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ORIGINAL RESEARCH

Environmental Persistence of High-Risk KPC-Producing ST463 *Pseudomonas aeruginosa* in Intensive Care Units in East China

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Purpose: This study aimed to investigate the distribution and characteristics of high-risk, difficult-to-treat resistant *Pseudomonas aeruginosa* (DTR-PA) sequence type (ST) 463 in intensive care units (ICUs) in East China.

Patients and Methods: This 11-month study investigated Carbapenem-resistant *Pseudomonas aeruginosa* (CRPA) in patients and environments across four ICUs in a large teaching hospital in East China. It involved whole-genome sequencing, antibiotic susceptibility testing, and biofilm-forming ability testing of all the isolates. Molecular epidemiology and phylogenetic relationships were assessed using multi-locus sequence typing (MLST) and core genome multi-locus sequence typing (cgMLST). Bacterial virulence was evaluated using *Galleria mellonella* infection model.

Results: A total of 54 CRPA were isolated from the environment and inpatients in ICUs and classified into 25 STs, with ST463 identified as the predominant ST (13/54, 24.07%). All 13 ST463 PA isolates belonged to DTR-PA, including 4 from patients and 9 from environments primarily from wastewater dumping pools. All ST463-DTR-PA isolates carried bla_{KPC-2} and exhibited *exoU+/exoS+* virulence genotype. There were 3 KPC-ST463-DTR-PA additionally carried bla_{AFM-1}, resulting in resistance to last-resort antibiotics. ST463-DTR-PA had higher biofilm-forming ability and mortality in infected larvae than non-ST463-DTR-PA. Both clinical and environmental strains showed high virulence. CgMLST results revealed that several ST463-DTR-PA isolated from different ICUs were genetically related.

Conclusion: KPC-ST463-DTR-PA can widely colonize in ICU environment. Contamination and incomplete disinfection in humid environments may provide evolutionary opportunities for ST463-DTR-PA. Hence, it's worth our vigilance about the growing prevalence of ST463-DTR-PA in East China's ICUs. Meanwhile, it is important to strengthen the management of the ICU environment, especially water reservoirs, and even to promote the management of patients without water.

Keywords: difficult-to-treat resistant *Pseudomonas aeruginosa*, ST463, virulence, resistance, ICU, transmission

Introduction

Pseudomonas aeruginosa (*P. aeruginosa*) is an opportunistic pathogen that can colonize a wide range of environments, including the human respiratory, digestive, and urinary tracts as well as hospital water systems. It is a major cause of hospital-acquired infections such as hospital-acquired pneumonia (HAP), ventilator-associated pneumonia (VAP), urinary tract infections, and bloodstream infections. These infections pose a significant threat to immunocompromised patients in intensive care units (ICUs), particularly those who require mechanical ventilation or invasive procedures.[1–4] Carbapenems are frequently used as the last line of defence against infections caused by multidrug-resistant *P. aeruginosa* (MDRPA) in ICU patients. However, the global incidence and mortality associated with carbapenem-

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resistant *P. aeruginosa* (CRPA) continue to increase.[5] A Consequently, ICUs are becoming focal points for the colonization and infection of CRPA, significantly increasing its clinical burden.

CRPA exhibits complex resistance mechanisms, including downregulation or loss of the OprD porin, hyperproduction of AmpC β-lactamase, overexpression of efflux systems such as MexAB-OprM and MexEF-OprN, and production of carbapenem-hydrolyzing enzymes, particularly metallo-β-lactamases (MBLs), such as IMP, VIM, and GIM enzymes.[5–8] Previously, there have been multiple outbreaks of CRPA in ICUs reported outside of China.[9] Moreover, the formation of dense and persistent biofilms reduces the efficacy of antibiotics and disinfectants, facilitating long-term colonization of hospital wastewater and medical equipment, thereby contributing to hospital-acquired infections (HAIs).[10]

Globally, high-risk CRPA clones, particularly sequence types (ST) 111, 235, 175, 155, and 298, are frequently observed in regions such as America and Europe.[11,12] In contrast, the ST463 clone has emerged as the predominant strain in China, characterized by high antibiotic resistance and pathogenicity.[13] ST463 CRPA, which often carries bla_{KPC}, displays a difficult-to-treat resistance (DTR) profile and is not susceptible to drugs such as piperacillin/tazobactam, ceftazidime, cefepime, aztreonam, meropenem, imipenem, ciprofloxacin, and levofloxacin.[14] Recent epidemiological studies have indicated that approximately 40% of the CRPA isolates in East China are related to the KPC-producing ST463 clone.[15] Alarmingly, infections caused by KPC-producing ST463 PA are associated with prolonged hospital stays and increased mortality.[16]

However, the prevalence and characteristics of ST463 DTR-PA in ICU environments remain poorly understood. To investigate the distribution, genetic relationship, and characteristics of CRPA, especially ST463 strains isolated from environment, infected and colonized patients in ICU, we collected the CRPA strains from four different ICUs of a large tertiary teaching hospital in East China from September 2020 to July 2021 for further study.

We have previously reported the clinical characteristics and genomic features of KPC-producing ST463 P. aeruginosa isolates collected from ICU patients in the same hospital setting.[17] In contrast, the present study emphasizes the environmental persistence and potential transmission routes of these high-risk clones within the ICU environment. This work provides novel insights into the ecology of ST463 in non-clinical reservoirs, which were not addressed in our previous study.

Materials and Methods

Study Design and Sampling

The study was conducted over 11 months, from September 2020 to July 2021, in four intensive care units (ICUs) located in three different buildings of a large tertiary teaching hospital in China, with a total of 91 ICU beds. Each ICU was equipped with wash basins in the patient rooms and common areas, and patient wastewater was handled in dedicated sewage rooms. Disinfection was performed three times daily using 500 mg/L chlorinated disinfectant.

Weekly screening for intestinal carriage of Carbapenem-resistant Organism (CRO), including CRPA, was performed in all ICU patients. Clinical specimens, such as tracheal aspirations, sputum, and urine, were collected routinely, whereas additional samples (eg, blood, wound pus, and drainage fluid) were obtained when infection was suspected. Patients with both infection symptoms and positive CRPA cultures were classified as having CRPA-related infection.

Monthly environmental sampling in each ICU included ventilator condensate, stethoscopes, beddings, wash basin surfaces, wastewater dumping pools, medical workers' overalls, and air. Samples were cultured on cetrimide agar plates containing 2 μg/mL imipenem at 37°C and assessed for growth after 24 h. Condensate samples were filtered through 0.45 μm membranes to capture bacteria, and isolates were identified using the *VITEK* 2 system (BioMerieux, France).

Whole Genome Sequencing and Analysis

We used the Illumina HiSeq 2500 instrument (Qiagen, Valencia, CA, USA) to sequence all CRPA isolates and the RAST server to provide annotations. Multi-locus sequence typing (MLST) was conducted using MLST 2.0, through the online services of the Center for Genomic Epidemiology (http://www.genomicepidemiology.org). Antibiotic resistance and virulence factor genes were separately identified using CGE servers (https://cge.cbs.dtu.dk) and the Virulence Factor Database (http://www.mgc.ac.cn/VFs/).

Phylogenetic Analysis by Core-Genome Multi-Locus Sequence Typing (cgMLST) and Single-Nucleotide Polymorphisms

The phylogenetic tree was visualized using the Interactive Tree of Life (*iTOL)* v6.3 (http://itol.embl.de/). Single nucleotide polymorphism (SNP) analysis of ST463 was performed using *Snippy* to obtain additional genomic information.

Antimicrobial Susceptibility Testing

The broth dilution method was used to determine the sensitivity of the strains to 12 antipseudomonal agents (imipenem, meropenem, piperacillin-tazobactam, ceftazidime-avibactam, ceftazidime, cefepime, amikacin, gentamicin, ciprofloxacin, levofloxacin, aztreonam, and polymyxin), and the results were analyzed according to the guidelines formulated by the Clinical and Laboratory Standards Institute (CLSI, 2024). *P. aeruginosa* ATCC 27853 was used as a control.

Virulence Studies

We randomly selected the participants to conduct the experiment. *G. mellonella* larvae (10 in each group) were infected with bacteria resuspended in Phosphate Buffered saline (PBS) to reach 10^2 CFU, and 10μL of bacteria liquid was injected into the second right proleg. The infected larvae were incubated in 90-mm plastic culture dishes at 37°C and observed every 12 h for 36 h. PA14 and *P. aeruginosa* ATCC9027 were used as hypervirulent and hypovirulent reference strains, respectively.

Biofilm-Forming Ability Test

The bacterial solution was diluted after overnight culture in Luria-Bertani (LB) broth to an OD_{600} of 1.0 and then 200μL of each 100-fold diluted sample was transferred to a 96-well plate. After incubation at 37 °C for 24 h, the floating bacteria were removed and the wells were washed with PBS. The biofilm was then dyed with crystal violet for 30 min, washed again with PBS, and subsequently dissolved in 200μL of 95% ethanol. Absorbance was measured at 540 nm using a microplate reader. The cut-off optical density (ODc) was calculated based on the absorbance of the negative control wells (containing only LB broth) and was defined as the mean OD of these wells plus three times their standard deviation (SD). Strains were then classified based on their own absorbance (OD) as follows: non-adherent ($OD \leq ODc$), weakly adherent ($ODc < OD \leq 2\times ODc$), moderately adherent ($2\times ODc < OD \leq 4\times ODc$), or strongly adherent ($OD > 4\times ODc$), according to the method described by Stepanović et al.[18] All experiments were performed in triplicate.

Statistical Analysis

The chi-square test or Fisher's exact test was used to evaluate the statistical significance of the proportional differences. SPSS software (version 21.0; SPSS, Inc.) was used for data analysis. Survival curves were calculated using GraphPad Prism 9.0 (GraphPad Software, USA). Statistical significance was set at $p < 0.05$.

Results

Isolation of CRPA from Patients and Environments in ICU

During the 11 months monitoring period, 54 CRPA isolates were collected and equally divided into patient samples (n=27) and environmental sources (n=27) in the ICU (Table 1). No CRPA was screened from the stool specimens. Two CRPA strains were isolated from the sputum samples of two patients, but these were classified as colonization due to the absence of clinical symptoms, normal inflammatory markers, and unremarkable lung imaging. The remaining 25 CRPA strains were isolated from various clinical samples including sputum (13/25, 52%), blood (4/25, 16.00%), urine (3/25, 12.00%), bile (2/25, 8.00%), wound secretions (1/25, 4%), ascites (1/25, 4%), and pleural fluid (1/25, 4%).

In the ICU environment, CRPA was predominantly isolated from ventilator condensates (12/27, 44.44%), followed by wastewater dumping pools (8/27, 29.62%), wash basins (4/27, 14.81%), medical workers' clothing (1/27, 3.70%), patient bedding (1/27, 3.70%), and air (1/27, 3.70%).

Table 1 Distribution of 54 CRPA Strains from Patient and Environment in ICUs

Sample Source	Patient-Derived CRPA							Environmental CRPA					
	Sputum	Blood	Urine	Bile	Incision Secretion	Pleural Fluid	Ascites	Ventilator Condensate	Wastewater Dumping Pools	Washbasins	The Clothes of Medical Workers	Patient Beddings	Air
Number	15	4	3	2	1	1	1	12	8	4	1	1	1

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Antimicrobial Susceptibilities of CRPA Isolates

Overall, 42.59% (23/54) of the CRPA strains were resistant to ceftazidime/avibactam, with 69.57% (16/23) of these displaying high-level resistance (MICs ≥ 64 μg/mL) (Supplemental Table A). When comparing by sequence type, all 13 ST463 CRPA strains were categorized as DTR-PA, a significantly higher proportion than the 34.15% (14/41) observed among non-ST463 isolates ($p < 0.0001$) (Figure 1A). In contrast, no significant differences in resistance rates were observed between environmental- and patient-derived strains for most antimicrobials, except for aztreonam, for which patient-derived strains showed higher resistance ($p < 0.05$) (Figure 1B). The detailed susceptibility profile for each individual strain is presented in Figure 2, which shows that most isolates remained highly susceptible to polymyxin B, with the exception of one high-level resistant strain from ventilator condensate (MIC ≥ 64 μg/mL).

MLST, Antibiotic Resistance Genes and Virulence Factors of CRPA Isolates

The MLST, antibiotic resistance genes, and virulence factors of all the CRPA strains are summarized in Figure 2. MLST analysis of the 54 CRPA isolates identified 25 STs, with ST463 being the most common (13/54, 24.07%), followed by ST231 (5/54, 9.26%), ST244 (4/54, 7.41%), ST235 (2/54, 3.70%), ST275 (2/54, 3.70%), and other dispersed STs (51.85%). Nine ST463 isolates were environmental, predominantly from wastewater dumping pools in ICU sewage rooms (8/9), with one strain isolated from a medical worker's clothing. Four ST463 clinical isolates were recovered from sputum and urine samples of infected patients.

All 13 ST463 CRPA strains carried multiple antimicrobial resistance genes, including carbapenem resistance gene (bla_{KPC-2}), aminoglycoside resistance gene (*aph(3')-IIb*), cephalosporin resistance gene ($bla_{OXA-486}$), and fluoroquinolone resistance gene (*crpP*). Notably, these strains also harbored both *exoU+/exoS+* virulence genes, a combination not found in other STs. Twelve CRPA strains carried MBLs genes: bla_{AFM-1} (7/54, 12.96%), bla_{VIM-2} (4/54, 7.41%) and bla_{IMP-1} (1/54, 1.85%). The bla_{AFM-1} was present in ST463 (3/7), ST275 (2/7), and ST712 (2/7), whereas bla_{VIM-2} was detected in ST 244 (3/4) and ST155 (1/4), and bla_{IMP-1} was detected in ST606. Three ST463 strains co-harbored bla_{AFM-1} and bla_{KPC-2}, isolated from different ICUs' wastewater dumping pools (two strains) and urine sample (one strain), showing extensive antimicrobial resistance.

All CRPA strains carried disinfectant resistance genes (*triA, triC*, and *opmH*) against triclosan and the detergent sodium dodecyl sulfonate (SDS), whereas 11 strains harbored *qacEΔ1*, conferring resistance to quaternary ammonium compounds. None of the isolates carried *sugE* or *qacF*.

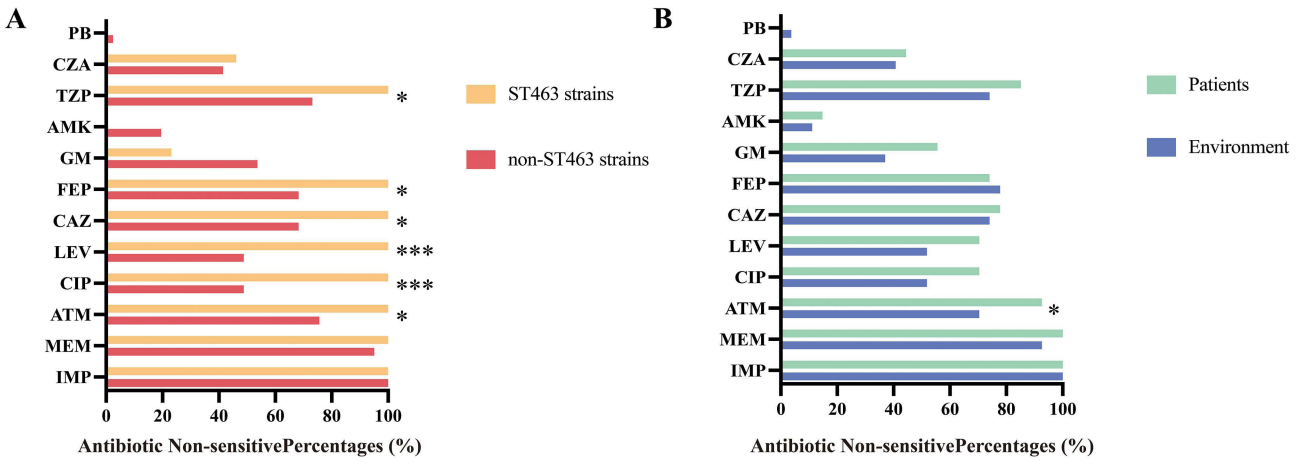

Figure 1 Overview of Antibiotic Resistance. The figure displays the rates of non-susceptibility (including intermediate and resistant) of 54 strains to antibiotics. The x-axis represents the non-susceptibility rate (%), defined as the percentage of isolates categorized as intermediate or resistant. Asterisks indicate statistically significant differences ($p < 0.05$: *, $p < 0.001$: ***). (**A**) compares the resistance rate differences between ST463 strains and non-ST463 strains; (**B**) compares the resistance rate differences between patient-derived strains and environmental strains.

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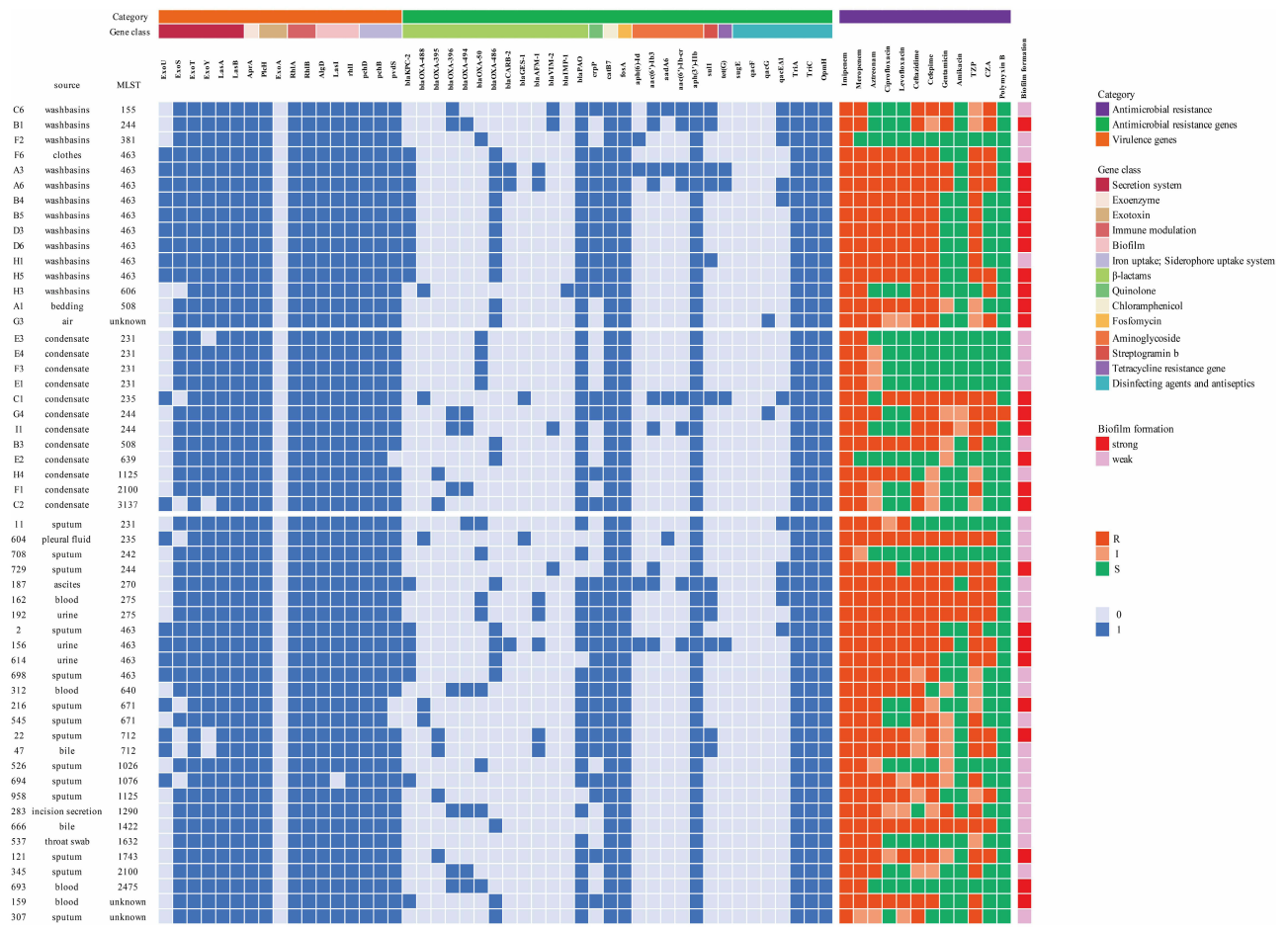

Figure 2 The results of MLST, virulence genes, resistance genes, antimicrobial susceptibilities and biofilm formation abilities among the 54 CRPA isolates. The names of the first line are virulence genes, drug resistance genes and drug sensitivity tests, while the gene categories of the second line represent the functional classification of virulence genes and drug resistance genes respectively. From left to right, the name, sample source, and ST typing of the strain are indicated respectively.

Distribution of CRPA Strains in Different ICUs

Figure 3 illustrates the temporal distributions of the 54 CRPA strains across four distinct ICUs (ICU1, ICU2, ICU3, and ICU4) located in three separate buildings. These four ICUs are located in three separate buildings within the hospital, with their spatial arrangement detailed in Figure 3A. During various periods, ST463 strains were consistently detected in the wastewater pools of sewage rooms in each ICU. In addition, one ST463 strain (PA-F6) was isolated from the clothing of a healthcare worker in ICU1. In addition to the dominant strain ST463-CRPA, other CRPA strains of different STs were also found to colonize different wash basins near patient beds in ICU4.

Patient P6 had an ICU stay of 11 months (October 2020 to September 2021). CRPA strains were first identified in sputum following respiratory infection symptoms and later detected in the ventilator condensate, with strain migration observed corresponding to bed location changes (Figure 3B). Four CRPA strains isolated from patient P6 were classified as ST231. Patient P4 experienced long-term CRPA colonization in the ventilator condensate over a 5-month period; however, no CRPA was detected in respiratory samples, where only carbapenem-sensitive P. aeruginosa was isolated. Additionally, for patients P8, P9, and P10, CRPA strains were isolated from both their sputum and ventilator condensate. For each patient, the isolates from both sources shared an identical sequence type—ST1125, ST244, and ST2100, respectively.

Patient P17, who was admitted to the ICU for duodenal perforation with abdominal infection, had CRPA strains isolated from the blood (JYPA162), urine (JYPA192J), and bile (YPA187) samples. The blood and urine isolates (JYPA162 and JYPA192J) were identified as ST275, whereas the bile isolate (YPA187) identified as ST270 (Supplemental Table B).

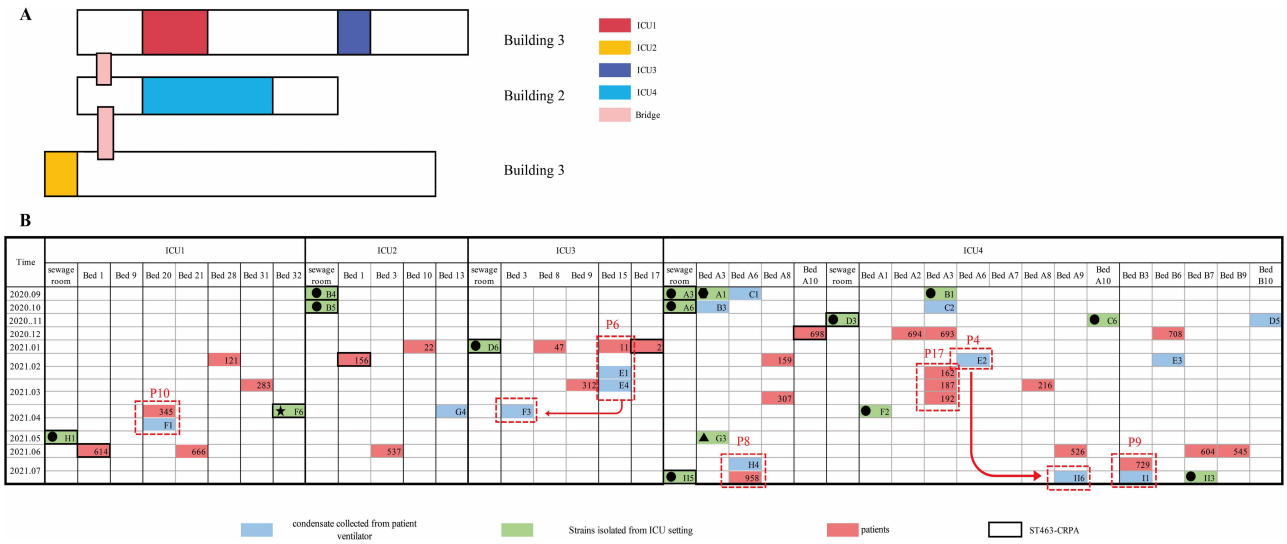

Figure 3 (**A**) Spatial location characteristics of the 4 ICUs in 3 different buildings. (**B**) Schematic diagram of CRPA strains collected from environments and patients in the ICU during the 11-month surveillance. The beds in a black box represent that they are arranged in the same room. Blue, green and red shade are used to represent CRPA strains isolated from ventilator condensate, ward environment and patients respectively. Different symbols are used to indicate the specific environmental source of the isolates: a star (clothes of a healthcare worker), a circle (wastewater dumping pool/wash basins), a triangle (air), and a hexagon (patient bedding). Strains belonging to the same patient are marked in the red dashed boxes and red arrows represent the transfer routes of patients. ST463-CRPA-positive sites are marked with black bolded boxes.

Genetic Correlation Analysis of ST463 CRPA Strains

The genetic relatedness of the ST463 CRPA strains was assessed by phylogenetic analysis (Figure 4A) and pairwise SNP comparisons (Figure 4B). The SNP differences in ST463 CRPA strains isolated from the same wastewater dumping pool at 1-month interval were not significant, for example, PA-A3 and PA-A6 (ICU4, 41 SNPs), PA-B4, and PA-B5 (ICU2, 22 SNPs). PA-D3 isolated from different floors in ICU4 had an SNP difference of 31–33 SNPs compared to the PA-A3 and PA-A6 in the same ICU. Two ST463 strains (PA-H1 and PA-D3), isolated from wastewater dumping pools in different buildings six months apart, showed a close genetic relationship, differing by only eight SNPs.

In addition, the SNP differences between JYPA156 isolated from the urine sample of an infected patient and the environmental strains PA-H1 and PA-D3 ranged from 22 to 30 SNPs. The isolation time of the patient strains was between that of the two environmental strains, and all three strains were isolated from different ICUs at 6-month intervals. The SNP differences among the three KPC-ST463-CRPA strains (JYPA156, PA-A3, and PA-A6) carrying $bla_{\text{AFM-1}}$ ranged from 41 to 59. These patient strains were isolated in ICU2, with a 5-month difference in isolation time compared to the other two environmental strains.

Virulence Assessment

The mortality rates of larvae infected with the two CRPA strains (JYPA121/ST1743 and JYPA526/ST1026) isolated from colonized patients were not significantly different from those of larvae infected with the low-virulence control strain ATCC9027 ($p > 0.05$). Additionally, when comparing CRPA strains from environmental samples with those from infected patients, both groups exhibited mortality rates similar to those of the high-virulence control strain PA14, with no significant differences ($p > 0.05$) (Figure 5A). Larval mortality caused by environmental CRPA strains was comparable to that caused by the strains isolated from infected patients (Figure 5B). However, larvae infected with ST463 strains showed significantly higher mortality rates than those infected with non-ST463 strains ($p < 0.05$) (Figure 5C).

Biofilm Forming Ability

The proportion of environmental CRPA strains exhibited strong biofilm-forming ability was significantly higher than that of patient-related CRPA strains (62.96% [17/27] vs 33.33% [9/27]; $p < 0.05$). Additionally, ST463 isolates exhibited a significantly higher biofilm-forming capacity than non-ST463 isolates (76.92% [10/13] vs 39.02% [16/41]; $p < 0.05$).

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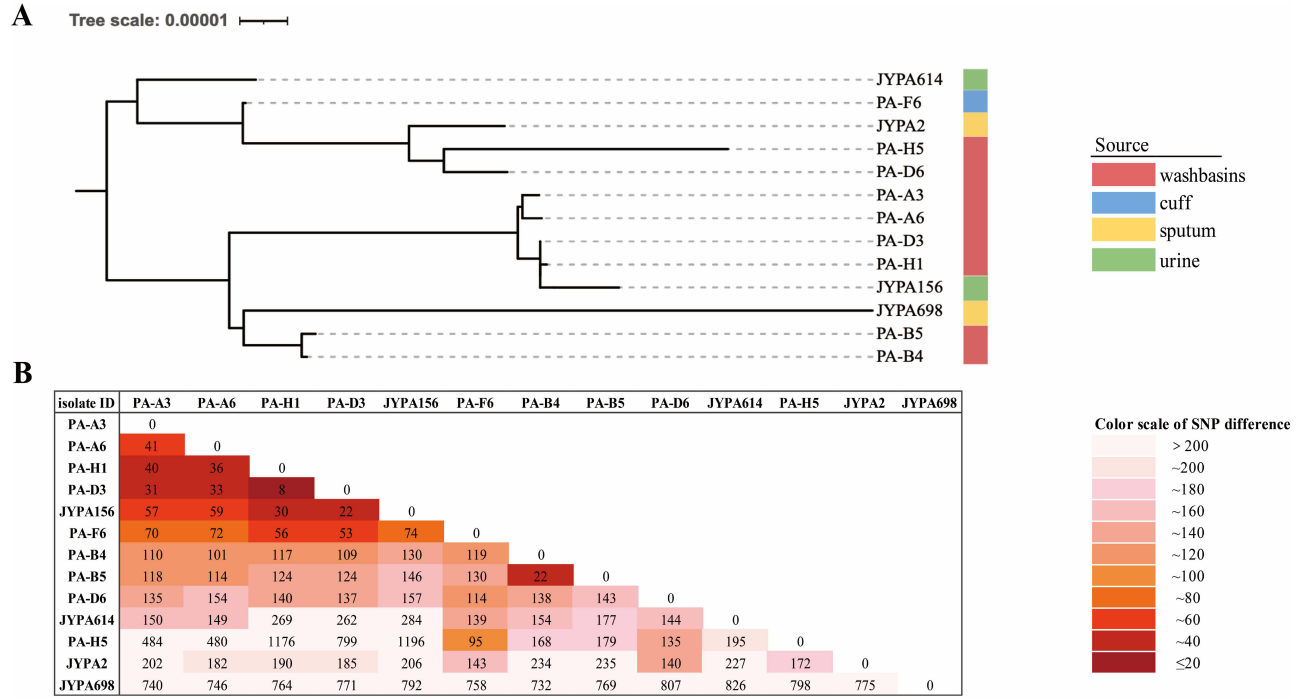

isolate ID	PA-A3	PA-A6	PA-H1	PA-D3	JYPA156	PA-F6	PA-B4	PA-B5	PA-D6	JYPA614	PA-H5	JYPA2	JYPA698
PA-A3	0												
PA-A6	41	0											
PA-H1	40	36	0										
PA-D3	31	33	8	0									
JYPA156	57	59	30	22	0								
PA-F6	70	72	56	53	74	0							
PA-B4	110	101	117	109	130	119	0						
PA-B5	118	114	124	124	146	130	22	0					
PA-D6	135	154	140	137	157	114	138	143	0				
JYPA614	150	149	269	262	284	139	154	177	144	0			
PA-H5	484	480	1176	799	1196	95	168	179	135	195	0		
JYPA2	202	182	190	185	206	143	234	235	140	227	172	0	
JYPA698	740	746	764	771	792	758	732	769	807	826	798	775	0

Figure 4 Genome phylogenetic tree of ST463 isolates generated by panaroo and iTOL. (**A**) The colored stripes represent the sample source. (**B**) SNP matrix diagram of ST463 strains.

Discussion

The emergence of DTR-PA has also become a global concern because it poses a significant constraint on clinical therapeutic strategies.[19] In China, the predominant DTR-PA clone is ST463, which commonly produces KPC and exhibits high virulence and mortality rates.[20,21] A recent epidemiological study confirmed that ST463 is the most prevalent CRPA clone in China (20%), with 91% of ST463-CRPA strains carrying the bla_{KPC-2} gene, while the detection rate of MBLs in CRPA was only 7.02%.[22] In our study, all ST463 strains isolated from ICU environments also harbored bla_{KPC-2} and were classified as DTR-PA, consistent with the clinical ST463 strains detected in the past.[21,23,24] However, we observed a significantly higher MBL detection rate (22.22%) in ICU-derived CRPA strains, which was significantly higher than the proportion of MBLs in CRPA in China.[22,25–27] Notably, our study identified ST463 strains co-harboring bla_{AFM-1} and bla_{KPC-2} not only in patient samples but also in ICU environments. These strains exhibited high-level resistance to all β-lactam antibiotics, including novel β-lactamase inhibitor combinations, indicating the emergence of a new "superbug" in ICU. We hypothesized that the widespread use of antibiotics, particularly carbapenems, in ICU settings and the strong colonization ability of CRPA may contribute to the acquisition of resistance genes and recombination events, facilitating adaptation to antibiotic pressure.

In our study, all ST463 strains isolated from patients and environments in ICUs co-carried *exoU* and *exoS* virulence genes encoding the type III secretion system in *P. aeruginosa* to deliver virulence factors into host cells.[23,28] While previous research has extensively reported on the virulence of ST463 strains,[16,21] our findings emphasize that environmental isolates share a similar high-virulence phenotype. *G. mellonella* virulence assays demonstrated that environmental ST463 strains exhibited virulence levels comparable to those of clinical strains, underscoring the persistence of these virulence factors outside the human host. Additionally, ICU environmental CRPA isolates, predominantly of the ST463 lineage, displayed enhanced biofilm-forming ability compared to the clinical strains. Biofilm formation facilitates long-term colonization by promoting bacterial adhesion, protecting cells from adverse conditions, and providing nutrients in nutrient-limited environments, thereby contributing to the survival and persistence of these bacteria on surfaces.[7]

This study identified ventilator condensate as a key colonization site for CRPA, likely originating from the respiratory tract, owing to the direct connection between ventilator tubes and patient airways. Some patients with CRPA-positive

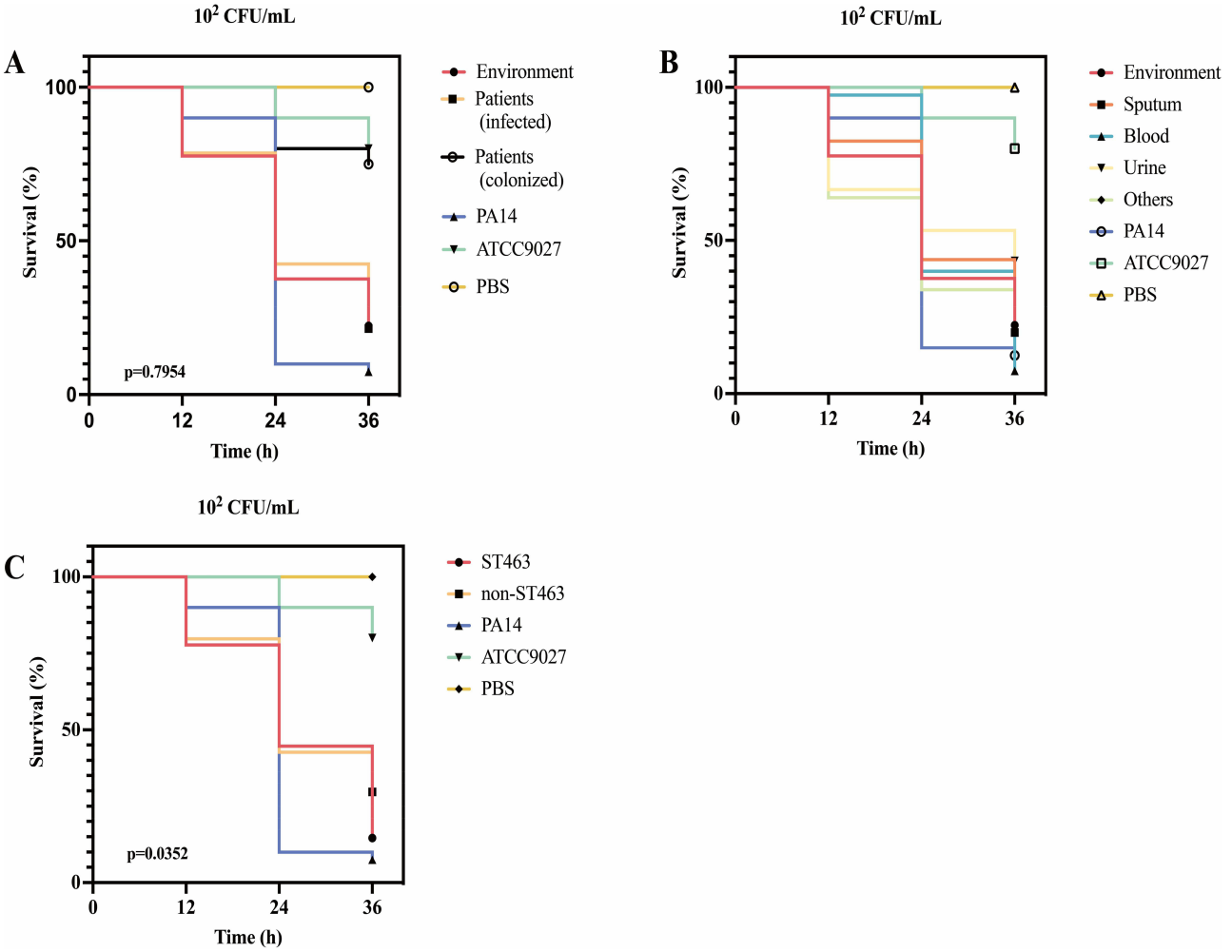

Figure 5 Virulence potential in a *G. mellonella* infection model. Survival curves compare virulence based on (**A**) general source categories (environment, infected, colonized), (**B**) specific isolation sources (eg, sputum, blood), and (**C**) genotype (ST463 vs non-ST463). PA14, ATCC9027, and PBS served as hypervirulent, hypovirulent, and negative controls, respectively.

ventilator condensate had carbapenem-sensitive *P. aeruginosa* detected in their sputum over extended periods, potentially reflecting the clonal diversity of *P. aeruginosa*. Contaminated condensates may contribute to the development of ventilator-associated or hospital-acquired pneumonia (VAP/HAP). Therefore, increased monitoring and more frequent handling of ventilator condensates are recommended for patients with respiratory *P. aeruginosa* colonization, to mitigate the spread of CRPA. Additionally, CRPA strains were isolated from ICU air in our study, suggesting a potential risk of aerosol dissemination and subsequent environmental contamination in ICU settings. This poses a threat to other patients by contaminating the wounds, skin, or catheters. Current ICU protocols that emphasize contact isolation may be insufficient in addressing aerosol dissemination. These findings underscore the need for single-room isolation of patients with antibiotic-resistant infections to mitigate broader ICU contamination and associated infection risks.

According to SNP analysis, representative clinically relevant P. aeruginosa was defined as bacteria with an SNP difference of less than 37.[29,30] In our study, the striking genomic similarity (differing by only eight SNPs) between two ST463-CRPA strains (PA-H1 and PA-D3) isolated from wastewater dumping pools in different buildings six months apart provides compelling evidence for the long-term persistence and inter-unit dissemination of a single high-risk clone. The powerful biofilm-forming ability of these strains may serve as a protective mechanism against disinfectant treatments. This finding underscores that routine disinfection may be insufficient to eradicate such entrenched biofilm-forming clones and highlights that transient vectors, such as the movement of healthcare workers, medical equipment,

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and patients are likely contributing to the spread of this strain across different regions. Furthermore, the genomic plasticity observed among these closely related isolates, even within a short time frame, suggests a capacity for rapid adaptation to environmental changes. Contamination in humid environments, such as wastewater dumping pools and inadequate disinfection, may provide opportunities for clonal spread and evolution of ST463 strains in the ICU.

Therefore, we emphasize the rigorous and effective disinfection of ICU environments to reduce the colonization of the emerging high-risk clone ST463 PA. It is crucial to standardize the use of water reservoirs in different areas of the ICU, such as prohibiting operations other than handwashing in wash basins near a patient's bed. Implementing waterless care for patients as much as possible, including removing wash basins from patient rooms, has proven effective in preventing outbreaks of VIM-producing CRPA in an ICU in another hospital setting.[9] In practice, this strategy involves using pre-packaged, disposable bathing products for patient hygiene and strictly prohibiting the use of tap water for direct patient care, thereby eliminating a major environmental reservoir for P. aeruginosa. Additionally, wastewater dumping pools, which are primarily used for handling patient secretions, have emerged as significant reservoirs for ST463 colonization. Introducing disinfection systems for fecal and urinary waste disposal could help mitigate environmental contamination and further reduce the risk of ICU colonization.

However, our study has certain limitations. First, it was a single-center study. Additionally, we did not perform phenotypic disinfectant susceptibility testing for the CRPA strains isolated from the ICU in our study. The presence of disinfectant resistance genes (eg, *qacEΔ1*) in our isolates suggests selection under environmental disinfectant pressure, and correlating these genotypes with phenotypic susceptibility to the specific biocides used in the hospital remains an important area for future investigation. Finally, the sampling frequency should be increased to adequately monitor the distribution and characteristics of the CRPA strains. In subsequent studies, we will systematically monitor and evaluate the distribution of CRPA strains in the ICU environments following the implementation of the aforementioned intervention measures.

Conclusion

In conclusion, this study identified the high-risk clone ST463 CRPA as frequently present in both ICU patients and environmental samples, with evidence supporting clonal transmission within the ICU. Our results also showed that ST463 strains in ICU environments had strong biofilm-forming ability and seemed to be more suitable for living in a hospital damp environment for a long time. This indicates that contamination and incomplete disinfection of environmental water reservoirs may lead to clonal dissemination and evolution of ST463 strains in the ICU. In addition to traditional infection control measures, targeted measures should be strictly implemented, such as standardizing the use of wash basins and wastewater dumping pools in different areas, thorough disinfection, and even implementing waterless care for patients to prevent and control potential outbreaks of ST463 strain infections in the ICU.

Accession Numbers

The whole-genome sequences of *Pseudomonas aeruginosa* isolates were submitted to NCBI GenBank under the BioProject ID PRJNA1206678. The BioSample accession numbers ranged from SAMN46080943 to SAMN46080991, and the Genome accession numbers ranged from JBKILN000000000 to JBKINI000000000. Detailed information is provided in Supplemental Table C.

Ethics Approval and Consent to Participate

Ethical approval was obtained from the institutional review board of the First Affiliated Hospital of Zhejiang University School in China (approval no. IIT20210120B). The need for individual informed consent was waived by the ethics committee because this study involved a retrospective analysis of clinical data and bacterial isolates, which posed no more than minimal risk to the subjects and did not adversely affect their rights and welfare. All patient data were fully anonymized before analysis to protect confidentiality. This study was conducted in compliance with the principles of the Declaration of Helsinki.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

This paper has been uploaded to Research Square as a preprint: https://www.researchsquare.com/article/rs-3239716/v1. The authors declare that they have no competing interests.

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