBEC)

Three antimicrobials that are commonly used for the treatment of *A. baumannii* infections were selected in the determination of MICs/MBCs and MBICs/MBECs. All of these nine strains were originally sensitive to tested antimicrobials. The MICs for ceftazidime, ciprofloxacin, and meropenem were assessed using broth microdilution method in 96-well plates according to the procedures described in CLSI 2020-M100 guideline.[15] After MIC determination, 10 μL aliquots from the wells with no observed bacterial growth were smeared on LB agar plates and incubated at 37 °C for 24 h, and the lowest concentration of antimicrobials that making no colony grows on

plates was considered as MBC. AST at biofilm stage was performed according to the procedures described previously.[22] Biofilm was established in 96-well plates for 24 h, and planktonic cells were removed by sterile deionized water. Each well was treated with 200 μL of LB broth containing serially diluted antimicrobials. The plates were incubated for 24 h at 37 °C. The minimum antimicrobial concentration at which $OD_{600} < 0.1$ was determined as the MBIC.

Following the MBIC examination, the plates were washed with sterile deionized water to remove planktonic cells and antibiotics. Two hundred microlitre of LB broth was added to each well, and the plates were incubated at 37 °C for 24 h. The lowest concentration at which $OD_{600} < 0.1$ was considered as the MBEC. All experiments were performed in triplicate.

Detection of Biofilm-Associated Genes by Polymerase Chain Reaction (PCR) and Real-Time Reverse Transcription PCR (RT-PCR)

The DNA template was prepared by boiling bacteria.[23] The biofilm-associated genes of all 104 clinical strains were detected by PCR, including *bap, ompA, bigR, bfmS, bfmR, csuA, csuB, csuC, csuD, csuE, csuAB, pgaD,* and *abaI* (Table S1). The procedures were followed by previous study with slight modifications.[24] Part of nucleotide sequences of PCR products were analyzed by Beijing Qingke Biotechnology Co. Ltd. The results were compared with sequences that retrieved from GenBank database by the BLAST online tool at www.ncbi.nlm.nih.gov. Moreover, eight strong biofilm producers were selected to detect the expression level of 13 biofilm-associated genes by RT-PCR according to the protocol described previously.[25] The fold change of mRNAs expression was calculated using the $2^{-\Delta\Delta Ct}$ method, and the relative expression of each gene was normalized to the control sample (planktonic stage), which was assigned a value of 1 arbitrary unit.[26] All assays were performed in triplicate.

Statistical Analysis

Statistical analyses and graphs were performed using GraphPad Prism 8.0.1 (GraphPad Software Inc, USA), Origin 2018 (OriginLab, USA) and SPSS 24.0 (SPSS Statistics, Inc., Chicago, IL, USA). Based on the distribution and homogeneity of the variances, the OD values were expressed as the mean ± SD or median (interquartile

range, IQR). Spearman's rank correlation test was used for intergroup comparison. Mann–Whitney U-test is used to analyze the carrying situation of 13 genes and biofilm formation capacity. Chi-square test was used to compare the positive rates of 13 genes and the number of genes carried in different resistance types of strains. Student's t-test was used for gene expression in planktonic stage and biofilm stage. In all analyses, a two-sided significance level of <0.05 was considered as statistically significant.

Results

Distribution and Antimicrobial Susceptibility Profiles of Clinical *A. baumannii* Strains

One hundred and four clinical *A. baumannii* isolates were collected from 18 departments within the major of intensive care unit (27, 26.2%) and department of neurology (22, 21.4%). Furthermore, the majority of specimens were obtained from sputum, accounting for 72.8%, while only 12.6%, 7.7%, 4.9%, and 2% specimens were isolated from secretion, blood, cerebrospinal fluid, and urine, respectively. The AST results for 18 antimicrobials are shown in Figure 1. Most of isolates (68, 65.4%) were resistance to ciprofloxacin and piperacillin/tazobactam, followed by levofloxacin (67, 64.4%), cefepime (67, 64.4%), meropenem (67, 64.4%), imipenem (67, 64.4%), ceftazidime (67, 64.4%), ceftriaxone (65, 62.5%), cefotaxime (65, 62.5%), piperacillin (62, 59.6%), ampicillin/sulbactam (61, 58.7%), ticarcillin/clavulanic acid (60, 57.7%), gentamicin (54, 51.9%), tobramycin (52, 50%), amikacin (52, 50%), and tetracycline (30, 28.8%). More than 65.4% of the isolates exhibited multidrug resistance (Figure 1B), but no strain was resistant to tigecycline and polymyxin B.

Biofilm Formation Ability and Growth Curve Analysis

The biofilm-forming capacity of each isolate was summarized. The OD_{570} values for the reference strain (ATCC19606) and negative control were 0.84 ± 0.12 and 0.14 ± 0.008, respectively (Table S2). The OD_{570} values for the clinical isolates ranged from 0.14 ± 0.01 to 2.54 ± 0.26, and the IQR was 0.58 (0.36, 1.04). It is worth noting that 99% of these strains can form biofilm, of which 51.5% were strong biofilm producers. In addition, 35.6% strains produced stronger biofilm than ATCC19606. No significant difference was observed between eight strong and eight weak biofilm producers in growth curves, indicating

that the difference in biofilm formation was not due to growth rate (Figure 2). Furthermore, four strains were selected to observe the biofilm formation ability using CLSM. The strongest biofilm-forming strain 20047 reflected the highest fluorescence intensity, followed by strain 20192 and ATCC19606, and biofilm-forming negative strain 19014 presented the lowest fluorescence intensity (Figure 3A). The fluorescence intensities under CLSM were consistent with the OD_{570} values obtained by crystal violet staining (Figure 3B).

Correlation Between Antimicrobial Resistance and Biofilm Formation Ability

Spearman's rank correlation analysis determined that non-MDR strains tended to form stronger biofilms than MDR strains (r_s = -0.212, $P = 0.03$; Table 1), suggesting a negative correlation between biofilm formation capacity and antimicrobial resistance phenotypes. Moreover, the results revealed that in addition to tigecycline and polymyxin B, susceptible strains could form stronger biofilm than resistant strains, indicating a negative correlation between biofilm-forming ability and resistance profiles to each of tested antimicrobials (r_s = 0.204 − 0.288, $P = 0.003 − 0.037$; Table 2)

Comparison of MICs/MBICs and MBCs/MBECs

The MICs and MBICs of three antimicrobials (ceftazidime, ciprofloxacin, and meropenem) were tested against nine strains with different biofilm-forming ability (three strong biofilm producers, three moderate biofilm producers, and three weak biofilm producers), and all strains were sensitive to these three antibiotics being investigated. MBICs for biofilms increased drastically compared with MIC for planktonic cells among all tested strains (Table 3). The increases ranged from 256- to 4096-fold for ciprofloxacin, 16- to 1024-fold for meropenem, and 128- to 1024-fold for ceftazidime. Indeed, when biofilms were established, the antimicrobial susceptibility profiles of all strains changed from sensitive to resistance.

Furthermore, MBCs and MBECs of these nine isolates were also evaluated. Undoubtedly, the MBECs were much higher than the MBCs among all tested strains (Table 4). Up to a 4096-fold increase in the concentration of ciprofloxacin and up to a 1024-fold increase in the concentration of ceftazidime and meropenem were required to eradicate biofilms compared with planktonic cells.
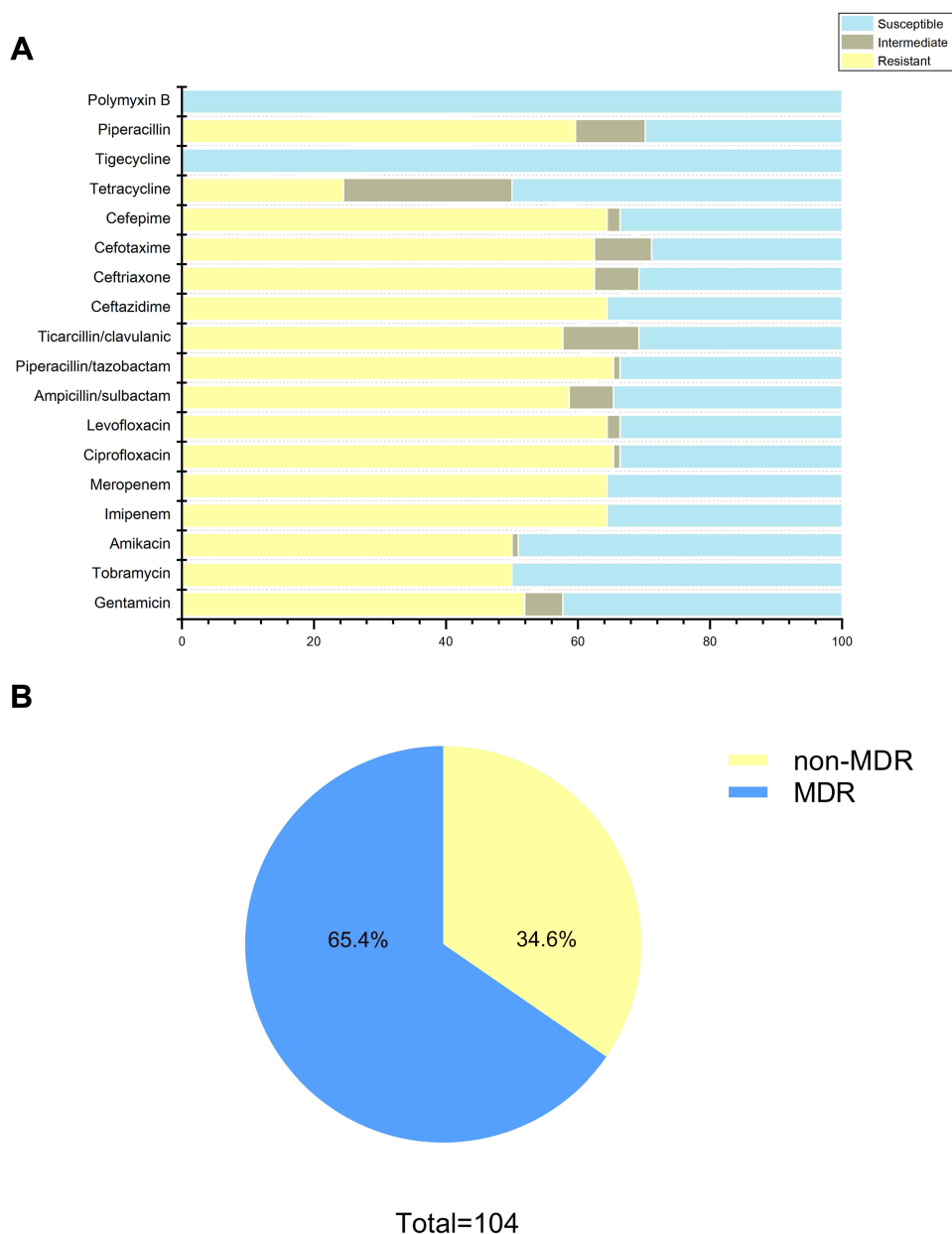
A

B

Total=104

Figure 1 Antimicrobial susceptibility profiles of clinical *A. baumannii* isolates. (**A**) Resistance rate of all strains to 18 antimicrobials; (**B**) approximately 65.4% of isolates exhibited MDR (including XDR).
Abbreviations: MDR, multiple drug resistance; XDR, extensively drug resistance.

Distribution and Expression of Biofilm-Associated Genes in Clinical *A. baumannii* Strains

In order to further explore the correlation between biofilm-forming ability and antimicrobial resistance, the presence of 13 reported biofilm-associated genes was detected by using PCR. The results showed that *bfmR* (98, 94.2%) was occurred mostly, followed by *csuC* (95, 91.3%), *csuE* (95, 91.3%), *bigR* (92, 88.5%), *csuAB* (91, 87.5%), *csuB* (90, 86.5%), *OmpA* (89,

85.6%), *bfmS* (88, 84.6%), *bap* (85, 81.7%), *pgaD* (80, 76.9%), *csuA* (76, 73.1%), *csuD* (73, 70.2%), and *abaI* (73, 70.2%). Twenty-five clinical isolates contained all 13 tested genes, including 23 strong biofilm producers and two moderate biofilm producers (Table S3). Remarkably, the biofilm formation ability of the *csuD*-positive strains was stronger than that of the *csuD*-negative strains (U = 365, *P* < 0.01).

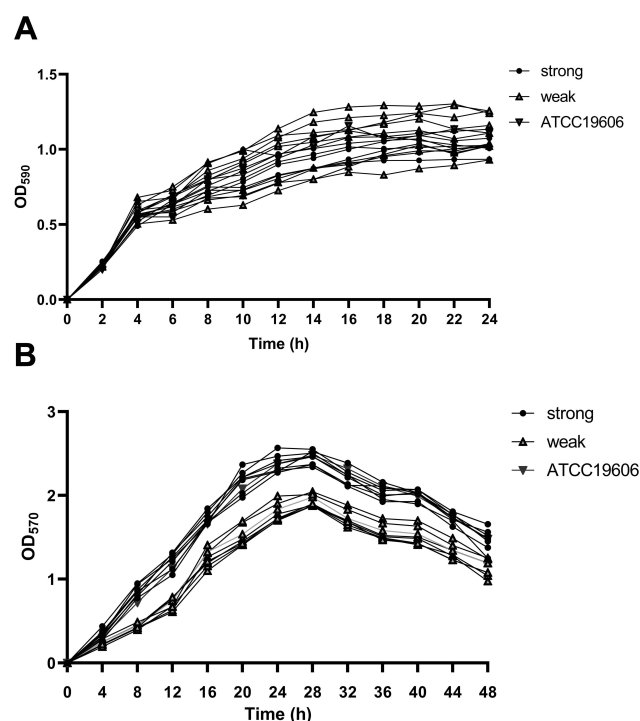The harboring rates of 13 biofilm-associated genes among two groups were evaluated by Chi-square test.

Figure 2 Growth curves and biofilm growth curves in representative *A. baumannii* strains. (**A**) Growth curves of 17 representative *A. baumannii* strains at planktonic stage, and OD_{590} values of each strain were measured every 2 h until 24 h; (**B**) growth curves for 17 representative *A. baumannii* strains at biofilm stage, and OD_{570} values of each strain were measured every 4 h until 48 h. The data were presented as the average of three measurements. Error bars (standard deviation <10%) were omitted for simplicity.

Three genes were significantly related to antimicrobial resistance. In detail, the strains in MDR group had higher carrying rate of *csuA* and *csuD* genes than non-MDR group ($P < 0.05$); however, the non-MDR strains possessed more *ompA* gene than MDR group ($P = 0.002$) (Figure 4A).

Eight strong biofilm-forming strains that carrying 13 biofilm-associated genes were selected to investigate the relationship between biofilm-associated genes and biofilm formation ability (Table S4). The RT-PCR results showed that the expression of *abaI* gene in the biofilm stage was significantly up-regulated compared with that in the planktonic stage ($P < 0.001$) (Figure 4B), while no significant difference was found for the rest of the 12 genes between planktonic stage and biofilm stage.

Discussion

Drug-resistant *A. baumannii* shows superior ability to disseminate worldwide, and the proportion of extreme drug resistance (XDR) *A. baumannii* has increased from less than 4% in 2000 to more than 60% recently, even close to 90% in some regional nosocomial settings.[27] According to the National Institutes of Health and the Center for Disease and Prevention, it is estimated that 65% to 80%
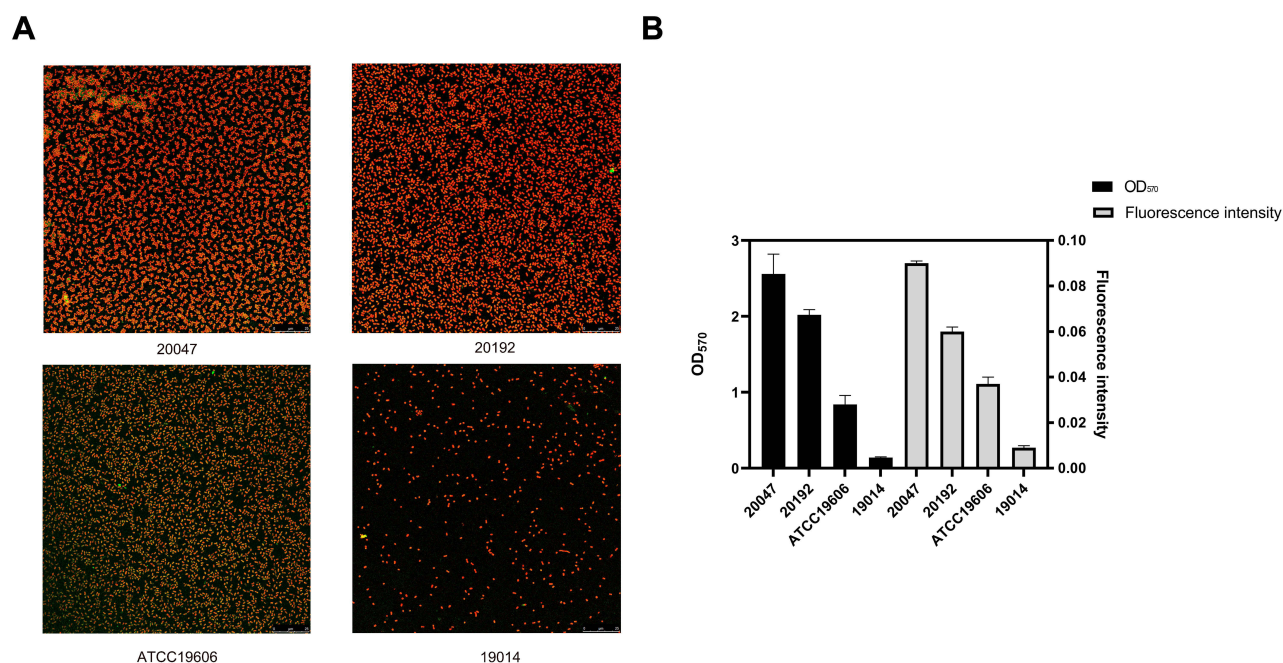

Figure 3 Evaluation of biofilm formation capacity of strains by confocal laser scanning microscopy. (**A**) Biofilm Images of four representative *A. baumannii* strains under CLSM (**B**) Comparison of biofilm formation capabilities between crystal violet and CLSM. Data were expressed as mean ± SD; fluorescence intensity represents CLSM results and OD_{570} indicates crystal violet results.

Table 1 Biofilm Formation Ability of Strains with Different Antimicrobial Resistance

Resistance Profile	N	OD$_{570}$	r_s	P
Non-MDR	36	0.98 (0.36, 1.47)	-0.212	0.03
MDR	68	0.55 (0.36, 0.85)		

Notes: OD$_{570}$: The absorbance value at the wavelength of 570 nm. Data was shown in median (interquartile range, IQR).

Table 2 Correlation Between Biofilm Formation Ability and Antimicrobial Resistance to 18 Antimicrobials

Antimicrobial Category	Antimicrobial Agent	OD$_{570}$		r_s	P
		S	R		
Aminoglycosides	Gentamicin	0.71 (0.34,1.28)	0.55 (0.38,0.84)	−0.211	**0.032**
	Tobramycin	0.82 (0.36,1.33)	0.53 (0.37,0.82)	−0.211	**0.032**
	Amikacin	0.84 (0.36,1.34)	0.53 (0.37,0.82)	−0.214	**0.03**
Carbapenemase	Imipenem	0.92 (0.36,1.43)	0.53 (0.37,0.85)	−0.215	**0.029**
	Meropenem	0.92 (0.36,1.43)	0.53 (0.37,0.85)	−0.215	**0.029**
Fluoroquinolones	Ciprofloxacin	0.92 (0.36,1.34)	0.55 (0.37,0.87)	−0.213	**0.031**
	Levofloxacin	0.92 (0.36,1.34)	0.55 (0.37,0.87)	−0.213	**0.031**
Penicillins + β-lactamase inhibitors	Ampicillin/sulbactam	0.98 (0.36,1.47)	0.69 (0.49,0.72)	−0.239	**0.018**
	Piperacillin/tazobactam	0.98 (0.36,1.47)	0.53 (0.36,0.85)	−0.235	**0.016**
	Ticarcillin/clavulanic acid	1.09 (0.37,1.52)	0.53 (0.36,0.84)	−0.288	**0.003**
Cephalosporins	Ceftazidime	0.92 (0.36,1.43)	0.53 (0.37,0.85)	−0.215	**0.029**
	Ceftriaxone	0.92 (0.36,1.43)	0.53 (0.37,0.85)	−0.215	**0.029**
	Cefotaxime	0.98 (0.37,1.38)	0.53 (0.36,0.78)	−0.263	**0.007**
	Cefepime	0.98 (0.36,1.47)	0.53 (0.36,0.85)	−0.235	**0.016**
Tetracyclines	Tetracycline	0.91 (0.38,1.25)	0.59 (0.36,1.00)	−0.209	**0.034**
	Tigecycline	0.57 (0.36, 1.1)	0	NA	NA
Penicillins	Piperacillin	0.88 (0.36,1,30)	0.53 (0.37,0.78)	−0.204	**0.037**
Lipopeptides	Polymyxin B	0.57 (0.36, 1.1)	0	NA	NA

Notes: S, sensitive; R, resistance; bold indicates P < 0.05.

Table 3 Comparison of MICs and MBICs of *A. baumannii* Strains

Strain	Ciprofloxacin			Meropenem			Ceftazidime		
	MIC for Planktonic Cells (µg/mL)	MBIC for Biofilm Cells (µg/mL)	Fold Change	MIC for Planktonic Cells (µg/mL)	MBIC for Biofilm Cells (µg/mL)	Fold Change	MIC for Planktonic Cells (µg/mL)	MBIC for Biofilm Cells (µg/mL)	Fold Change
20053[a]	0.125	32	256	0.5	8	16	8	1024	128
20061[a]	0.0625	32	512	0.25	16	64	4	1024	256
20068[a]	0.0625	32	512	0.5	16	32	2	1024	512
20098[b]	0.0625	64	1024	0.25	16	64	4	1024	256
20101[b]	0.125	32	256	0.25	16	64	2	1024	512
20110[b]	0.125	32	256	1	16	16	4	1024	256
20047[c]	0.125	512	4096	0.5	512	1024	4	4096	1024
20180[c]	0.125	64	512	0.5	16	32	8	2048	256
20250[c]	0.0625	64	1024	1	16	16	8	2048	256

Notes: [a]Indicates weak biofilm producer, [b]Indicates moderate biofilm producer, [c]Indicates strong biofilm producer.

Table 4 Comparison of MBCs and MBECs of *A. baumannii* Strains

Strain	Ciprofloxacin			Meropenem			Ceftazidime		
	MBC for Planktonic Cells (µg/mL)	MBEC for Biofilm Cells (µg/mL)	Fold Change	MBC for Planktonic Cells (µg/mL)	MBEC for Biofilm Cells (µg/mL)	Fold Change	MBC for Planktonic Cells (µg/mL)	MBEC for Biofilm Cells (µg/mL)	Fold Change
20053[a]	0.25	128	512	1	128	128	16	2048	128
20061[a]	0.125	64	512	0.5	32	64	8	2048	256
20068[a]	0.125	64	512	1	32	32	4	2048	512
20098[b]	0.125	128	1024	0.5	32	64	8	2048	256
20101[b]	0.25	64	256	0.5	128	256	4	2048	512
20110[b]	0.25	64	256	2	256	128	8	2048	256
20047[c]	0.25	1024	4096	1	1024	1024	8	8192	1024
20180[c]	0.25	128	512	1	256	256	16	4096	256
20250[c]	0.125	128	1024	2	64	32	16	4096	256

Notes: [a]Indicates weak biofilm producer, [b]Indicates moderate biofilm producer, [c]Indicates strong biofilm producer.

of the human infectious are caused by biofilm-forming bacteria.[28] The lifestyle changes from planktonic phenotype to biofilm is a highly regulated developmental process that depends on species-dependent environmental and genetic factors.[4]

This study evaluated the biofilm-forming ability of 104 clinical *A. baumannii* strains, and explored the effect of biofilm production on antimicrobial susceptibility. The MDR strains have reached 65.4%, which increased the difficulty of infection treatment. The AST results revealed

that all tested strains remained sensitive to polymyxin B and tigecycline, and it seemed that these two antimicrobials were the most effective strategy for MDR-*A. baumannii* infections. However, two meta-analyses discourage using tigecycline for the treatment of MDR-*A. baumannii* infections due to the higher in-hospital mortality and longer hospitalization when compared with other active antimicrobials.[29,30] Moreover, polymyxin B rarely used in clinical treatment because of the lack of pharmacokinetic/pharmacodynamic studies and highly renal toxicity.[31]
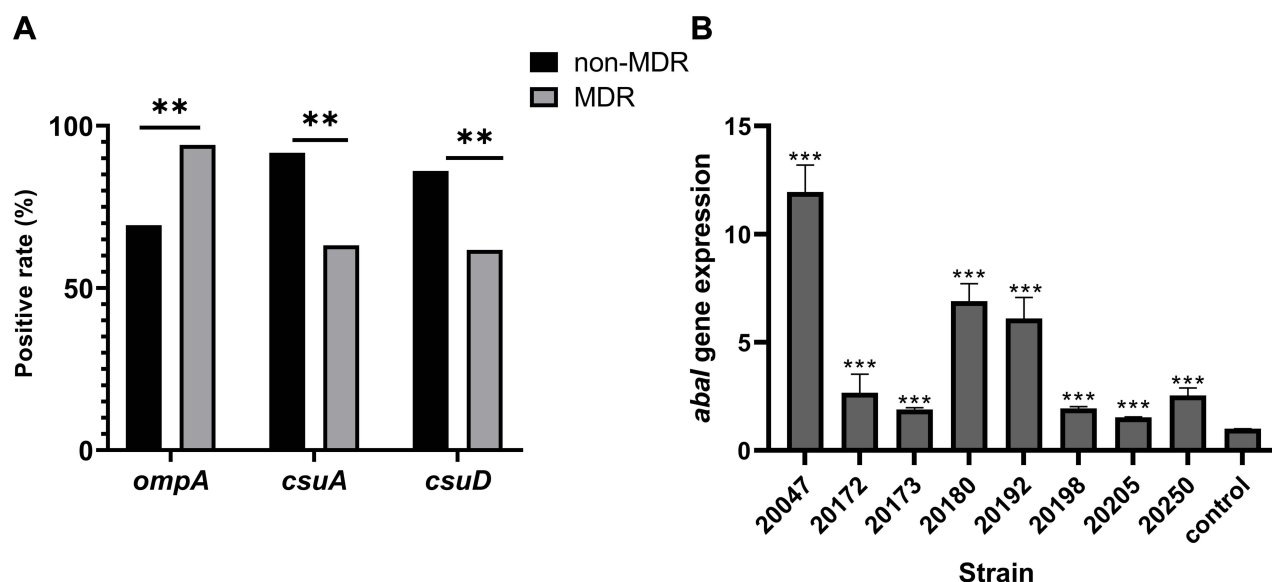
Figure 4 Role of biofilm-associated genes in antimicrobial resistance and biofilm of clinical *A. Baumannii*. (**A**) Percentage of biofilm-associated gene in different antimicrobial-resistant strains; (**B**) the relative expression of *abaI* in eight selected *A. Baumannii* strains. Data were shown as fold changes between planktonic and biofilm stage, and control represented planktonic cells.
Notes: **P<0.01, ***P<0.001.

Therefore, the optimal treatment for MDR-*A. baumannii* infections have not been established so far.[32]

Then, the crystal violet staining results demonstrated that 99% strains have biofilm-forming ability and nearly one-half of strains can produce strong biofilms, which was consistent with the previous reports.[24,33] Up to date, there is still no regular pattern in the relationship between biofilm formation ability and bacterial antimicrobial resistance, and the conclusions between different researches were even contradictory.[24,33–36] In our research, the non-MDR strains could produce stronger biofilm compared with MDR strains, indicating that the antimicrobial resistance and biofilm formation ability were negatively correlated in *A. baumannii*. Rodriguez-Bano et al have illustrated that biofilm-forming *A. baumannii* isolates were more sensitive to ciprofloxacin and imipenem,[37] suggesting that these strains were not as dependent on antimicrobial resistance as non-biofilm-forming isolates for survival, which was consistent with our result. Moreover, a research has found that the biofilm-forming ability of ciprofloxacin-sensitive *Pseudomonas aeruginosa* was significantly promoted after exposure to ciprofloxacin,[38] highlighting the importance of biofilms for the survival of sensitive bacteria. Therefore, it seems that the biofilm formation is another survival mechanism besides antimicrobial resistance. Furthermore, MIC/MBIC and MBC/MBEC values confirmed that the resistance of isolates was greatly enhanced after biofilm formation, regardless of whether the isolates were originally sensitive or resistant to this antimicrobial. Remarkably, antimicrobials inhibit biofilms mainly through a complex penetration process,[39] however, this still cannot complete remove the bacteria in the biofilm stage due to multifactorial tolerance of biofilms.[40] For example, beta-lactam antimicrobials can be inactivated by beta-lactamases in the biofilm matrix, which restricts their penetration. Biofilm-stage bacteria with low metabolic activity can resistant to kinds of antimicrobials that target processes that occur in growing bacteria, such as cell wall synthesis and DNA replication.[41] In addition, the expression of specific genes and dormant state persister cells are also responsible for the failure inhibition of biofilms.[40,42]

In addition to the correlation between antimicrobial resistance and biofilm formation, the relationship between biofilm formation and biofilm-associated genes expression was also evaluated in this study. All the biofilm-associated genes were detected in more than 70% of tested strains, bring with the most frequent gene *bfmR* (98, 94.2%) as well as the least genes *csuD and abaI* (70.2%). It has been known that the chaperone usher pili assembly system is essential for the initial steps of biofilm formation.[43] Tomaras et al have certified that the inactivation of the *csuE* gene resulted in the disappearance of both pili formation and biofilm formation in *A. baumannii*.[44] The gene *csuD* is part of the CsuA/BABCDE chaperone-usher pili assembly system, in this study, *csuD*-positive strains can form stronger biofilm than *csuD*-negative strains. This result was consistent with the previous study that indicating *csuD* was highly expressed in stronger biofilm producers.[45] Moreover, in this work, three genes had a obviously distributed regularity among strains with different antimicrobial-resistant profiles. With the increase of resistance, there was a growing positive rate of *ompA* and a decreasing positive rate of *csuA* and *csuD*. Although CsuA/BABCDE system is able to enhance biofilm formation, the relationship between this system and antimicrobial resistance still not clear. OmpA is a major component of outer membrane proteins (OMPs) in *A. baumannii*, which is related to antimicrobial resistance, aggressiveness, and biofilm formation.[13] Smani et al first determined that the deletion of *ompA* decreased the MICs of chloramphenicol, nalidixic and aztreonam by 8-, 2.67-, and 8-fold, respectively.[46] Sanchez-Encinales et al demonstrated that overproduction of OmpA was an independent risk factor for nosocomial pneumonia, bacteremia, and higher mortality caused by *A. baumannii* infections.[47] A previous study confirmed that *ompA* measured by RT-PCR can be used as a rapid diagnostic factor for antimicrobial-resistant *A. baumannii* due to the high consistency with MICs.[48] In *A. baumannii*, QS system is consists of AbaI/AbaR system, which is closely interconnected with biofilm development.[12] The *abaI* gene encodes the self-induced synthase which activates the synthesis of AHL signals, and *abaI* mutant leads a greatly reduction of biofilm formation.[12,34] In this study, *abaI* was the only gene that up-regulated in biofilm stage; however, other tested genes did not show regular changes between planktonic and biofilm stages. A recent study has shown that the absence of AHL signal in shaking cultures (planktonic) seemed to be the result of low expression of *abaI*, while the AHL signal was significantly up-regulated under static conditions (biofilm).[49] Niu et al have demonstrated that the biofilm formation ability of wild type and *abaI* mutant was no difference at 8 h, but the biofilm formation ability of the mutant was significantly lower than that of wild type at 16 h and 24 h, which was 40% and 41%, respectively.

These results suggest that the *abaI*-directed QS pathway is required for the later stages of biofilm maturation.[12] The biofilms were established for 24 h in this study, and it might be the reason why only *abaI* was overexpressed and the expressions of other genes that contributed to the initial steps of biofilm formation were not significantly changed.[1] Indeed, the mechanism of biofilm-associated genes in biofilm formation and antimicrobial resistance needed to be further explored.

Conclusion

In this study, most of *A. baumannii* have biofilm formation ability and high carrying rate of biofilm-associated genes. There was a negative correlation between biofilm formation ability and antimicrobial resistance, which was stronger biofilm formation occurring in non-MDR strains. Moreover, once the biofilm was formed, whether the biofilm was strong or weak, the antimicrobial resistance can be greatly improved. In addition, presence of *csuD* was significantly associated with biofilm-forming capacity. The *csuA* and *csuD* genes more occured in non-MDR strains, but *ompA* was more common in MDR strains. Notably, the expression of QS-related gene *abaI* up-regulated in biofilm stage. In fact, the number and type of biofilm-associated genes carried by biofilm-forming strains are quite different among different regions,[11,50,51] indicating that the biofilm-forming ability is not only determined by the presence of biofilm-associated genes but a complex process depended on multiple factors. Deeper exploration of mechanisms of biofilm formation and biofilm-specific resistance in *A. baumannii* are highly needed.

Ethics Approval and Consent to Participate

This study was approved by the Institutional Review Board of affiliated hospital of southwest medical university (KY2020043). Written informed consent was obtained from all participants.

Acknowledgments

Author Contributions

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

Disclosure

The authors declare that they have no conflicts of interest in this work.

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