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The virulence trait and genotype distribution amongst the *Pseudomonas aeruginosa* clinical strains

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Abstract

Background *Pseudomonas aeruginosa* is notorious for its complex virulence system and rapid adaptive drug resistance. This study aimed to compare the prevalence and genotype distribution of virulence genes in multidrug-sensitive and multidrug-resistant clinical strains of *Pseudomonas aeruginosa*. It is possible to better understand the genetic characteristics of *Pseudomonas aeruginosa* and carry out effective treatment and prevention measures.

Methods The genes *phzS*, *aprA*, *plcH*, *toxA*, *pilA* and *exoU* were detected amongst 184 clinical strains, whose cytotoxicity and biofilm formation ability were evaluated as well. Phenotypic screening for drug susceptibility was conducted by standard antimicrobial susceptibility test and interpreted according to standards established by CLSI.

Results A total of 94 multidrug-sensitive and 90 multidrug-resistant isolates were included in this study. Statistically significant relationship was observed in the frequency of the *toxA* ($p=0.002$) and *plcH* ($p=0.001$) genes between multidrug-resistant and multidrug-sensitive strains. Moreover, thirteen genotypes were observed in multidrug-sensitive strains, and seven of them were included in multidrug-resistant groups. There was statistically significant correlation found between the presence of genotype IV ($p=0.001$) and genotype VII ($p=0.001$) in two subgroups. Additionally, It was found that genotype III isolates exhibited most obvious cytotoxicity, and multidrug-resistant isolates of genotype III showed the most significant cytotoxicity. Moreover, the strains of strong biofilm-formation accounted for a relatively high proportion in genotype III and VI groups.

Conclusion These virulence genes could form abundant genotype varieties, whose overall number is greater in multi-sensitive strains. In addition, particular genotypes were characteristically distributed and exhibited different cytotoxicity and biofilm-formation abilities.

Keywords *Pseudomonas aeruginosa*, Genotype, Resistance, Virulence, Biofilm

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Introduction

Pseudomonas aeruginosa (*P. aeruginosa*) can survive under a wide range of environmental conditions and utilize various sources of carbon, nitrogen, and energy, except that its growth temperature range is quite limited [1]. Therefore, it ubiquitously exists in both terrestrial and aquatic ecosystems. As one of the common pathogenic bacteria in clinical practice, *P. aeruginosa* is considered to be a pathogen of major medical importance that can cause acute or chronic infections in various immunocompromised patients [2]. Epidemiological information shows that the isolation rate of *P. aeruginosa* from clinical infected specimens reached about 10% [3].

P. aeruginosa can become resistant to conventional antimicrobial drugs due to its quick adaptability and high intrinsic resistance, thereby increasing the difficulty of treatment. Additionally, expressions of numerous acquired antimicrobial resistance mechanisms, virulence factors and mechanisms for evading host defences (including biofilm formation) of *P. aeruginosa* make the clinical treatment even more challenging and infections are often life-threatening [4]. In 2017, *P. aeruginosa* was listed by the WHO (World Health Organization) as one of the most life-threatening bacteria and a key pathogen in the development of new antibiotics [5]. Besides, in 2021, *P. aeruginosa* was considered one of the six "ESKAPE" pathogens listed by the WHO as a key pathogen for antibiotic resistance [6].

P. aeruginosa can adapt to the adverse environment of its host by secreting multiple virulence factors, which contribute to successful infection and pathogenesis. Some factors have been confirmed to contribute to the virulence of *P. aeruginosa*. For instance, Exotoxin U (encoded by the *exoU* gene) was reported to induce highly rapid lysis on the host cell membrane, leading to necrosis of epithelial cells, macrophages and neutrophils [7], and its presence can lead to more severe disease progression [8]. Moreover, other factors of cellular components (such as pili), exogenous toxin A and some enzymes have also been proven to be associated with bacterial virulence. The *pilA* gene encodes bacterial pili proteins, which are structural proteins synthesized by *P. aeruginosa* and closely related to bacterial adhesion and biofilm formation [9, 10]. Meanwhile, the *toxA* gene encodes the exogenous toxin A, which is a protein involved in stimulating the synthesis of pro-inflammatory cytokines, thereby being responsible for the cytotoxicity [9, 10]. It was reported that hemolytic phospholipase C (encoded by the *plcH* gene) preferentially hydrolyzes sphingomyelin and phosphatidylcholine to cause tissue damage and inflammation and interferes with the oxidative burst of immune cells [11]. Besides, the *aprA* gene is involved in the synthesis of zinc metalloproteinase that degrades several components of the host immune system, such as the complement C1q and C3, or cytokines, such as IFN-γ and TNF-α [12]. Additionally, *phzS* gene mainly plays a role in inducing the synthesis of flavin dependent hydroxylase and mediating the conversion of phenazine-1-carboxylic acid to pyocyanin and 1-hydroxyphenazine (1-HP) [13], which contributes to pathogenicity and host tissue damage by producing reactive oxygen species and mediating the production of superoxide radicals [14, 15]. Genes encoding virulence factors of *P. aeruginosa* may be located on bacterial chromosome or other genetic elements of the genome, such as plasmids and pathogenic islands [9].

In general, the overall infection status of *P. aeruginosa* is to some extent determined by the presence of virulence factors as well as its capacity to resist many antimicrobial drugs [16, 17]. Pathogenic bacteria that develop resistance to three or more classes of antibacterial drugs are called multidrug resistance (MDR) [18]. It was reported that *P. aeruginosa* has developed a large number of MDR strains, and these strains are associated with a significantly high incidence rate and mortality [19]. Therefore, studies focused on the MDR strains of *P. aeruginosa*, such as epidemiological information, virulence feature, evolutionary strategies, etc., have now become national and international important research subjects [20].

This study aimed to compare the prevalence and genotype distribution of some virulence genes in clinical multidrug sensitive (MDS) and MDR strains of *P. aeruginosa* from clinical sources. The presence of six virulence genes (*exoU*, *pilA*, *toxA*, *plcH*, *aprA* and *phzS*) of those strains was investigated, and their presence in clinical strains with different phenotypes of susceptibility were analysed as well. Moreover, We also compared the cellular virulence activity and the ability of biofilm formation among MDS and MDR strains of each genotype of virulence genes.

Materials and methods

Isolates and their identification

A total of 94 MDS and 90 MDR clinical isolates of *P. aeruginosa* strains from the Microbiology Department of Laboratory Medicine in Zhongshan Hospital Affiliated to Fudan University in China were included in the study. Clinical samples are processed according to the standard procedures of the microbiology laboratory, then, the preliminary identification of *P. aeruginosa* cultured from clinical samples was based on the typical colony morphology in Columbia agar with 5% sheep blood, chocolate agar and MacConkey agar plates at aerobic conditions. The final verification of each isolate was performed by utilizing MALDI-TOF/TOF (bioMérieux, Craponne, France) to single colony with VITEK® MS CHCA (bioMérieux, Craponne, France). All *P. aeruginosa*

strains involved in this study were nonrepetitive and separately originated from different patient individuals.

Antimicrobial susceptibility test

During the antimicrobial susceptibility testing, the following steps were used: fresh bacterial suspension was prepared by selecting three or more purified bacterial colonies, minimum inhibitory concentration (MIC) of each drug was acquired through Vitek2 Compact system using GN 335 cards (bioMérieux, Craponne, France). Doubtful results will be verified by the disc diffusion method on Mueller–Hinton agar (Becton Dickinson). Results were interpreted according to standards established by the Clinical and Laboratory Standards Institute (CLSI. Performance standards for antimicrobial susceptibility testing.).

According to the international definition from European Centre for Disease Prevention and Control [18], MDR strains were non-sensitive to at least one of the representatives of at least three independent classes of antimicrobial drugs. Whereas isolates that did not meet above criteria were assigned as MDS strains (sensitive to most groups of the antimicrobial drugs).

Bacterial genomic DNA isolation

A well-grown *P. aeruginosa* strain was cultivated in LB medium at 37 °C overnight with constant shaking (200 rpm). After centrifugation, the bacteria at the bottom of the tube were used for nucleic acid extraction. Then, the genomic DNA of *P. aeruginosa* strain was isolated using EasyPure ® TransGen Biotech (TransGen Biotech, Beijing, China) according to the manufacturer's protocol. The DNA sample is stored at -20 ℃ for polymerase chain reaction (PCR) detection.

Virulence genes detection

Six virulence genes were examined in this research. In brief, the DNA products were detected using 2×TransTaq®-T PCR SuperMix kit (TransGen Biotech, Beijing, China), and the detection procedure was as follows: 94 °C, 5 min; 35 cycles: 94 °C, 30 s and 50–60 °C, 30 s; 72 °C, 30 s; 72 °C, 10 min. The PCR product was separated into individual bands using agarose gel electrophoresis, and was visualized under gel imaging system (Bio-Rad, Feldkirchen, Germany) with Nucleic Acid Gel Stain (Yeasen Biotechnology, Shanghai, China). Meanwhile, strains of *P. aeruginosa* carrying the particular virulence gene and PAO1 were assigned as positive or negative controls. Primers used in this study were included in Table 1.

Biofilm formation assay

Biofilm formation assay was conducted as previously described [23]. Bacterial suspension of 0.5 McFarland standard was prepared and then added to 96-well microtiter plates (100 μL/well). After incubation at 37 °C for 24 h, the supernatant and floating bacteria were discarded. Then, each well was washed gently 3 times by PBS. The biofilm at the bottom of the plate well was fixed with methanol and stained with 0.1% crystal violet solution for 20 min. Afterwards, the stained wells were washed 3 times by PBS and dissolved with glacial acetic acid solution. The OD of each well was measured at 570 nm by using an automated Microplate reader (Bio-Rad, USA), the value of OD_{570} represents bacterial biofilm. The OD_{570} value of wells only containing LB broth without bacteria (OD_C) was designed as negative control. *P. aeruginosa* isolates with OD_{570} value > 4 OD_C were classified into strong biofilm-formation strains [24].

Cell culture and cytotoxicity assay

Immortalized human bronchial epithelial cells (BEAS-2B cells) were purchased from Shanghai Institute of Cell Biology, Chinese Academy of Sciences. In study, cells were cultured in DMEM/F12 (Zhong Qiao Xin Zhou Biotech, Shanghai, China) containing 10% fetal bovine serum (FBS; Zhong Qiao Xin Zhou Biotech, Shanghai,

Table 1 Primer sequences in the study and expected sizes of amplification products

Gene detected	Primer	Primer sequence (5′→3′)	Annealing Temperature (◦C)	Product Size (bp)	References
exoU	F	ATGCATATCCAATCGTTG	58	2000	[21]
	R	TCATGTGAACTCCTTATT			
pilA	F	ACAGCATCCAACTGAGCG	59	1675	[10]
	R	TTGACTTCCTCCAGGCTG			
toxA	F	GGTAACCAGCTCAGCCACAT	52	352	[10]
	R	TGATGTCCAGGTCATGCTTC			
plcH	F	GAAGCCATGGGCTACTTCAA	52	307	[22]
	R	AGAGTGACGAGGAGCGGTAG			
aprA	F	TGTCCAGCAATTCTCTTGC	50	1017	[22]
	R	CGTTTTCCACGGTGACC			
phzS	F	TCGCCATGACCGATACGCTC	63	1752	[10]
	R	ACAACCTGAGCCAGCCTTCC			

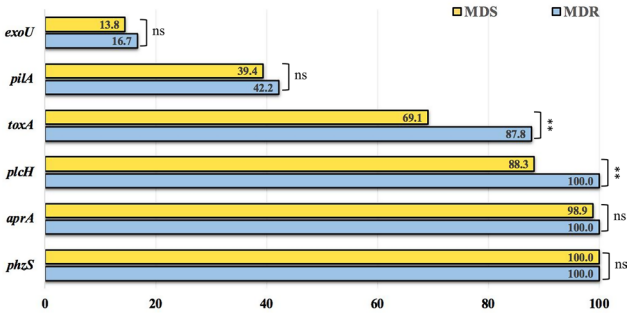

Fig. 1 Prevalence of detected virulence genes amongst clinical strains of *P. aeruginosa* included in the study according to antimicrobial susceptibility profiled (multidrug-sensitive, MDS, $n = 94$ vs. multidrug-resistant, MDR, $n = 90$)

	phzS	*aprA*	*plcH*	*toxA*	*pilA*	*exoU*	MDS n	MDS %	MDR n	MDR %
I	■	■	■	■	■	■	3	3.2	6	6.7
II	■	■	■	■	■		26	27.6	30	33.3
III	■	■	■	■		■	7	7.4	5	5.6
IV	■	■	■	■			18	19.1	38	42.2
V	■	■	■		■		1	1.1	2	2.2
VI	■	■	■			■	3	3.2	4	4.4
VII	■	■	■				23	24.4	5	5.6
VIII	■	■		■	■		5	5.3	0	0.0
IX	■	■		■			4	4.3	0	0.0
X		■	■	■	■		1	1.1	0	0.0
XI		■	■				1	1.1	0	0.0
XII		■					1	1.1	0	0.0
XIII				■	■		1	1.1	0	0.0

Fig. 2 Distribution of genotypes detected amongst examined *P. aeruginosa* strains (multidrug-sensitive, MDS, $n = 94$ vs. multidrug-resistant, MDR, $n = 90$)

China) and supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37 °C with 5% CO_2.

The cytotoxicity of each *P. aeruginosa* strain was detected as follows [25]: BEAS-2B cells were seeded in 96-well plates overnight and formed a dense monolayer. *P. aeruginosa* strains were cultured in Luria-Bertani (LB) broth (Solarbio, Shanghai, China) at 37 °C with shaking of 200 rpm overnight. A small volume of bacterial suspension was added to new LB broth and grew to logarithmic phase. The bacterial suspensions were centrifuged and the pellets were resuspended in sterile phosphate buffered saline (PBS). Each well of cells was infected with *P. aeruginosa* strain at a multiplicity of infection (MOI) of 200:1 and were incubated at 37 °C for 2 h. Cytotoxicity was detected using LDH Cytotoxicity Assay Kit (beyotime, Shanghai, China) according to manufacturer's instructions.

Statistical analysis

Spearman's rank correlation coefficient was calculated to study the correlations between particular genes in MDS and MDR groups. The chi-square ($\chi 2$) test and Fisher's two-rank exact test were used to investigate the differences between the presence of particular genes and genotypes among MDS and MDR strains. Data were represented as means ± SD. In addition, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$ were considered as significant difference.

Results

The origin and prevalence of virulence genes amongst MDS and MDR strains

Based on the results of the antimicrobial susceptibility testing (supplementary Table S1), a total of 94 MDS and 90 MDR clinical strains of *P. aeruginosa* were incorporated into this study. Their origins of specimen types and units were respectively presented in supplementary Figure S1.

The evaluation of frequency of virulence genes reveals the extensive diversity of genes' distribution. The gel electrophoresis results of all tested genes of some tests were shown in supplementary Figure S2. The genes *phzS*, *aprA* and *plcH* were detected in all tested MDR strains of *P. aeruginosa*, whereas *exoU* and *pilA* genes presented with lower frequency amongst both MDS and MDR isolates, being below 20% and 50% for *exoU* and *pilA* respectively. The prevalence of *toxA* gene displayed the most significant difference between MDS and MDR subgroups of strains. There was a statistically significant relationship present in the frequency of the *toxA* ($p = 0.002$) and *plcH* ($p = 0.001$) genes between MDR and MDS strains. The incidence of detected virulence genes amongst clinical strains of *P. aeruginosa* included in the study is shown in Fig. 1.

The distribution of observed genotypes amongst MDR and MDS strains

Assessment of detected virulence genes in this study demonstrated a wide variety of genotype prevalence. Generally, thirteen genotypes were observed in all examined clinical strains of *P. aeruginosa* and their distribution was shown in Fig. 2.

In the MDS group of *P. aeruginosa* strains, there were thirteen genotypes observed. The most prevalent genotype II, including detected genes of *phzS*, *aprA*, *plcH*, *toxA* and *pilA*, was observed among 26 (27.6%) of the isolates. In addition, genotypes VII (23, 24.4%) and IV (18, 19.1%) were also the major genotypes in MDS strains. Meanwhile, five (5.3%) strains presented genotype VIII. Seven genotypes were observed among the MDR strains. Genotype IV, including *phzS*, *aprA*, *plcH* and *toxA* genes, was observed among 38 (42.2%) of the isolates. Secondly, genotype II is more prevalent in MDR isolates than in MDS isolates. While other five genotypes are relatively rare. There was statistically significant correlation found

between the presence of genotype IV ($p = 0.001$) and genotype VII ($p = 0.001$) in the MDS or MDR groups of strains.

In the group of MDS strains, two pairs of genes showed a moderate positive correlation: *phzS* and *aprA* ($r = 0.492$), *pilA* and *toxA* ($r = 0.491$). Moreover, other two pairs of genes presented weak positive correlation: *aprA* and *plcH* ($r = 0.285$), *phzS* and *plcH* ($r = 0.251$). In the present study, there was no negative correlation found between these genes pairwise. Interestingly, no correlation was discovered between any two genes among MDR strains. The detailed statistical calculation of Spearman's rank correlation coefficient for the particular gene pairs, with respect to the antimicrobial susceptibility profiles of the *P. aeruginosa* strains included in the study, is presented in supplementary Table S2.

The pathogenic feature of *P. aeruginosa* strains with different genotypes

Induction of cytotoxicity and biofilm formation are important pathogenic strategies for *P. aeruginosa* during the course of infection [26, 27]. To evaluate the pathogenic potential of *P. aeruginosa* strains of each genotype, biofilm formation and cytotoxicity experiments were performed.

In the present study, according to the results of the biofilm formation assay (supplementary Figure S3), a total of 49 *P. aeruginosa* isolates were classified as strong biofilm-formation strains (supplementary Table S3), among which there were 16 MDS and 33 MDR strains. Compared with the MDS group, the MDR group contains a higher number or proportion of strong biofilm-formation strains. The proportion of strong biofilm-formation strains in each genotype of MDS and MDR groups was shown in Fig. 3. The proportion of *P. aeruginosa* strains with strong biofilm formation is relatively high in genotypes III and VI. It should be particularly noted that in these two genotypes, all MDR strains are strong biofilm-formation strains.

As shown in Fig. 4, all genotype III isolates exhibited obvious cytotoxicity. Notably, MDR isolates of genotype III showed the most significant cytotoxicity.

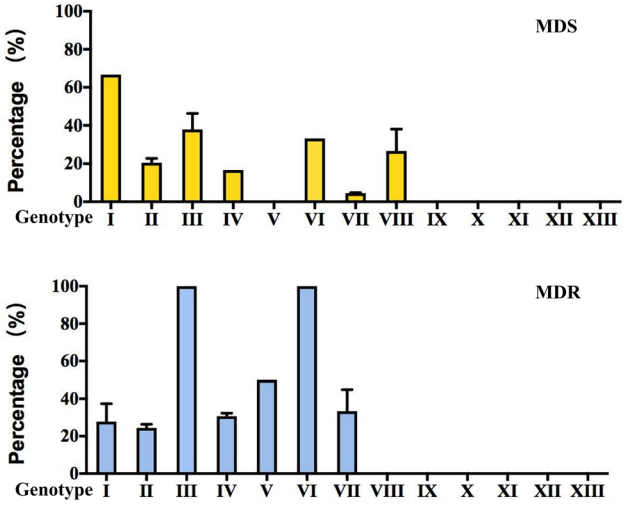

Fig. 3 The percentages of strong biofilm-formation amongst *P. aeruginosa* strains of different genotypes. *P. aeruginosa* isolates with OD_{570} value > 4 OD_C were classified into strong biofilm- formation strains. The percentage refers to the proportion of strong biofilm-formation strains among the strains of each genotype in MDS or MDR group. (multidrug-sensitive, MDS, $n = 94$ vs. multidrug-resistant, MDR, $n = 90$)

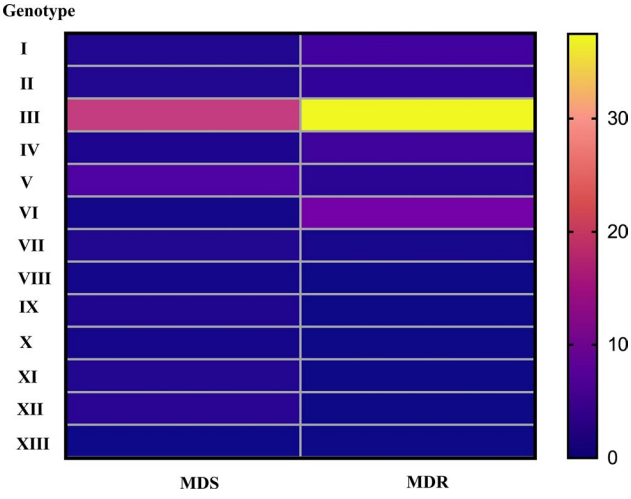

Fig. 4 The cytotoxicity of *P. aeruginosa* strains of different genotypes against epithelial cells BEAS-2B. The indicator colors for each genotype represent the average cytotoxicity of the bacteria in that group. The colors range from bottom (dark blue) to top (bright yellow), indicating increasingly stronger cytotoxicity. (multidrug-sensitive, MDS, $n = 94$ vs. multidrug-resistant, MDR, $n = 90$)

Discussion

P. aeruginosa has been associated with nosocomial infection and thus has been the subject of many studies. Its global threat is significant and the combination of antibiotic resistance mechanisms and virulence factors often determines the severity of the infection [28]. *P. aeruginosa* infections of MDR strains were increasingly prevalent, making continuous observation of this pathogen necessary [29]. It is valuable to monitor the variability of drug resistance and virulence genotypes of *P. aeruginosa*, which could provide useful tools for more effective control against the infection.

The genes detected in this study encoding many virulence factors with different activities, such as typical toxins, the most important enzymes, and structure-dependent factors, involving well-known and little-studied genes. To our knowledge, this study is one of the largest in terms of the number of strains included in the study and the number of different virulence factors investigated.

In the present study, three important virulence genes (*plcH*, *aprA* and *phzS*) were only identified in all MDR isolates, and they may serve as detection markers for drug resistance. Some researchers also reported high proportions of the *phzS*, *aprA* and *plcH* genes among both MDS strains and MDR strains [10, 30]. Moreover, a study reported that some virulence factor genes, including *plcH*, *aprA* and *phzS*, were detected in all CRPA isolates [31]. Additionally, it was found that there were important epidemiological characteristics of carrying resistance genes and virulence genes (*plcH* and *aprA*) among carbapenem non-susceptible *P. aeruginosa* [32]. The co-existence of virulence factors and multidrug resistance determinants merits close attention, considering that this would become an emerging threat.

In this study, multiple pairs of genes showed a positive correlation, namely *phzS - aprA*, *phzS - plcH*, *plcH* - aprA, *toxA - pilA*. The phenomenon suggests a certain interaction between them in the regulation of the virulence gene network of *P. aeruginosa* infection [33, 34]. Furthermore, the distribution of *toxA* gene between groups of MDR and MDS strains was statistically significant, and the detection rate was higher in MDR strains. The positive correlation between *toxA* and *pilA* may endow strains with more comprehensive pathogenic ability. Given the role of exogenous toxin A in cytotoxicity and the importance of pili proteins in adhesion and biofilm formation, it is plausible that the coordinated action of these two gene products could enhance the overall pathogenicity of the strains. This study found that low levels of *exoU* gene were present in both MDS and MDR strains, occurring in 13.8% and 16.7% of the strains, respectively. There are significant differences in the detection rates of *exoU* gene in studies conducted in different *P. aeruginosa* isolates worldwide, and it was demonstrated to be more likely to fluoroquinolone resistance caused by gyrA mutation and efflux pump overexpression [35].

The different distribution of virulence profiles among the tested strains allows for analysis of dominant genotypes. Genotype II was the dominant genotype among MDS isolates (27.6%) and the second most dominant in MDR strains (33.3%), with genotype IV being the most significant dominant genotype (42.2%). Both genotypes contained genes of *phzS*, *aprA*, *plcH* and *toxA*, and genotype II had an additional *pilA* gene than genotype IV. And this result demonstrates the versatility of the occurrence of these genes, regardless of the origins and resistance profiles of the investigated isolates. In addition, genotypes IV and VII with statistical significance in the distribution of MDS and MDR strains may become representative genotypes of particular resistance phenotype. The continuous monitoring of the dynamics of gene ratio changes in the *P. aeruginosa* strains may contribute to the development of an effective predictive tool based on the most common genes in both the MDR and MDS strains [36].

P. aeruginosa is able to evolve towards a phenotype favorable for survival through genetic adaptation, resulting in a highly diverse population of pathogens within and between patients [37]. Some genes related to virulence have been discovered in different phenotypes or origins of *P. aeruginosa* strain. *exoU*-positive genotype was demonstrated to be associated with worse prognosis [38]. It was reported that higher levels of *lasB* gene expression were related to early-stage infection in cystic fibrosis patients [39]. In addition to the clinical phenotype, it is important to understand the roles and differences of genes related to virulence in the drug resistance phenotype. This understanding allows us to better comprehend the evolutionary strategy and pathogenicity of *P. aeruginosa*. In the study conducted by Tomasz et al. [40], a higher prevalence of genes determining the virulence factors was observed in the MDR strains. Moreover, some researches had revealed a significantly positive correlation between virulence factors and antimicrobial resistance marker profiles of the tested MDR isolates [41, 42]. In the present study, genes of *toxA* and *plcH* and genotypes of IV and VII were differentially distributed between two groups of MDS and MDR isolates. Significantly, *P. aeruginosa* isolates of genotype III had been proven to have prominent cytotoxicity, especially the MDR strains with this genotype possessed strong ability of biofilm formation. The isolate that possesses the feature of both high virulence and resistance might become the strains that require special attentions in clinical practice [43, 44]. Understanding such associations might help us better predict the clinical outcomes of infections caused by different genotypes of *P. aeruginosa* and potentially guide more personalized treatment strategies [45]. Further research is needed to more thoroughly reveal the interaction between resistance and virulence factors, as this is crucial for addressing bacterial infections and formulating management strategies.

Conclusion

In this study, specific virulence genes or genotypes displayed statistical differences among strains with different drug susceptibility phenotypes, making it possible to develop an effective prediction tool based on the most common genes in multidrug-sensitive or multidrug-resistant strains. Significantly, particular MDR strains with high virulence characteristics may constitute the key focus that requires special attention in clinical practice.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12866-025-03754-6.

Supplementary Material 1

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Author contributions
Conceptualisation: YS, WG and XW; Data curation: XW and KG; Formal analysis: XW and KG; Funding acquisition: YS and WG; Investigation: XW, KG, BW and BP; Methodology: XW and KG; Project administration: YS and WG; Resources: BW and BP; Software: XW and KG; Supervision: BW and BP; Validation: BW and BP; Visualisation: XW and KG; Writing - original draft: XW and KG; Writing - review & editing: XW, KG, YS and WG.

Data availability
The datasets generated or analyzed during this study available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
The ethic statement was approved by the Ethics Committee at Zhongshan Hospital Affiliated to Fudan University (B2022-044R) (Approved on 25 February 2022), and informed consent was acquired for clinical and biological information.

Consent for publication
Not applicable.

Clinical trial number
Not applicable.

Competing interests
The authors declare no competing interests.

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