

Molecular Characteristics, Antimicrobial Resistance, and Biofilm Formation of *Pseudomonas aeruginosa* Isolated from Patients with Aural Infections in Shanghai, China

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Purpose: To investigate molecular characteristics, antimicrobial resistance, and biofilm formation ability of *Pseudomonas aeruginosa* strains isolated from patients with aural infections.

Methods: Isolates (n = 199) were collected from ear discharges of patients with aural infections from January 2019 to December 2020. Antimicrobial susceptibility testing was performed according to the Clinical and Laboratory Standards Institute guidelines. All isolates were subjected to multilocus sequence typing (MLST) with amplification and sequencing of seven housekeeping genes. Biofilm formation and eradication were quantitatively assessed in microtiter plates. Genes associated with biofilm formation and the quinolone-resistance-determining region (QRDR) of genes *gyrA* and *parC* were investigated using polymerase chain reaction amplification and sequencing.

Results: Of the 199 *P. aeruginosa* strains isolated, 109 (54.77%) were from females and 90 (45.23%) were from males. The isolates exhibited very low rates of resistance to most antibiotics tested, including piperacillin (1.51%), ceftazidime (0.50%), and imipenem (3.52%); however, the quinolones ciprofloxacin (80.40%) and levofloxacin (82.91%) were notable exceptions. The QRDR sequence results of the quinolone-resistant *P. aeruginosa* isolates showed Thr83Ile (n = 155) was the most common amino acid mutation in *gyrA* (n = 165), while Ser87Leu (n = 157) was widely detected in *parC* (n = 165). MLST analysis identified 34 sequence types (STs) with most isolates belonging to ST316 (73.87%). Almost all of the *P. aeruginosa* isolates (96.98%) produced biofilms and biofilm-forming genes *algD* (98.49%), *pslD* (96.98%), and *pelF* (96.48%) were highly prevalent.

Conclusion: The *P. aeruginosa* strains isolated from aural discharges in this study exhibited very low rates of resistance to most antibiotics tested, except for the resistance rates to quinolones, which were relatively high. The isolates also exhibited a strong biofilm formation ability and low susceptibility to eradication, indicating that more effective drugs and treatment methods are needed to combat these infections.

Keywords: *Pseudomonas aeruginosa* isolate, ear infection, antimicrobial resistance, ST316, biofilm

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Introduction

Aural infections are one of the most common diseases of the head and neck and are predominantly caused by bacterial infections of the ear canal. Several studies from different countries have reported that *Pseudomonas aeruginosa* is the most common

pathogen isolated from ear canal secretions, followed by *Staphylococcus aureus*.¹ For instance, in a cross-sectional, descriptive study carried out in Iraq, *P. aeruginosa* (57.5%) and *S. aureus* (16.8%) were the two most commonly isolated microorganisms.² Similarly, in another study conducted in India, the predominant isolates were again *P. aeruginosa* (49%) and *S. aureus* (18%).³

P. aeruginosa aural infections can result in considerable hearing loss with life-long sequelae occurring more frequently in high-risk populations of both developing and developed countries.⁴ Serious extracranial and intracranial complications are also possible;⁵ however, treatment of *P. aeruginosa* aural infection is often recurrent and refractory, probably because *P. aeruginosa* can thrive in the ear and is difficult to eradicate.⁶ According to results from 2005 to 2017 from the CHINET surveillance system, *P. aeruginosa* showed a relatively low resistance profile and exhibited trends of decreasing resistance to the five most commonly used antimicrobials, amikacin, ceftazidime, ciprofloxacin, piperacillin/tazobactam, and imipenem.⁷ However, resistance data regarding *P. aeruginosa* isolated from ear canal discharge samples are not clear.

P. aeruginosa biofilms potentially play an important role in patients with aural infection. The treatment of *P. aeruginosa* aural infection may be hindered by the ability of *P. aeruginosa* to form biofilms, which protect the bacteria from surrounding environmental stresses and impedes phagocytosis, thereby conferring the potential for colonization and long-term persistence.⁸ Biofilms are microbial communities encased in extracellular polymeric substances (EPS), which consist of polysaccharides, extracellular DNA (eDNA), and proteins.⁹ The biofilm matrix in *P. aeruginosa* is composed of at least three distinct exopolysaccharides, alginate, Psl, and Pel.¹⁰ Alginate, which is encoded by *algD*, is a polymer consisting of β -D-mannuronic acid and α -L-guluronic acid and provides structural stability for *Pseudomonas* biofilm formation.¹¹ Psl is produced from a sugar nucleotide pool of precursors, including GDP-D-mannose, UDP-D-glucose, and dTDP-L-rhamnose.¹² PslD is a secreted protein encoded by *PslD* and is required for biofilm formation, presumably for its role in exopolysaccharide export.¹³ In addition, the gene product of *PelF* has been suggested to be a soluble glycosyltransferase that uses UDP-glucose as a donor substrate in the biosynthesis of Pel exopolysaccharide.¹⁴

Multiple studies have reported the characteristics of *P. aeruginosa* isolated from ear canal discharges;¹⁵

however, few studies have focused on *P. aeruginosa* aural infections in Chinese populations. Therefore, we conducted a study of 199 *P. aeruginosa* strains isolated from patients with aural infections at two hospitals in Shanghai, China, from January 2019 to December 2020. This study aimed to evaluate the molecular characteristics, antimicrobial resistance, and biofilm formation ability of these strains to better understand the underlying factors that may lead to aural infection associated with *P. aeruginosa*.

Materials and Methods

Clinical Isolates

A total of 199 non-duplicate sequential isolates were collected from January 2019 to December 2020 from ear discharges of patients with aural inflammation at the Eye and ENT Hospital, Fudan University and the Huashan Hospital, Fudan University, Shanghai, China. Species identification was performed and confirmed using a VITEK 2 automated system (bioMérieux, France). The study was approved by the Institutional Review Boards of Huashan Hospital (number: KY2017-274) and the Eye and ENT Hospital (number: EENT2015011).

Multilocus Sequence Typing (MLST)

MLST of all isolates was performed according to previously described methods¹⁶ based on polymerase chain reaction (PCR) amplification and sequencing of seven housekeeping genes (*acsA*, *aroE*, *guaA*, *mutL*, *nuoD*, *ppsA*, and *trpE*). Primers used for the PCR amplification are summarized in Table 1. The nucleotide sequences of the above noted genes were submitted to the MLST database (<http://pubmlst.org/paeruginosa/>) to determine the allelic numbers and sequence types (STs). A minimum-spanning tree (MST) was inferred using PHYLOViZ 2.0 software based on the MLST allelic profiles.

Antimicrobial Susceptibility Testing

Minimum inhibitory concentrations (MICs) of the isolates exposed to different antibiotics were determined using either the broth microdilution or agar dilution method. *P. aeruginosa* ATCC 27853 was used as the control strain. Quality control and interpretation of the results were based on the Clinical and Laboratory Standards Institute (CLSI) 2021 breakpoints for all antimicrobial agents,¹⁷ except cefoperazone and cefoperazone/sulbactam for which CLSI criteria were not available. Therefore, the

Table 1 Primers Used for Amplification

Targets	Primers	Sequence (5' -to 3')	Amplified Product (bp)
<i>acsA</i>	<i>acsA-F</i> <i>acsA-R</i>	ACCTGGTGTACGCCTCGCTGAC GACATAGATGCCCTGCCCTTGAT	842
<i>aroE</i>	<i>aroE-F</i> <i>aroE-R</i>	TGGGGCTATGACTGGAAACC TAACCCGGTTTTGTGATTCCTACA	825
<i>guaA</i>	<i>guaA-F</i> <i>guaA-R</i>	CGGCCTCGACGTGTGGATGA GAACGCCTGGCTGGTCTTGTGGTA	940
<i>mutL</i>	<i>mutL-F</i> <i>mutL-R</i>	CCAGATCGCCGCCGGTGAGGTG CAGGGTGCCATAGAGGAAGTC	940
<i>nuoD</i>	<i>nuoD-F</i> <i>nuoD-R</i>	ACCGCCACCCGTA CTG TCTCGCCCATCTTGACCA	1042
<i>ppsA</i>	<i>ppsA-F</i> <i>ppsA-R</i>	GGTCGCTCGGTCAAGGTAGTGG GGGTTCTCTTCTCCGGCTCGTAG	989
<i>trpE</i>	<i>trpE-F</i> <i>trpE-R</i>	GCGGCCAGGGTCGTGAG CCC GGCGCTTGTGATGGT	811
<i>gyrA</i>	<i>gyrA-F</i> <i>gyrA-R</i>	GTGTGCTTTATGCCATGAG GGTTTCCTTTCCAGGTC	287
<i>parC</i>	<i>parC-F</i> <i>parC-R</i>	CATCGTCTACGCCATGAG AGCAGCACCTCGGAATAG	267
<i>algD</i>	<i>algD-F</i> <i>algD-R</i>	CTACATCGAGACCGTCTGCC GCATCAACGAACCGAGCATC	593
<i>pelF</i>	<i>pelF-F</i> <i>pelF-R</i>	GAGGTCAGCTACATCCGTCG TCATGCAATCTCCGTGGCTT	789
<i>pslD</i>	<i>pslD-F</i> <i>pslD-R</i>	TGTACACCGTGCTCAACGAC CTCCGGCCCCGATCTTCATC	369

cefoperazone and cefoperazone/sulbactam MICs were estimated using the CLSI 2021 breakpoints for *Enterobacterales*.

Identification of Quinolone-Resistant Determination Regions (QRDR) Mutations

Quinolone-resistant strains of *P. aeruginosa* were determined according to CLSI guidelines. Quinolone resistance presents mainly as a result of mutations in the QRDR of *gyrA*, which codes for DNA gyrase subunits, and the QRDR of *parC*, which codes for topoisomerase IV subunits.¹⁸ The *gyrA* and *parC* genes in resistant strains were PCR amplified using the primers listed in Table 1 and sequenced to detect any point mutations.¹⁹ The resulting DNA sequences were compared with those of wild-type *P. aeruginosa* PAO1 using the Basic Local Alignment

Search Tool of the National Center for Biotechnology Information (Bethesda, MD, USA).

Biofilm Formation Assay

Quantitative assessment of biofilm formation was performed as described by Taha et al²⁰ using overnight cultures of *P. aeruginosa* with their concentration adjusted to that of the turbidity of a 0.5 McFarland standard. The suspensions were then diluted 1:100 with fresh Luria-Bertani (LB) broth, seeded into sterile flat-bottomed 96-well microplates, and incubated for 24 h at 37°C. The microplate wells were then gently washed three times with sterile phosphate-buffered saline (PBS) to remove non-adherent bacteria. The adherent biofilms were fixed with 99% methanol for 15 min, the solution removed, and the plates air-dried. The biofilms were then stained with 100 µL of crystal violet (0.1%) for 15 min at room

temperature, rinsed with water, and allowed to dry. Acetic acid (33%) was used to extract the crystal violet adhered to the biofilm. Aqueous acetic acid (33%) was used as the negative control. For quantitative assays, each experiment was performed in triplicate wells. The optical density (OD) was measured at 570 nm using a microtiter plate reader. The OD cut-off value (OD_c) for biofilm formation was calculated using the following equation: OD_c = average OD of the negative control + (3 × SD of the negative control). For interpretation of the results, the strains were divided into four categories: non-biofilm producer (OD < OD_c), weak biofilm producer (OD_c < OD < 2 × OD_c), moderate biofilm producer (2 × OD_c < OD < 4 × OD_c), and strong biofilm producer (OD > 4 × OD_c).²¹

Biofilm Eradication Assay

P. aeruginosa strains with different biofilm-forming abilities were selected to evaluate the eradication effect of distinct concentrations of ceftazidime (National Institutes for Food and Drug Control, China) on the mature biofilm, as described previously.²² First, diluted *P. aeruginosa*

suspensions were incubated in 96-well microtiter plates at 37°C for 24 h to allow mature biofilm formation. The established mature biofilms were then washed three times with sterile PBS. The surface-attached cells were then treated with different concentrations of antibiotic-containing LB Broth (1 × MIC, 2 × MIC, 4 × MIC) and incubated for 24 h at 37°C. Biofilm formation was measured as described above.

Detection of Biofilm-Related Genes

All strains were evaluated for three genes encoding biofilm formation, *algD*, *pslD*, and *pelF*. Each gene was PCR-amplified as previously described using the gene-specific primers listed in Table 1.²³

Statistical Analysis

Statistical analysis was performed using SPSS version 21.0 software (SPSS Inc., Chicago, IL, USA) or Prism version 9.0 software (GraphPad Software, San Diego, CA, USA). The chi-squared test was performed to determine the relationship between categorical variables,

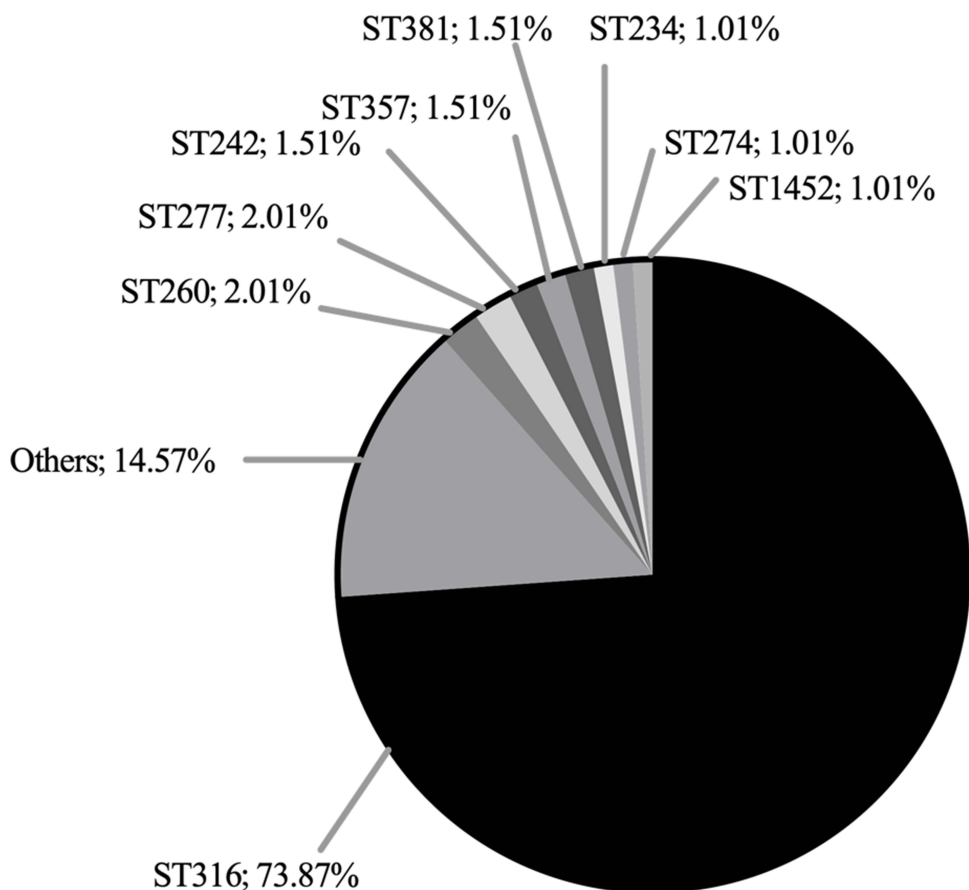


Figure 1 Distribution of sequence types (STs) among *P. aeruginosa* isolates.

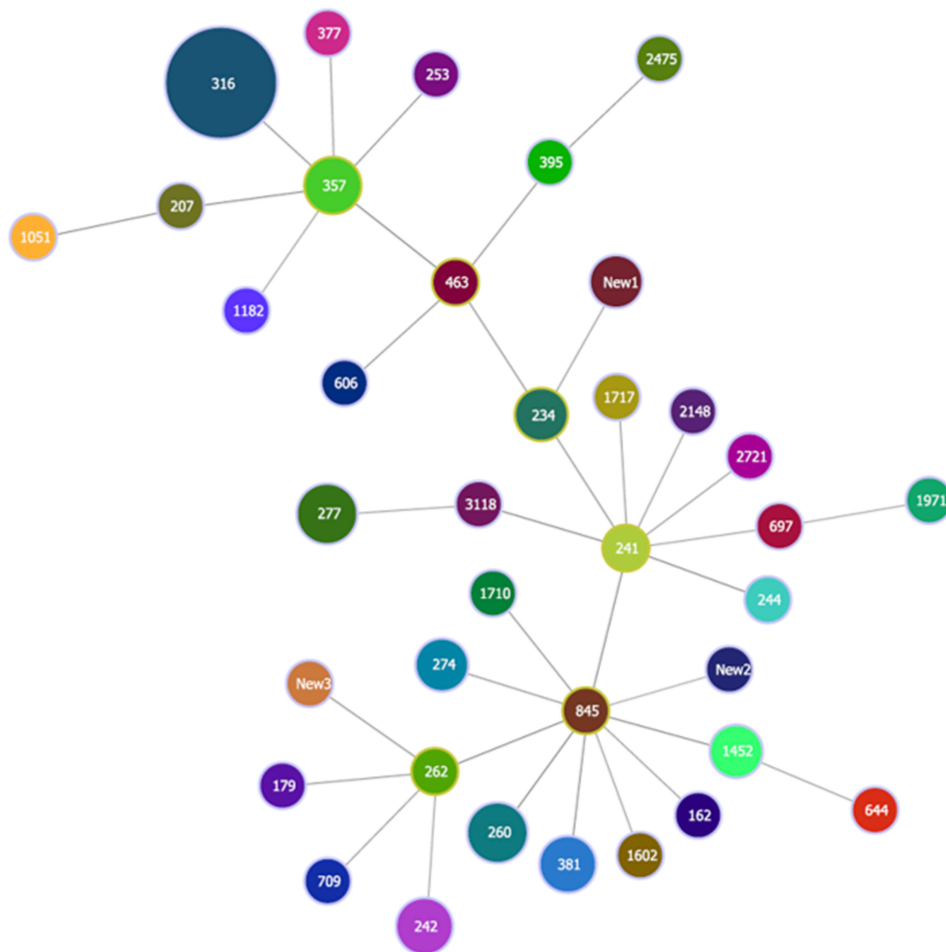


Figure 2 Relationship of the 199 *P. aeruginosa* isolates based on multilocus sequence typing (MLST) allelic profiles inferred from the minimum-spanning tree (MST). **Notes:** Each circle represents a single sequence type (ST); the size of the circle is relative to the number of isolates.

including biofilm characteristics and antimicrobial resistance. The *t*-test was used for analyses of biofilm formation with or without ceftazidime. A *p*-value <0.05 was considered statistically significant.

Results

A total of 199 *P. aeruginosa* isolates were included in this study. The median age of the patients was 49 years with the proportion of female patients (54.77%) being slightly higher than that of the male patients (45.23%). MLST analysis revealed a total of 34 STs among the 199 *P. aeruginosa* isolates based on MST inference (Figures 1 and 2). Most isolates belonged to ST316 (73.87%), followed by ST260 (2.01%), ST277 (2.01%), ST242 (1.51%), ST357 (1.51%), and ST381 (1.51%). Four isolates were designated as belonging to a new ST.

Overall, the *P. aeruginosa* isolates exhibited very low rates of resistance to most of the antibiotics tested, such as

piperacillin (1.51%), ceftazidime (0.50%), and imipenem (3.52%) (Table 2). However, the resistance rates to quinolone antibiotics, including ciprofloxacin (80.40%) and levofloxacin (82.91%), were significantly higher than those to other antibiotics. The QRDR sequence changes of the quinolone-resistant *P. aeruginosa* isolates (*n* = 165) revealed that 95.76% had an amino acid mutation in either *gyrA* or *parC*. The most common mutations in the quinolone-resistant strains occurred in codon 83 of *gyrA* (Thr83Ile; *n* = 155), followed by Asp87Tyr (*n* = 1), and Gly254Ser (*n* = 1). Most *parC* point mutations encoded amino acid changes at positions 87 and 91: Ser87Leu (*n* = 157) and Glu91Lys (*n* = 7). Mutations Val646Leu (*n* = 2), His262Gln (*n*=1), and Pro752Thr (*n* = 1) were also detected in *parC*.

Biofilm formation capacity among the 199 isolates quantified using the crystal violet assay was distributed as follows: 16.08% (*n* = 32) were strong biofilm producers,

Table 2 Minimum Inhibitory Concentration (MIC) Characteristics and Resistance Rates of *Pseudomonas aeruginosa* Isolates

Antimicrobial Agents	<i>Pseudomonas aeruginosa</i> (n=199)				
	S	I	R	MIC ₅₀ (µg/mL)	MIC ₉₀ (µg/mL)
Piperacillin	80.90%	17.59%	1.51%	16	32
Piperacillin-tazobactam	87.94%	11.56%	0.50%	16/4	32/4
Ceftazidime-avibactam	100.00%	-	0.00%	2/4	8/4
Ceftazidime	98.49%	1.01%	0.50%	4	8
Cefepime	91.46%	7.54%	1.01%	4	8
Aztreonam	35.18%	27.64%	37.19%	16	32
Imipenem	95.98%	0.50%	3.52%	1	2
Meropenem	95.48%	0.50%	4.02%	0.25	8
Gentamicin	44.22%	6.53%	49.25%	8	128
Amikacin	97.99%	0.50%	1.51%	4	8
Ciprofloxacin	16.58%	3.02%	80.40%	>128	>128
Levofloxacin	15.08%	2.01%	82.91%	>128	>128
Cefoperazone	58.29%	38.69%	3.02%	16	32
Cefoperazone/sulbactam	61.81%	36.18%	2.01%	16/8	32/16

Abbreviations: S, sensitive; I, intermediary; R, resistant.

56.28% (n=112) were moderate biofilm producers, 24.62% (n=49) were weak biofilm producers, and 6 isolates were classified as non-biofilm producers. Meanwhile, PCR amplification of biofilm-associated genes in the *P. aeruginosa* isolates revealed a high prevalence of *algD*

(98.49%), *pslD* (96.98%), and *pelF* (96.48%) (Supplementary Figure S1 and Table S1). All three high-prevalence genes (*algD*⁺/*pslD*⁺/*pelF*⁺) were detected in 95.98% of the isolates, whereas 1.51% of the isolates were *algD*⁻/*pslD*⁻/*pelF*⁻. Three *P. aeruginosa* strains

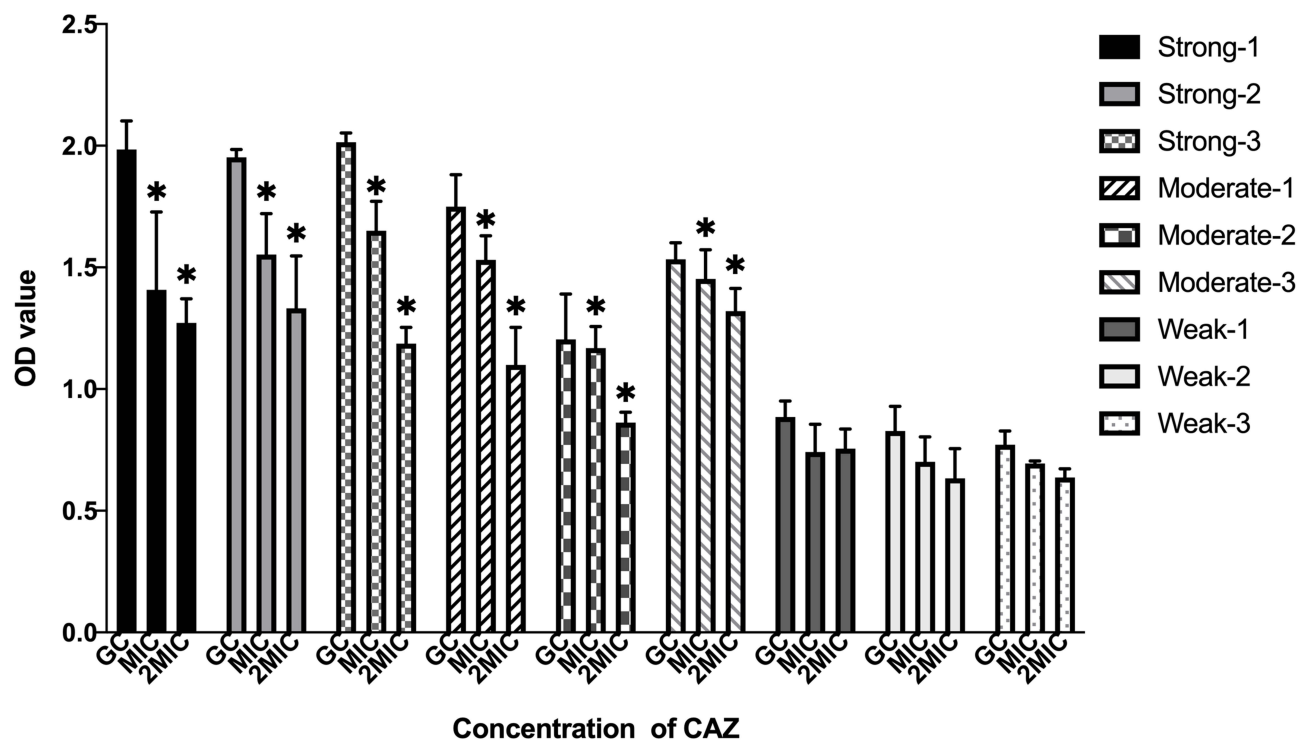


Figure 3 Effect of different ceftazidime concentrations on biofilm eradication of *P. aeruginosa* isolates.

Notes: Strong-1, Strong-2, and Strong-3 refer to strong biofilm-producing isolates; Moderate-1, Moderate-2, and Moderate-3 refer to moderate biofilm-producing isolates; and Weak-1, Weak-2, and Weak-3 refer to weak biofilm-producing isolates. The error bars represent the standard deviations. *P < 0.05.

Abbreviation: GC, growth control.

with different biofilm-forming abilities were selected for subsequent biofilm eradication assays. Of the established mature biofilms, no isolates could be fully eradicated using 1× MIC or 2× MIC ceftazidime (Figure 3). Based on biofilm-forming ability, the *P. aeruginosa* strains were divided into biofilm-positive and biofilm-negative groups. There was no significant correlation between *P. aeruginosa* drug resistance for most antimicrobial agents and biofilm positivity ($p > 0.05$; Table 3).

Discussion

Aural infections caused by *P. aeruginosa* are an ongoing source of concern. Hu et al found that the resistance rates of *P. aeruginosa* to ceftazidime, piperacillin-tazobactam, and ciprofloxacin were 21.4%, 13.4% and 14.8%, respectively, in 2017, according to CHINET surveillance data.⁷ However, our current results showed that *P. aeruginosa* strains isolated from ear canal discharge had significantly lower average resistance rates to antibiotics tested, except for levofloxacin and ciprofloxacin. For instance, the resistance rates for ceftazidime and piperacillin-tazobactam each were only 0.50%. Although the resistance rates of most antimicrobial agents tested in the present study were relatively low, resistance to fluoroquinolones was considerably higher than previously reported.²⁴ This may hint at an alarming situation reflecting the threat of limited treatment options for aural infections. In China, levofloxacin ear drops are used widely, which may be the main cause of induced quinolone resistance. In agreement with previous

reports, our results also showed that *P. aeruginosa* quinolone resistance may be because of point mutations in *gyrA* and *parC*.²⁵

MLST analysis showed that ST316 was the predominant ST of *P. aeruginosa* among the ear discharge isolates analyzed, accounting for 73.87% of all *P. aeruginosa* strains. Zowawi et al found that ST316 clones are less predominant in carbapenem-resistant *P. aeruginosa*²⁶ while Khan et al reported ST316-related clones of *P. aeruginosa* isolates from patients with keratitis in India.²⁷ Furthermore, the molecular typing results of *P. aeruginosa* isolated from a Chinese burn center from 2011 to 2016 showed that ST316, ST111, ST360, ST244, and ST1158 are dominant STs with at least 15 isolates found in each type, accounting for 51.53% of the total isolates.²⁸ However, little data are currently available describing the relationship between *P. aeruginosa* ST316 strains and aural infections, thus further study is required.

Antimicrobial treatments of *P. aeruginosa* infections are challenging, mostly because of the ability of *P. aeruginosa* to form dense and persistent biofilms.²⁹ Here, 96.98% of the *P. aeruginosa* isolates studied were biofilm producers, a percentage suggesting stronger biofilm formation in the ear canal than that reported in other studies,^{30,31} which may contribute to recurrent ear infections. In our study, the results of biofilm-related gene detection indicated that 95.98% of *P. aeruginosa* strains were *algD*⁺/*pslD*⁺/*pelF*⁺, which is consistent with a strong biofilm forming capacity, a finding similar to that of other studies.^{23,31}

Table 3 Comparison of the Antimicrobial Susceptibilities of Biofilm-Forming and Non-Biofilm-Forming Isolates

Antimicrobial Agents	Biofilm-Forming Isolates (n=193)	Non-Biofilm-Forming Isolates (n=6)	p value
	Sensitivity, no. (%)	Sensitivity, no. (%)	
Piperacillin	157 (78.89%)	5 (83.33%)	0.22
Piperacillin-tazobactam	173 (86.93%)	3 (50.00%)	0.02
Ceftazidime-avibactam	193 (96.98%)	6 (100.00%)	1.00
Ceftazidime	190 (95.48%)	6 (100.00%)	1.00
Cefepime	177 (88.94%)	5 (83.33%)	0.42
Aztreonam	70 (35.18%)	1 (16.67%)	0.42
Imipenem	185 (92.96%)	6 (100.00%)	1.00
Meropenem	184 (92.46%)	6 (100.00%)	1.00
Gentamicin	85 (42.71%)	3 (50.00%)	1.00
Amikacin	189 (94.97%)	6 (100.00%)	1.00
Ciprofloxacin	33 (16.58%)	1 (16.67%)	1.00
Levofloxacin	29 (14.57%)	1 (16.67%)	1.00
Cefoperazone	114 (57.29%)	2 (33.33%)	0.24
Cefoperazone/sulbactam	120 (60.30%)	3 (50.00%)	0.68

Biofilm eradication experiments showed that once a biofilm was formed, it was very hard to entirely eradicate it. This may explain the poor efficacy of antimicrobial therapy for ear canal infections caused by *P. aeruginosa*. Furthermore, biofilm-forming bacteria can tolerate antimicrobials and components of the host immune system.³² Accordingly, biofilms play an important role in hindering the treatment of *P. aeruginosa* aural infections, but also provide potential treatment targets for aural infections caused by *P. aeruginosa*. Thus, the development of drugs that destroy biofilms or inhibit their formation may increase the efficiency of antibacterial drugs.

Conclusion

Our results provide valuable information regarding the molecular characteristics, antimicrobial resistance, and biofilm-forming ability of *P. aeruginosa* strains isolated from patients with aural infections in China. Although the rate of resistance to multiple antibiotics among the *P. aeruginosa* isolates was relatively low, our results showed that *P. aeruginosa* strains isolated from patients with aural infections had a strong capacity for biofilm formation and a high tolerance to biofilm eradication. Therefore, more effective drugs and treatment methods for the eradication of biofilms are urgently needed to effectively combat these infections.

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Disclosure

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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