Emerging and re-emerging KPC-producing hypervirulent *Pseudomonas aeruginosa* ST697 and ST463 between 2010 and 2021

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ABSTRACT

Carbapenem-resistant *Pseudomonas aeruginosa* (CRPA) has been a major threat to human health due to its increased morbidity and mortality in clinical settings. Carbapenemase genes are less frequently found in CRPA compared with carbapenem-resistant *Enterobacteriaceae*, of which carbapenemase producers are common. In this study, we identified 11 *blaKPC-2*-harbouring *P. aeruginosa* isolates from 139 carbapenemase-insensitive *P. aeruginosa* isolates collected between 2010 and 2021 in a tertiary hospital in China. Nine isolates belonged to ST697, while the other two were ST463. The antibiotic susceptibility testing showed that all the isolates were multidrug resistant, including resistance to imipenem, meropenem, ceftazidime, and tigecycline. Patients with *Klebsiella pneumoniae* carbapenemase-2 (KPC-2)-producing *P. aeruginosa* infections were mostly associated with complicated diseases and prolonged hospital stay, with 30% deterioration. The whole-genome sequencing analysis showed that these isolates carried multiple antibiotic resistance genes and virulence genes, and the KPC-2 genetic elements were highly related in ST697 isolates. The complete sequencing of ST697 isolate SES416 showed that the harbouring of *blaKPC-2* resulted from complex transposition and homologous recombination of an IncP-1 plasmid and other mobile elements. The *Galleria mellonella* infection model experiment showed that these KPC-2-producing *P. aeruginosa*-infected larvae had low survival rates and high virulence. The present study revealed the shifting of CRPA from ST697 to ST463 in East China; ST463 had higher drug resistance, posing greater challenges for clinical management.

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Introduction

*Pseudomonas aeruginosa* is one of the most common Gram-negative bacteria in China [1]. *P. aeruginosa* can easily colonize in the human body and invade the respiratory tract, urinary tract, surgical site, blood, abdominal cavity, and skin/soft tissues, causing hospital-acquired pneumonia [2,3]. *P. aeruginosa* has the characteristics of high mutation rates with complex antibiotic resistance mechanisms [4]. As an opportunistic pathogen, it is one of the main causes of morbidity and death in patients with cystic fibrosis and individuals with weakened immune functions [5]. In the last two decades, the spread of multidrug-resistant (MDR) *P. aeruginosa*, including those resistant to third-generation cephalosporins and carbapenem antibiotics, has greatly increased worldwide [6,7].

Carbapenems are important antibiotics for treating MDR *P. aeruginosa* infections [8]. Unlike carbapenem-resistant *Enterobacteriaceae* in which the carbapenem resistance is mainly caused by the production of different carbapenemases (Ambler class A, B, and D), carbapenem resistance in *P. aeruginosa* is more commonly associated with intrinsic resistance mechanisms, including overexpression of the efflux pump system, overexpression of chromosomal cephalosporinase, and reduction or loss of OprD outer membrane protein expression [9]. In addition, among clinical carbapenem-producing *P. aeruginosa* (CPPA), Ambler class B metal-β-lactamases (MBLs, e.g. VIM and IMP) were more commonly reported, whereas class A carbapenemases [e.g. *Klebsiella pneumoniae* carbapenemase (KPC)] were less frequently described, especially in China [10]. In 2007, scientists from Columbia first...
discovered a *P. aeruginosa* isolate harbouring the *bla*KPC-2 gene [11]; since then, additional reports on such isolates in other foreign countries have been published [12,13]. Subsequent studies found that the *bla*KPC gene was mobilized on the 10-kb Tn3-family active transposon Tn4401, which was delimited by two 39-bp inverted-repeat sequences [14]. Currently, ST235, ST111, and ST175 are high-risk *P. aeruginosa* clones in clinical isolates worldwide, often associated with multidrug resistance or extensively drug resistance [6].

The present 12-year retrospective study was conducted in a tertiary hospital, and 11 KPC-2-producing *P. aeruginosa* isolates were identified. Most of these isolates belonged to an uncommon ST697 clone, whereas the remaining recently isolated clones belonged to the emerging high-risk clone ST463. *In vitro* phenotypic experiments and *in vivo* Galleria mellonella infection model results suggested that these KPC-2-producing *P. aeruginosa* isolates were highly resistant and virulent, and required more attention and further research for their prevention and control.

Materials and methods

Collection of isolates

A total of 139 *P. aeruginosa* isolates that were not sensitive to carbapenem antibiotics (minimum inhibitory concentration of meropenem ≥4 μg/mL and/or imipenem ≥4 μg/mL) were collected from the Second Affiliated Hospital of Soochow University from 2010 to 2021. All isolates were speciated using the MALDI-TOF MS apparatus (Bruker Microflex LT, Bruker Daltonik GmbH, Bremen, Germany). The isolates were cultured using lysogeny broth (LB) solid medium (pH = 7.0) with subinhibitory meropenem/imipenem concentration (0.5 μg/mL) at 37°C.

Molecular detection

*Bla*KPC was screened using polymerase chain reaction, followed by Sanger sequencing [15]. The DNA template was prepared by the boiling method. In brief, a loopful of bacteria were added to 100 μL of ddH2O and boiled for 10 min, followed by centrifugation at 5000 rpm and 4°C for 5 min. The supernatant was then diluted at a ratio of 1:10 in fresh ddH2O and used as a DNA template.

Antibiotic sensitivity testing

The antimicrobial susceptibility testing was performed using the standard broth microdilution method, and the results were interpreted following the 2020 CLSI breakpoints [16]. The experiment was performed in three biological replicates on two different dates. *P. aeruginosa* ATCC27583 was used as the quality control.

Genome sequencing analysis

Whole-genome sequencing was performed on all KPC-2-producing *P. aeruginosa* isolates. The maximum-likelihood phylogenetic tree of KPC-producing *P. aeruginosa* isolates based on recombination-free core genome single-nucleotide polymorphisms (SNPs) was conducted with MUMmer 3.0, ClonalFrameML, and MEGA7, as described in a previous study [17]. The sequence of *P. aeruginosa* PAO1 (GenBank accession no. NC_002516) was used as the reference, and the sequence of *Pseudomonas putida* strain 14164 (GenBank accession no. NC_021505) was used as the out-group.

G. mellonella infection model

All isolates were grown in 1 mL of LB liquid medium (pH = 7.0) and cultured with shaking at 200 rpm overnight at 37°C. The next day, the cultures were diluted at a ratio of 1:200 in 10 mL of fresh LB liquid medium (pH = 7.0) and grown for another 1.5–2 h at 37°C to mid-log phase (∼10⁸ CFU/mL, 0.3 OD at 600 nm). Then, all cultures were washed and adjusted to ∼10⁵ CFU/mL with normal saline before infection. The culture was then injected through the last left pro-leg with 10 μL of bacteria (10⁵ CFU) [18]. Preliminary experiments showed that the concentration of bacteria could better reflect the difference in virulence in these isolates. After infection, the number of survivors was recorded at 37°C for three consecutive days. A saline control group was included, and 20 larvae were used in the control and experimental groups. This experiment was divided into three groups: the experimental group, the normal saline group, and the blank control group. This experiment was performed in three biological replicates on two different dates.

Ethical approval

The ethical approval was obtained from the ethics committee of the Second Affiliated Hospital of Soochow University. The isolates used in this study were collected previously from routine microbiological specimens, while all the microbiological specimens were anonymized. The patients were not physically involved in this study. Therefore, no consent was needed for this study.

Results

Clinical and molecular characteristics of the isolates

A total of 139 clinical carbapenemase-insensitive *P. aeruginosa* isolates were collected from the Second Affiliated Hospital of Soochow University between
2010 and 2021. Molecular testing showed that 11 isolates harboured the bla\textsubscript{KPC-2} gene. These isolates were recovered from the respiratory tract (n = 7, 63.6%), skin/soft tissue (n = 2, 18.2%), and urinary tract (n = 2, 18.2%). Among the patients, 70.0% (n = 7) were male and 30.0% (n = 3) were female (two isolates were recovered from the same patient during different periods). The median age was 58 years. Seven (70.0%) patients improved upon their discharge, while the condition of three (30%) of them deteriorated (Table 1).

Information on the medical history of seven patients showed that all patients had a long hospital stay of more than 30 days, with the longest being 110 days; five patients had received surgical treatment. These patients suffered from a variety of diseases, were in poor health conditions, and had been hospitalized in the intensive care unit (Table S1). They all received invasive ventilation and central venous catheterization, adjuvant therapy, and three of them also received closed thoracic drainage (data not shown).

### Antimicrobial susceptibility

The antibiotic susceptibility testing of these 11 KPC-2-\textit{P. aeruginosa} isolates showed that they were nonsensitive to carbapenem antibiotics (meropenem and/or imipenem) and highly resistant to most antibiotics tested (Table 2). Ten of these isolates showed resistance rates of more than 85% to the 23 antibiotics tested, with most of them displaying resistance rates of more than 90%. The results showed that the resistance rates of these isolates to cefazolin, ceftriaxone, nitrofurantoin, tetracycline, minocycline, and sulbactam, ––, fosfomycin, and aminoglycosides —— was related to tetracycline resistance [19]. CmlA1, catB7, fosA, aac (3)-IId, and aph(3’)-IIb encoded resistance to chloramphenicol, fosfomycin, and aminoglycosides [20,21]. Tet(G) was related to tetracycline resistance [22,23]. Sul1 was related to sulfa antibiotic resistance [24]. In addition, genes such as sul2, armA, and mphA were located in the bacterial resistance determinant cluster (AbGRI1 and AbGRI3) [25,26].

In silico screening showed that the isolates harboured more than 200 virulence genes. The total number of each was as follows: SE5463, SE5465, SE5470, SE5460, SE5441, SE5433, SE5416, SE5419, and SE5400 (n = 229); SE9002 (n = 228); and SE9005 (n = 214) (Figure 1). These virulence genes primarily encode function or pathway of type II, III and V1 secretion systems, flagellar and pili biosynthesis, exopolysaccharide, siderophore and lipopolysaccharide formation and secretion etc., contributing to the invasion and destruction of host tissues (Table S2). All these genes were chromosome borne but were not carried by antibiotic resistance plasmids.

The phylogenetic analysis of bla\textsubscript{KPC-2} isolates showed that the ST697 and ST463 isolates formed two separate clusters (Figure 2). The 9 ST697 isolates differed with an average of 26 core SNPs (range 5–45), while 2 ST463 isolates differed with an average of 78 core SNPs, suggesting that the spread of CPPA in the hospital was mainly associated with the clonal expansion of 2 KPC-2-producing \textit{P. aeruginosa} isolates.

### Inc\textsubscript{pRBL16} plasmid pSE5416-KPC

SE5416 was selected for complete genome sequencing using Illumina and PacBio RSII. The bla\textsubscript{KPC-2} was harboured by a 510.71-kb Inc\textsubscript{pRBL16} plasmid, which was assigned the name pSE5416-KPC (the eight isolates of ST697 carry the similar plasmid) (data not shown). A detailed genetic dissection analysis was applied to this plasmid. The modular structure of pSE5416-KPC was divided into the backbone and eight accessory modules (defined as exogenous DNA regions inserted at different sites of the backbone) (Figure 3). The backbone of pSE5416-KPC shared 98.56% nucleotide identity and

### Table 1. Information and molecular characteristics of the isolates.

| Isolates number | ST typing | Patient age | Gender | Isolate source | Separation time | Carbapenemase gene | ESBLs gene | Disease outcome |
|-----------------|-----------|-------------|--------|----------------|-----------------|---------------------|------------|----------------|
| SE5463          | ST697     | 68          | Male   | Respiratory tract | 2010            | \textit{bla}\textsubscript{KPC-2} | \textit{bla}\textsubscript{TEM-1A} | Healed         |
| SE5465          | ST697     | 36          | Female | Respiratory tract | 2010            | \textit{bla}\textsubscript{KPC-2} | \textit{bla}\textsubscript{TEM-1A} | Deteriorated   |
| SE5470          | ST697     | 77          | Male   | Respiratory tract | 2010            | \textit{bla}\textsubscript{KPC-2} | \textit{bla}\textsubscript{TEM-1A} | Improved       |
| SE5460          | ST697     | 26          | Male   | Skin/Soft tissue | 2010            | \textit{bla}\textsubscript{KPC-2} | \textit{bla}\textsubscript{TEM-1A} | Improved       |
| SE5441          | ST697     | 79          | Male   | Respiratory tract | 2012            | \textit{bla}\textsubscript{KPC-2} | \textit{bla}\textsubscript{TEM-1A} | Deteriorated   |
| SE5433          | ST697     | 60          | Male   | Respiratory tract | 2012            | \textit{bla}\textsubscript{KPC-2} | \textit{bla}\textsubscript{TEM-1A} | Improved       |
| SE5416          | ST697     | 30          | Male   | Respiratory tract | 2013            | \textit{bla}\textsubscript{KPC-2} | \textit{bla}\textsubscript{TEM-1A} | Improved       |
| SE5419          | ST697     | 47          | Female | Skin/Soft tissue | 2013            | \textit{bla}\textsubscript{KPC-2} | \textit{bla}\textsubscript{TEM-1A} | Improved       |
| SE5400          | ST697     | 48          | Female | Respiratory tract | 2014            | \textit{bla}\textsubscript{KPC-2} | \textit{bla}\textsubscript{TEM-1A} | Improved       |
| SE9002          | ST463     | 80          | Male   | Urinary tract    | 2020            | \textit{bla}\textsubscript{KPC-2} | –           | Deteriorated   |
| SE9005          | ST463     | 80          | Male   | Urinary tract    | 2021            | \textit{bla}\textsubscript{KPC-2} | –           | Deteriorated   |
Table 2. In vitro susceptibility of different clinical antibiotics against 11 KPC-2-\-P. aeruginosa\ isolates.

| Antimicrobial agent | MIC (μg/ml) | Sensitivity | Resistance ratea | (n/ Total, %) |
|---------------------|-------------|-------------|------------------|---------------|
| **β-lactams**       |             |             |                  |               |
| First generation cephalosporins |             |             |                  |               |
| Cefazolin | >16, R | >16, R | >16, R | >16, R | >16, R | >16, R | >16, R | >16, R | >16, R | >16, R | 11/11, 100 |
| Second generation cephalosporins |             |             |                  |               |
| Cefuroxime | >16, R | >16, R | >16, R | >16, R | >16, R | >16, R | >16, R | >16, R | >16, R | >16, R | 11/11, 100 |
| Third generation cephalosporins |             |             |                  |               |
| Ceftriaxone | >32, R | >32, R | >32, R | >32, R | >32, R | >32, R | >32, R | 16, R | >32, R | >32, R | 11/11, 100 |
| Fourth generation cephalosporins |             |             |                  |               |
| Cefepime | >32, R | >16, R | >16, R | >16, R | >16, R | >16, R | >16, R | >16, R | 8, S | >16, R | 10/11, 90.9 |
| **Carbapenems** |             |             |                  |               |
| Meropenem | >8, R | >8, R | >8, R | >8, R | >8, R | >8, R | >8, R | 2, S | >8, R | >8, R | 10/11, 90.9 |
| Ertapenem | >2, R | >2, R | >2, R | >2, R | >2, R | >2, R | >2, R | >2, R | >2, R | >2, R | 11/11, 100 |
| **Monocyclic β-lactams** |             |             |                  |               |
| Aztreonam | 4, S | >32, R | >32, R | >32, R | >32, R | >32, R | >32, R | >32, R | 8, S | >16, R | 9/11, 81.8 |
| **Aminoglycosides** |             |             |                  |               |
| Gentamicin | >8, R | >8, R | >8, R | >8, R | >8, R | >8, R | >8, R | >8, R | >8, R | >8, R | 11/11, 100 |
| Tobramycin | 8, I | 8, I | 8, I | >8, R | 8, I | 8, I | 4, S | 8, I | 2, S | >8, R | 10/11, 90.9 |
| Amikacin | ≤8, S | ≤8, S | ≤8, S | ≤8, S | ≤8, S | ≤8, S | ≤8, S | ≤8, S | ≤8, S | 32, R | 2/11, 18.2 |
| **Quinolones** |             |             |                  |               |
| Ciprofloxacin | >4, R | >4, R | >4, R | >4, R | >4, R | >4, R | >4, R | <0.5, S | >4, R | >4, R | 10/11, 90.9 |
| Levofloxacin | >8, R | >8, R | >8, R | >8, R | >8, R | >8, R | >8, R | ≤1, S | >8, R | >8, R | 10/11, 90.9 |
| **Nitrofurans** |             |             |                  |               |
| Nitrofurantoin | >64, R | >64, R | >64, R | >64, R | >64, R | >64, R | >64, R | >64, R | >64, R | >64, R | 11/11, 100 |
| **Tetracyclines** |             |             |                  |               |
| Tetracycline | >8, R | >8, R | >8, R | >8, R | >8, R | >8, R | >8, R | >8, R | >8, R | >8, R | 11/11, 100 |
| Minocycline | >16, R | >16, R | >16, R | >16, R | >16, R | >16, R | >16, R | >16, R | >16, R | >16, R | 11/11, 100 |
| Tigecycline | >8, R | >8, R | >8, R | >8, R | >8, R | >8, R | >8, R | >8, R | >8, R | >8, R | 11/11, 100 |
| **Chloramphenicol** |             |             |                  |               |
| Chloramphenicol | 16, R | >16, R | >16, R | >16, R | >16, R | >16, R | >16, R | >16, R | >16, R | >16, R | 11/11, 100 |
| **β-lactam/β-lactamase inhibitors** |             |             |                  |               |
| Ampicillin—sulbactam | >16/8, R | >16/8, R | >16/8, R | >16/8, R | >16/8, R | >16/8, R | >16/8, R | >16/8, R | >16/8, R | >16/8, R | 11/11, 100 |
| Amoxicillin—clavulanic acid | >32/16, R | >32/16, R | >32/16, R | >32/16, R | >32/16, R | >32/16, R | >32/16, R | >32/16, R | >32/16, R | >32/16, R | 11/11, 100 |
| Piperacillin—tazobactam | >64/4, R | >64/4, R | >64/4, R | >64/4, R | >64/4, R | >64/4, R | >64/4, R | >64/4, R | >64/4, R | >64/4, R | 11/11, 100 |
| Cefoperazone-sulbactam | >32/8, R | >32/8, R | >32/8, R | >32/8, R | >32/8, R | >32/8, R | >32/8, R | >32/8, R | >32/8, R | >32/8, R | 11/11, 100 |
| **Resistance rateb** |             |             |                  |               |
| (n/ Total, %) | 20/23, 87.0 | 21/23, 91.3 | 21/23, 91.3 | 22/23, 95.7 | 21/23, 91.3 | 21/23, 91.3 | 21/23, 91.3 | 11/23, 47.8 | 23/23, 100 | 22/23, 95.7 |

aCLSI M100-S30 interpretive criteria were used in all cases.
Table 3. Whole genome sequencing results of antibiotic resistance genes and plasmids.

| Isolates number | ST typing | Other resistance genes | Plasmid types |
|-----------------|-----------|------------------------|---------------|
| SE5463          | ST697     | (may be present on the plasmid) aadA1, qnrS2, mph(A), cmlA1, tet(G), sul1, aph(3')-Ila, blaoxa-399m, aac(3)-IId, blaoxo-268m, crpP, fosA, catB7 | IncPCRBL16, IncU |
| SE5465          | ST697     | (may be present on the plasmid) aadA1, qnrS2, mph(A), cmlA1, tet(G), sul1, aph(3')-Ila, blaoxa-399m, aac(3)-IId, blaoxo-268m, crpP, fosA, catB7 | IncPCRBL16, IncU |
| SE5470          | ST697     | (may be present on the plasmid) aadA1, qnrS2, mph(A), cmlA1, tet(G), sul1, aph(3')-Ila, blaoxa-399m, aac(3)-IId, blaoxo-268m, crpP, fosA, catB7 | IncPCRBL16, IncU |
| SE5460          | ST697     | (may be present on the plasmid) aadA1, qnrS2, mph(A), cmlA1, tet(G), sul1, aph(3')-Ila, blaoxa-399m, aac(3)-IId, blaoxo-268m, crpP, fosA, catB7 | IncPCRBL16, IncU |
| SE5441          | ST697     | (may be present on the plasmid) aadA1, qnrS2, mph(A), cmlA1, tet(G), sul1, aph(3')-Ila, blaoxa-399m, aac(3)-IId, blaoxo-268m, crpP, fosA, catB7 | IncPCRBL16, IncU |
| SE5433          | ST697     | (may be present on the plasmid) aadA1, qnrS2, mph(A), cmlA1, tet(G), sul1, aph(3')-Ila, blaoxa-399m, aac(3)-IId, blaoxo-268m, crpP, fosA, catB7 | IncPCRBL16, IncU |
| SE5416          | ST697     | (may be present on the plasmid) aadA1, qnrS2, mph(A), cmlA1, tet(G), sul1, aph(3')-Ila, blaoxa-399m, aac(3)-IId, blaoxo-268m, crpP, fosA, catB7 | IncPCRBL16, IncU |
| SE5419          | ST697     | (may be present on the plasmid) aadA1, qnrS2, mph(A), cmlA1, tet(G), sul1, aph(3')-Ila, blaoxa-399m, aac(3)-IId, blaoxo-268m, crpP, fosA, catB7 | IncPCRBL16, IncU |
| SE5400          | ST697     | (may be present on the plasmid) aadA1, qnrS2, mph(A), cmlA1, tet(G), sul1, aph(3')-Ila, blaoxa-399m, aac(3)-IId, blaoxo-268m, crpP, fosA, catB7 | IncU |
| SE9002          | ST463     | (may be present on the plasmid) aadA1, qnrS2, mph(A), cmlA1, tet(G), sul1, aph(3')-Ila, blaoxa-399m, aac(3)-IId, blaoxo-268m, crpP, fosA, catB7 | IncU |
| SE9005          | ST463     | aac(6)-Ila, ant(2')-Ia, aph(3')-Ib, aph(3')-VI, sul1, blaOXY, crpP, blaoxo-268m, fosA, catB7 | IncU |

Figure 1. The relationship between the number of virulence genes carried by the isolates. The virulence genes of the remaining 8 isolates were consistent with SE5463, so they were not shown here.

96% coverage with the IncPCRBL16 reference plasmid pRBL16 (GenBank accession number CP015879) [27]. Among the eight accessory modules pSE5416-KPC carried, only the Tn6411-related region harboured antimicrobial resistance genes [28], mediating resistance to aminoglycosides (aadC2), β-lactams (blaKPC-2), fluoroquinolones (qnrS2), and macrolides (mph(A)). The Tn6411-related region could be preliminarily divided into several truncated Tn6411 fragments and a 41.99-kb MDR region, which was embedded in one of the truncated Tn6411 fragments (neatly carrying the aacC2 gene) with 5-bp direct repeats (DRs; target site duplication signals for transposition) (Figure 4). The MDR region was composed of two repeat Tn1403-related regions [carrying the mph(A) gene] [29], and the Tn6296-related blaKPC-2 region (carrying the blaKPC-2 and qnrS2 genes) [30] inserted between two repeat regions with 8-bp DRs. Additionally, the blaKPC-2 region contained a partial plasmid sequence 100% identical to the IncU plasmid pAH227 with 36% coverage (GenBank accession number KT315926) [31]. This was a new blaKPC-2 region surrounded by ΔIS Kpn6 and IS Kpn27. This structure indicated that the harbouring of blaKPC-2 in pSE5416-KPC resulted from the complex transposition and homologous recombination of an IncPCRBL16 plasmid and other mobile elements. Additionally, blaKPC-2 genes from SE9002 and SE9005 were located on chromosome. Further analysis showed that both these two blaKPC-2 genes were also carried by the Tn6296-related blaKPC-2 regions, which were highly similar to the one from pSE5416-KPC (data not shown).
Survival rates of G. mellonella infection

We selected G. mellonella larvae of similar weight (190–220 mg) to minimize the differences in body weight and standardize the age of the larvae to evaluate the virulence level of these isolates [32]. We injected 10^3 CFU of different-numbered clinical isolates into the larvae and monitored the survival of the larvae every 24 h [33].

Isolate SE5352 was an ST235 P. aeruginosa carrying exoU virulence gene, and SE5356 was an ST697 P. aeruginosa without blakPC-2 (carrying 227 virulence genes, data not shown), collected by our research group during the same period. The exoU-harbouring ST235 P. aeruginosa was often accompanied by high virulence [34]. The SE5333 selected in the experiment was an ST508-type P. aeruginosa without major virulence genes. SE5352 and SE5333 were used as the high- and low-virulence controls in the G. mellonella infection model.

The results showed that, compared with the two control groups (blank group and physiological saline group) and the low-virulence group (ST508–SE5333), the larvae infected by eight isolates, including ST697 isolates (SE5416, SE5433, SE5460, SE5465, SE5470, and SE5356), ST463 isolates (SE9002 and SE9005), and hypervirulent ST235 isolates (SE5352) had a survival rate of 0% after 24 h. The survival rate of larvae infected by ST697 SE5441 was 0% after 48 h, and that by ST697 SE5419 was 10% after 72 h. On the contrary, the survival rates of larvae infected by ST697 SE5400 and SE5463 were as high as 100% and 95% after 72 h, respectively. The low-virulence control strain ST508 SE5333 had the same survival rate as ST697 SE5463 (Figure 5). The results suggested a higher virulence in the ST697 and ST463 P. aeruginosa isolates, and the high virulence of these clinically isolated KPC-2–P. aeruginosa had strong harmfulness.

Discussion

Today, P. aeruginosa nosocomial infections have become a global healthcare problem because they are largely associated with hospital-acquired infections, including ventilator-associated pneumonia, central catheter–associated bloodstream infections, catheter-associated infections, and surgical/transplant infections [35,36]. Especially in adults with cystic fibrosis, P. aeruginosa colonization ranged from 31% to 48%, and the development of antimicrobial resistance made it increasingly difficult to treat and eradicate [37]. The overall mortality rate of P. aeruginosa bacteremia was more than 30% [38,39], the resistance rate to one or more antibiotic groups was 38% [40], and the sensitivity to carbapenems decreased [41]. Importantly, carbapenem-resistant bacterial infections caused by Acinetobacter baumannii or P. aeruginosa accounted for 82.3% of all Gram-negative bacteria [42], which should not be underestimated. In addition, P. aeruginosa showed a higher propensity of mutations compared with other Gram-negative bacterial species, making P. aeruginosa more difficult to treat. The World Health Organization has classified...
Carbapenem-resistant *Pseudomonas aeruginosa* (CRPA) as one of the pathogens for which new alternative antibiotics are urgently required [43]. At present, KPC-producing *P. aeruginosa* isolates are alarmingly increasing worldwide, which has aroused the attention and vigilance of clinical researchers [44–46].

In this study, 11 *bla*KPC-2-harbouring *P. aeruginosa* isolates and other resistance genes were identified from the 139 carbapenemase-insensitive *P. aeruginosa* isolates collected in China from 2010 to 2021. These KPC-2-*P. aeruginosa* isolates were collected from different clinical sites of the respiratory tract, skin/soft tissue, and urinary tract, with a large time span (Table 1). Different from the dominant clones ST235 and ST111 found in previous global studies [47], we found the evolutionary trend of KPC-producing hypervirulent *P. aeruginosa* superior clone ST697 (first report) to ST463, which has become a potential high-risk clone in China in recent years [48]. Not to be underestimated, these isolates were all MDR-PA, resulting in refractory patients with a mortality rate of up to 30% (Table 2). KPC β-lactamases, encoded by *bla*KPC-2, hydrolyze β-lactams of all classes and are highly efficient in hydrolyzing carbapenem antibiotics [49]. The shifting of *bla*KPC-2-positive

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**Figure 3.** Schematic diagram of the plasmid pSE5416-KPC. Genes of different functions are denoted by arrows and presented in various colours. The circles show (from outside to inside): predicted coding sequences, scale, backbone (black) and accessory module (gray) regions, GC content and GC skew [(G−C)/(G + C)].
carbapenemase-insensitive *P. aeruginosa* isolates in this study significantly increased the level of resistance to carbapenems, other β-lactams and quinolones, especially the recently isolated ST463 strains that showed higher antibiotic resistance rates, resulting in greater challenges to treatment; hence, it should be closely monitored in hospitals in the future (Table 2, Table S3).

Besides intrinsic resistance and chromosomal mutations, mobile genetic elements such as plasmids and integrating conjugative elements are responsible for transmitting resistance genes to *P. aeruginosa* strains [50]. Further whole-genome sequencing and analysis found that all these 11 isolates harboured multiple antibiotic resistance genes, leading to the ineffective treatment using antibiotics such as quinolone, aminoglycoside, tetracycline, and sulfa (Table 3), as demonstrated by the clinical antibiotic susceptibility testing results. The structure analysis indicated that the harbouring of \(\text{bla}_{\text{KPC}-2}\) in pSE5416-KPC resulted from the complex transposition and homologous recombination of an Incp\textsubscript{RBL16} plasmid and other mobile elements (Figures 3 and 4). The antibiotic resistance gene \(\text{bla}_{\text{KPC}}\) is most commonly harbourd by plasmids. The NCBI GenBank database has listed 17 complete *P. aeruginosa* plasmids encoding KPC, of which three have been reported in China [51]. Incp\textsubscript{RBL16} is a 370.3-kb plasmid first reported in *Pseudomonas citellella* SJTE-3, which was isolated from the activated sludge from a sewage treatment plant in China, but did not carry the resistance gene \(\text{bla}_{\text{KPC}-2}\) [52]. One of the most important vectors for the widespread \(\text{bla}_{\text{KPC}-2}\) gene transfer is Tn\textsubscript{6296}, which was originally discovered in the MDR plasmid pKP048 from *Klebsiella pneumoniae* [51]. In this study, complex transposition and homologous recombination of Tn6296 and other related mobile elements promoted the expression of \(\text{bla}_{\text{KPC}-2}\) in the isolates, and was involved in influencing antibiotic sensitivity and even clinical symptoms of patients.

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**Figure 4.** Linear Comparison of Tn6411-related region and related regions. Genes are denoted by arrows. Genes, mobile elements, and other features are coloured based on their functional classification. Shading denotes regions of homology (nucleotide identity ≥95%). Numbers in brackets indicate nucleotide positions within the plasmid pSE5416-KPC. The accession number of Tn6411, Tn1403, Tn6296, and Tn5563 used as reference are CP024477, AF313472, FJ628167, and U88088, respectively.

**Figure 5.** Survival rates of *G. mellonella* infection.
A variety of virulence factors lead to the pathogenesis of P. aeruginosa infection, including a variety of toxins, motor systems, and pigments. One of the most relevant P. aeruginosa virulence factors is the type III secretion system, which can directly transfer effector toxins (exoT, exoY, exoS, and exoU) into host cells [53]. The clinically isolated hypervirulent KPC-2-P. aeruginosa in this study carried exoT, exoY, and exoS genes, and the infected G. mellonella larvae had low survival rates. Surprisingly, the 24-h mortality rates of infected larvae of ST463 isolates were 100% compared with those of ST697 isolates (56%) (Figure 5), leading to severe and complex clinical symptoms and even poor clinical outcomes. Among these, exoU has been associated with a high-virulence phenotype and poor prognosis in patients with pneumonia and bacteremia [54]. This may be because ExoU-positive P. aeruginosa isolates are more likely to resist multiple antibiotics, such as carbapenems, cephalosporins, fluoroquinolones, and aminoglycosides, which can exacerbate infection and increase mortality. Interestingly, a previous study reported exoU and exoS as mutually exclusive in P. aeruginosa [48]. ST235, a high-risk MDR clone worldwide, highly correlated with exoU [55], which was also the largest proportion (9%) of multilocus sequence typing in this study (Figure S1), all carrying exoU gene (data not shown). The clone of ST235/ST463 (with blaKPC-2) carrying exoU/exoS gene appeared and spread in East China instead of ST697, with higher virulence, and may become a new clinical threat. Although most of these virulence genes were found to be on the chromosome, the possibility of plasmid-borne virulence factors cannot be ruled out. For example, the 510.71 kb pSE5416-KPC plasmid harbours over 637 genes, and accounts for ~5% total P. aeruginosa genomes, and could potentially contain uncharacterized virulence factors that contribute to the increased virulence. The plasmid-borne virulence factors in P. aeruginosa deserve further studies.

Currently, treatment options for KPC-2-producing CRPA infections are very limited. Some novel antibiotics, such as ceftazidime-avibactam, ceftolozane-tazobactam, cefiderocol, and imipenem-clastatin/relebactam showed promising activity against CRPA in certain studies [56]. However, resistance to these agents have already emerged. For example, a recent study described the emergence of ceftazidime-avibactam resistant CRPA ST463 isolates in China, likely as a consequence of treatment selection pressure [51]. Adequate empiric and definitive therapeutic decisions should be carefully considered during CRPA treatment to minimize selected resistance. Additional antibiotic development is urgently needed to provide sufficient options to successfully manage these CRPA infections.

This study was novel in reporting KPC-2-producing ST697-type P. aeruginosa, which was associated with high virulence in the G. mellonella infection model. KPC-2-producing P. aeruginosa ST463 was first reported in China in 2015 and has rapidly spread in Zhejiang province in recent years [57]. In this study, we analyzed KPC-2-producing carbapenemase-insensitive P. aeruginosa isolates from 2010 to 2021 in East China and revealed that the shifting of CRPA from ST697 to ST463, which was more antibiotic resistant, posing greater challenges for clinical management, resulting in poor therapeutic efficacy. The clinical management should be alert to the emergence and spread of these MDR and potentially high virulent P. aeruginosa strains.

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