

# Characterization of ST11 and ST15 Carbapenem-Resistant Hypervirulent *Klebsiella pneumoniae* from Patients with Ventilator-Associated Pneumonia

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**Background:** The prevalence of carbapenem-resistant hypervirulent *Klebsiella pneumoniae* (hv-CRKP) is a serious public threat globally. Here, we performed clinical, molecular, and phenotypic monitoring of hv-CRKP strains isolated from the intensive care unit (ICU) to offer evidence for prevention and control in hospitals.

**Methods:** Data analysis of ICU patients suffering from ventilator-associated pneumonia (VAP) because of hv-CRKP infection, admitted at the Chinese Teaching Hospital between March 2019 and September 2021 was performed. Patients' antibiotic-resistance genes, virulence-associated genes, and capsular serotypes of these isolates were detected. Homology analysis of the strains was performed by MLST and PFGE. Six different strains were tested for their virulence traits using the serum killing test and the *Galleria mellonella* infection assay. For whole genome sequencing, KP3 was selected as a representative strain.

**Results:** Clinical data of 19 hv-CRKP-VAP patients were collected and their hv-CRKP were isolated, including 10 of ST11-KL64, 4 of ST15-KL112, 2 of ST11-KL47, 1 of ST15-KL19, 1 of ST17-KL140, and 1 of ST48-KL62. Four ST15 and 8 ST11 isolates revealed high homology, respectively. Most strains carried the carbapenemase gene *bla<sub>KPC-2</sub>* (14/19, 73.68%), followed by *bla<sub>OXA-232</sub>* (4/19, 21.05%). All strains were resistant to almost all the antibiotics except polymyxin and tigacycline. Ten patients were treated with polymyxin or tigacycline based on their susceptibility results, and unfortunately 6 patients died. All strains exhibited a hyper-viscous phenotype, and the majority (17/19, 89.47%) of them contained *rmpA* and *rmpA2*. The serum killing test showed that KP9 was resistant to normal healthy serum, others were intermediately or highly sensitive. *G. mellonella* larvae infection assay suggested that the strains in this study were hypervirulent.

**Conclusion:** This study highlights the dominant strain and molecular epidemiology of hv-CRKP in a hospital in China. We should pay more attention to the effect of hv-CRKP on VAP, strengthen monitoring and control transmission.

**Keywords:** *Klebsiella pneumoniae*, hypervirulent, carbapenem-resistant, ventilator-associated pneumonia

## Introduction

*Klebsiella pneumoniae* is a gram-negative opportunistic pathogen that can cause both community and hospital-acquired pneumonia, urinary tract infections, and bacteremia primarily in immunocompromised patients.<sup>1</sup> Most experts agree that carbapenems are an effective treatment for serious infections brought on by *Enterobacteriaceae* that produce extended-spectrum beta-lactamases (ESBLs).<sup>2-4</sup> Because of the wide and irrational use of antibiotics, the number of carbapenemase-resistant *Enterobacteriaceae* (CRE) infections is increasing per year and antibiotic resistance has become an international problem.<sup>5,6</sup> During 2005–2020, the China Antimicrobial Surveillance Network (CHINET, <http://www.chinets.com>) reported

that *K. pneumoniae* resistance rates to imipenem and meropenem have rapidly increased from 3% and 2.9% to 23.2% and 24.2% respectively. There has been a significant increase in respiratory tract infections in intensive care units (ICUs) due to carbapenem-resistant *K. pneumoniae* (CRKP), which is considered a severe danger to public health as a pathogen associated with hospital-acquired infections (HAIs).<sup>7–10</sup> It is reported that 9–27% of ICU patients suffer from ventilator-associated pneumonia (VAP).<sup>11</sup> Meanwhile, treatment of CRKP-induced VAP in ICU patients is difficult because of the lack of multiple therapeutic drugs.

Hypervirulent *K. pneumoniae* (hvKP) is the primary cause of community-acquired illnesses such as meningitis, necrotizing fasciitis, endophthalmitis, liver abscesses, and pneumonia in people of all ages and health statuses. It is associated with severe morbidity and mortality and has been reported to have an increasing incidence rate globally.<sup>12–15</sup> When the string test is positive for hvKP, it is defined to have a hyper-viscous phenotype. K1 and K2 are the most frequent capsular loci for hvKP.<sup>12</sup> ST23 and ST258 strains account for the majority of hvKP strains.<sup>16</sup> Antibiotics usually work well on hvKP, however infections brought on by carbapenem-resistant hvKP (hv-CRKP) have recently been documented increasingly frequently.<sup>17,18</sup> To the best of our knowledge, the emergence of such a high-virulent, drug-resistant pathogen poses an even more significant challenge to clinical patient safety.

This investigation was conducted on the VAP outbreaks caused by ST11 and ST15 hv-CRKP in a teaching hospital's Emergency Intensive Care Unit (EICU) in China. Furthermore, the clinical characteristics of patients as well as the outcome, resistance mechanisms, virulence characteristics, and genomic features of these strains were analyzed.

## Materials and Methods

### Isolates Collection and Identification

From March 2019 to September 2021, 19 hv-CRKP-VAP cases were admitted to the EICU of the Affiliated Hospital of Xuzhou Medical University. 19 non-repeated isolates of hv-CRKP were collected from these patients. Matrix-Assisted Laser Desorption / Ionization Time of Flight Mass Spectrometry (Bruker Daltonics, Billerica, MA, USA) was used to recheck the isolates of *K. pneumoniae*. All isolates were resistant to at least one carbapenems (imipenem, meropenem, ertapenem) and were identified as CRKP by the VITEK-2 compact automated microbiology analyzer (Biomerieux, Marcy L'Etoile, France). String tests were carried out to see if hvKP had the hypermucoviscosity phenotype. Furthermore, patients' clinical data including gender, age, isolation date, sampling site, comorbidity, invasive operation, antibiotic treatments, and outcomes were also collected. This investigation was authorized by the Ethics Committee of the Affiliated Hospital of Xuzhou Medical University XYFY2020-KL084.

### Testing for Antimicrobial Susceptibility

The minimal inhibitory concentrations of antibiotics, including amikacin, aztreonam, ceftazidime, ciprofloxacin, cefotaxime, cefepime, imipenem, meropenem, polymyxin B, sulfamethoxazole, tigecycline, and piperacillin/tazobactam were determined by VITEK-2 Compact system. The guidelines provided by the Clinical and Laboratory Standards Institute (CLSI, 2021) were followed for the interpretation of the results.<sup>19</sup> *Pseudomonas aeruginosa* ATCC27853 and *Escherichia coli* ATCC25922 were used as quality control strains.

### Detection of Antimicrobial Resistance Genes

For extracting the genomic DNA of all the isolates, the boiling method was applied. The ESBL genes (*bla*<sub>TEM</sub>, *bla*<sub>SHV</sub>, *bla*<sub>CTX-M-1</sub> group, *bla*<sub>CTX-M-2</sub> group, *bla*<sub>CTX-M-8</sub> group, and *bla*<sub>CTX-M-9</sub> group), carbapenemase genes (*bla*<sub>KPC</sub>, *bla*<sub>NDM</sub>, *bla*<sub>VIM</sub>, *bla*<sub>IMP</sub>, and *bla*<sub>OXA-48</sub>) and AmpC-lactamase genes (*bla*<sub>CMY</sub> and *bla*<sub>DHA</sub>) were amplified by polymerase chain reaction (PCR) as described previously and the PCR products were sequenced and then on the sequencing results BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) was run.<sup>20,21</sup> The primer sequences are enlisted in [Table S1](#).

## Detection of Capsular Serotyping and Virulence-Associated Genes

*Wzi* gene was amplified by PCR and sequenced as previously described.<sup>22</sup> To compare the findings and identify the capsular type of *K. pneumoniae*, the database (<https://bigsdbs.pasteur.fr>) was consulted. 12 virulence-associated genes including *aerobactin*, *rmpA2*, *rmpA*, *magA*, *kfu*, *allS*, *fimH*, *ybtS*, *entB*, *mrkD*, *iroN*, and *iucA* were also amplified by PCR.<sup>23,24</sup> The primer sequences of *Wzi* and virulent genes are enlisted in [Table S1](#).

## Multilocus Sequence Typing (MLST)

*gapA*, *infB*, *mdh*, *pgi*, *phoE*, *rpoB*, and *tonB* are the seven housekeeping genes (expressed by *K. pneumoniae*) that were amplified by PCR and sequenced for all isolates by the MLST website's methodology (<https://bigsdbs.pasteur.fr/klebsiella/primers-used>). The sequencing results were compared with the database to identify sequence types.

## Pulsed-Field Gel Electrophoresis (PFGE)

The homology of all strains was analyzed by PFGE assay.<sup>25</sup> Genomic DNA was digested with XbaI for 2 h at 37°C and the fragments were separated using the CHEF Mapper XA System (Bio-Rad) for 18h at 14°C. The molecular identifier was determined to be a *Salmonella* serotype Braenderup strain H9812.<sup>26</sup> Bionumerics software was utilized for the unweighted pair-group technique with arithmetic means (UPGMA) for cluster analysis. A greater than 85% similarity was considered as a closely related.<sup>20,27</sup>

## String Test

Isolates of *K. pneumoniae* were plated on blood agar and grown overnight at 37°C. A single colony was contacted and pushed outward using an inoculation loop. The string test was considered positive and the isolate showed a hypervirulent phenotype if the length of the viscous string was greater than 5 mm.<sup>28</sup> This experiment was repeated thrice.

## Serum-Killing Assay

Three ST11-KL64 strains (KP3, KP9, KP13) and 3 ST15-KL112 strains (KP6, KP15, KP18) of bacteria were randomly selected for serum killing assay. The test was chosen in accordance with a previously reported study.<sup>28,29</sup> Serum was collected from 10 healthy individuals and was kept at -80°C. In phosphate-buffered saline (PBS), the mid-log phase bacteria were suspended in a concentration of 1×10<sup>6</sup> CFU/mL and then combined with normal serum in a ratio of 1:3. The mixture was immediately plated on Mueller-Hinton agar after being incubated at 37°C for 0, 1, and 3 hours in order to count the number of bacteria. The results were graded on six levels, and an isolate was declared serum sensitive at grade 1 or 2, intermediately sensitive at 3 or 4, and resistant at 5 or 6. The experiment was repeated thrice. For hyper and low virulence control, ATCC43816 and ATCC700603 were employed, respectively.<sup>27,30,31</sup>

## Galleria Mellonella Larvae Infection Assay

*Galleria mellonella* larvae infection assay was performed to further assess bacterial pathogenicity.<sup>20,32-34</sup> The isolates and controls were the same as those in the serum-killing assay. PBS was used to adjust the overnight culture to the concentrations of 1×10<sup>5</sup> to 1×10<sup>8</sup> CFU/mL. *Galleria mellonella* larvae weighing 250–350 mg were used in this experiment. Ten microliter of various concentrations of each bacterium strain suspension was injected in 10 larvae through last proleg. Furthermore, 10 µL PBS was injected into 10 wax moths as an experiment control. Each larva was put onto a clean Petri plate and incubated at 37°C. For three days, the larval survival rates every 12 hours were recorded. This experiment was performed in three replicates.

## Whole Genome Sequencing and Analysis

BGI Technology Service Co., Ltd sequenced the whole genome of strain KP3 in Shenzhen. The PacBio Sequel II system was used for third-generation sequencing. The whole genome was submitted to GenBank. BLAST (<http://blast.ncbi.nlm.nih.gov>) was utilized to compare plasmid sequences and the genes associated with drug resistance and virulence were predicted using ResFinder4.1 and VFDB 2019 databases. Map plasmid genome circles were assessed via the Gcview website.

## Results

### Patient's Clinical Features and CR-hvKP Isolates

Nineteen isolates of hv-CRKP-VAP were collected from different patients during their stay at the EICU between March 2019 to September 2021. Before strain isolation, all the patients underwent invasive operations and received antibiotic treatment. The mortality rate was 73.68% (14/19). The isolates were acquired from different specimens including sputum (11/19, 57.89%), blood (4/19, 21.05%), bone marrow (1/19, 5.26%), cerebrospinal fluid (1/19, 5.26%), fibro bronchoscope lotion (1/19, 5.26%), and hydrothorax (1/19, 5.26%). The specifics of each clinical feature are listed in [Table 1](#).

### Antibiotic Sensitivity and Genetic Characteristics of Resistance Genes

Except for tigecycline and colistin, all the organisms were highly resistant to the tested antibiotics ([Table 2](#)). Carbapenemase genes were detected from all 19 isolates, including *bla*<sub>KPC-2</sub> (14/19, 73.68%), *bla*<sub>OXA-232</sub> (4/19, 21.05%), and *bla*<sub>NDM-1</sub> (2/19, 10.53%). KP19 contained both *bla*<sub>KPC-2</sub> and *bla*<sub>NDM-1</sub>. Seven strains (36.84%) harbored the *bla*<sub>CTX-M-1</sub> group and the profitable products were identified as *bla*<sub>CTX-M-107</sub>. The *bla*<sub>CTX-M-65</sub> variant was present in 12 (63.16%) of the strains that owned the *bla*<sub>CTX-M-9</sub> group. The ratios of *bla*<sub>SHV</sub> and *bla*<sub>TEM</sub> expression by CR-hvKP bacteria were 100% (19/19) and 73.68% (14/19), respectively. No isolate expressed *bla*<sub>VIM</sub>, *bla*<sub>IMB</sub>, *bla*<sub>CMY</sub>, *bla*<sub>DHA</sub>, *bla*<sub>CTX-M-2</sub>, and *bla*<sub>CTX-M-8</sub> groups ([Figure 1](#)).

### Virulence-Associated Genes and Capsular Serotyping

[Figure 1](#) displays the outcomes of 12 virulence-related genes. The *fimH* and *entB* genes were present in all 19 isolates. The following genes were *mrkD* (18/19, 94.74%), *ytbS* (18/19, 94.74%), *iucA* (17/19, 89.47%), *rmpA2* (16/19, 84.21%), *rmpA* (11/19, 57.89%), *iroN* (10/19, 52.63%), and *kfu* (5/19, 26.32%). No isolate in this study expressed *aerobactin*, *magA*, and *allS*. Six distinct capsular serotypes for strains were found by PCR and sequencing of the *Wzi* gene, including KL64 (10/19, 52.63%), KL112 (4/19, 21.05%), KL47 (2/19, 10.53%), KL19 (1/19, 5.26%), KL62 (1/19, 5.26%), and KL140 (1/19, 5.26%) ([Figure 2](#)).

### MLST and PFGE

In total four STs were identified, where ST11 (12/19, 63.16%) accounted for the majority of the strains, followed by ST15 (5/19, 26.32%), ST17 (1/19, 5.26%), and ST48 (1/19, 5.26%). In the PFGE experiment, 4 ST15 and 8 ST11 isolates indicated high homology (>85%), respectively ([Figure 2](#)).

### Virulence-Related Characteristics

The string test revealed that all strains were positive ([Figure 1](#)). KP9 and the hypervirulence standard strain ATCC43816 are resistant to healthy, normal serum (grade 6). KP15 and KP18 strains were moderately sensitive (grade 4) to healthy serum, however, KP3, KP6, and KP13 as well as the classical *K. pneumoniae* isolate ATCC700603 were all susceptible to healthy serum (grade 1 or 2) ([Figure 3](#)). To further evaluate the virulence of these strains in-vivo, *G. mellonella* larvae were infected with the experimental strains and their survival rate was calculated after 72h of incubation. When the concentration of bacteria suspension was  $1 \times 10^6$  CFU/mL, the survival rate of classic *K. pneumoniae* isolates ATCC700603 was 73.33%, and that of hypervirulence control ATCC43816 was 6.67%, whereas, the survival rates of KP3, KP6, and KP13 were 16.67%, 6.67%, and 13.33%, respectively at 72h. KP9 and KP15 had a survival rate of 10% after 72h. Survival of *G. mellonella* reached 0% with KP18 at 24h ([Figures 4](#) and [S1](#)). The result of the *G. mellonella* larvae infection assay suggested the strains in this study were hypervirulent.

### Whole Genome Sequencing and Analysis

The genome sequence identified in this investigation was submitted to GenBank with the accession number CP116491, CP116492, CP116493, and CP116494. KP3 was discovered to have a 5521665bp chromosome and three plasmids

**Table 1** Patient's and Hv-CRKP Isolate's Clinical Features

Isolate	Patient No.	Gender	Age (Years)	Specimen	Comorbidity	Surgery	Invasive Operation	History of Antibiotic Use	Antibiotic Treatment	Outcome
KP1	1	M	55	Blood	Cancer	Y	Urinary catheter, Central venous catheter	CTS	CTS	Death
KP2	2	F	76	Bone marrow	Lung disease	N	Urinary catheter, Central venous catheter	BIA	POL, FOS, BIA	Death
KP3	3	F	63	Cerebrospinal fluid	Neurological diseases	Y	Urinary catheter, Central venous catheter, Tracheotomy	CTS	MEM	Death
KP4	4	M	51	Blood	Neurological diseases	Y	Urinary catheter, Central venous catheter, Tracheotomy	CTX, CTS, MEM	MEM	Death
KP5	5	F	68	Sputum	Diabetes mellitus, Hypertension, Neurological diseases, Heart diseases	N	Urinary catheter, Central venous catheter, Tracheotomy	CTS, AMK	TGC, IPM	Death
KP6	6	M	56	Sputum	Diabetes mellitus, Heart diseases, Neurological diseases, Renal diseases	Y	Urinary catheter, Central venous catheter, Tracheotomy	CFP/TAZ, CDZ, MFX, TZP, IPM	IPM	Death
KP7	7	F	47	Sputum	Hypertension, Neurological diseases	Y	Urinary catheter, Central venous catheter, Tracheotomy	CFP/TAZ	TGC	Improvement
KP8	8	F	67	Sputum	Hypertension, Diabetes mellitus, Heart diseases, Neurological diseases, Renal diseases	N	Urinary catheter, Central venous catheter, Tracheotomy	TZP, BIA	BIA	Death
KP9	9	M	79	Sputum	Diabetes mellitus, Hypertension, Multiple injury	N	Urinary catheter, Central venous catheter, Tracheotomy	CTS	BIA, TGC	Death
KP10	10	M	53	Sputum	Liver disease, Neurological diseases	N	Urinary catheter, Central venous catheter, Tracheotomy	MSU, CSL, TGC, AMK, FEP	TGC, AMK, FEP	Death
KP11	11	M	53	Blood	Cancer, Diabetes mellitus	Y	Urinary catheter, Central venous catheter, Tracheotomy	CTS, CFP/TAZ, MEM	MEM, TGC	Death
KP12	12	M	74	Sputum	Neurological diseases	Y	Urinary catheter, Tracheotomy	CTS, MEM, BIA, MFX	BIA	Death
KP13	13	F	63	Sputum	Hypertension, Neurological diseases, Heart diseases	Y	Urinary catheter, Central venous catheter, Tracheotomy	CTS, TZP, AMK, TGC, MEM	MEM, LVX	Improvement
KP14	14	M	63	Fibrobronchoscope lotion	Diabetes mellitus, Hypertension, Neurological diseases	Y	Urinary catheter, Central venous catheter, Tracheotomy	CFP/TAZ, IPM, AMK, TGC, ETM	BIA, TGC, TZP	Improvement
KP15	15	M	46	Sputum	Multiple injury	Y	Urinary catheter, Central venous catheter, Tracheotomy	MSU, IPM, TGC	IPM, TGC, MFX	Improvement
KP16	16	M	42	Sputum	Hypertension, Neurological diseases	Y	Urinary catheter, Central venous catheter, Tracheotomy	CTX, CFP/TAZ	CFP/TAZ, AMK	Death
KP17	17	M	69	Blood	Diabetes mellitus, Hypertension, Neurological diseases	N	Urinary catheter, Central venous catheter, Tracheotomy	CTS, IPM	IPM	Death
KP18	18	F	67	Hydrothorax	Hypertension, Neurological diseases, empyema	Y	Urinary catheter, Central venous catheter, Tracheotomy	CFP/TAZ, AMK, TGC, IPM	TGC, IPM	Improvement
KP19	19	F	71	Sputum	Diabetes mellitus, Hypertension, Renal diseases, Heart diseases, Neurological diseases	N	Urinary catheter, Central venous catheter, Tracheotomy	BIA, IPM, LVX	MFX, TGC, IPM	Death

**Abbreviations:** F, female; M, man; Y, yes; N, no; AMK, Amikacin; BIA, Biapenem; CDZ, Cefodizime; CEP/TAZ, Cefoperazone tazobactam; CSL, Cefoperazone/Sulbactam; CTS, Cefotaxime/Sulbactam; CTX, Cefotaxime; ETM, Etimicin; FEP, Cefepime; IPM, Imipenem; MEM, Meropenem; FOS, Fosfomicin; MFX, Moxifloxacin; LVX, Levofloxacin; MSU, Mezlocillin/Sulbactam; POL, Polymyxin B; TGC, Tigecycline; TZP, Piperacillin/Tazobactam.

**Table 2** Antibiotic Resistance Characteristics

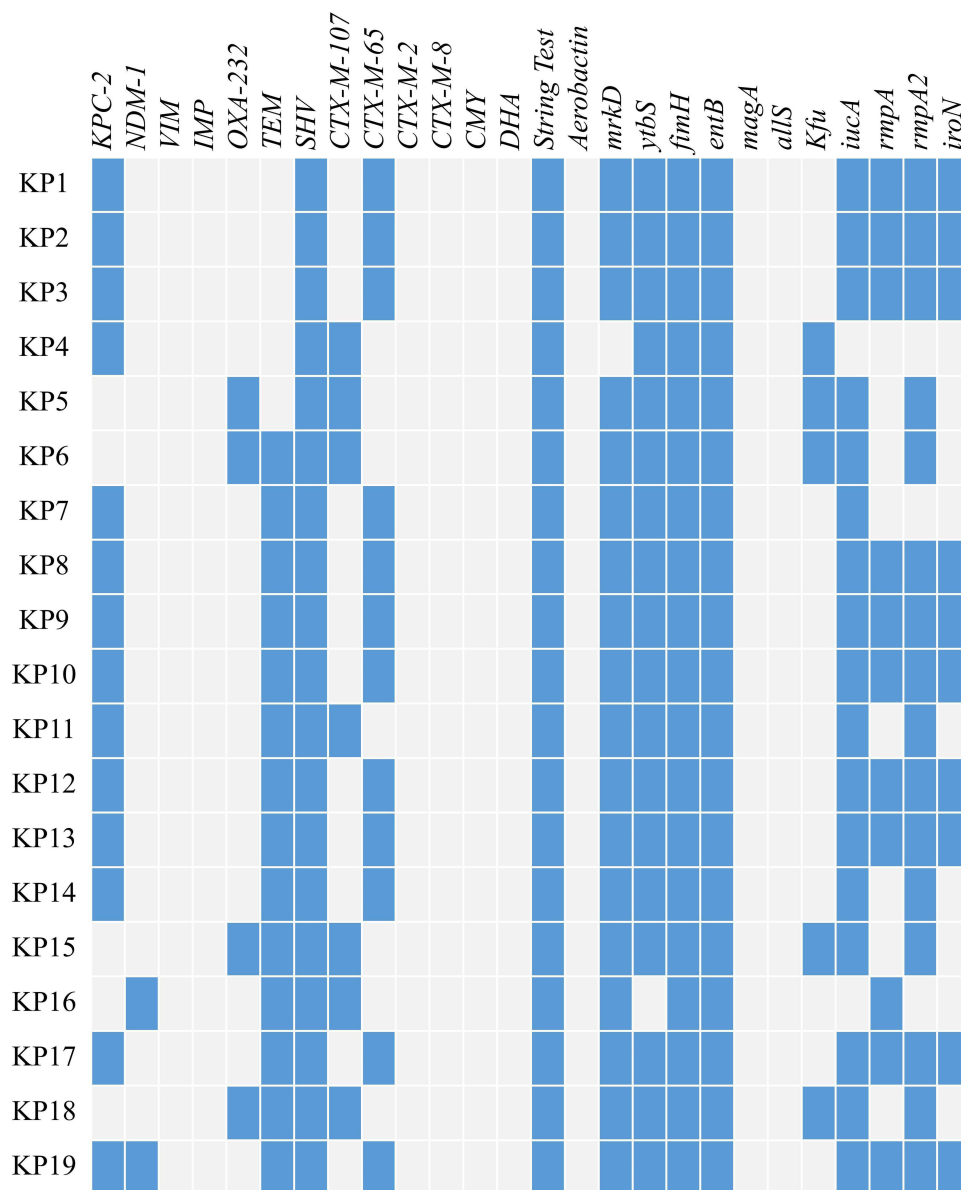
Isolate	MIC (mg/L)											
	AMK	ATM	SXT	CIP	TZP	FEP	CTX	CAZ	IPM	MEM	TGC	POL
KP1	<=2	>32	>8	>2	>64	>16	>32	>32	>8	>8	<=0.5	<=0.5
KP2	<=2	>32	>8	>2	>64	>16	>32	>32	>8	>8	<=0.5	<=0.5
KP3	<=2	>32	>8	>2	>64	>16	>32	>32	>8	>8	<=0.5	<=0.5
KP4	4	>32	>8	>2	>64	>16	>32	32	>8	>8	<=0.5	<=0.5
KP5	4	>32	4	>2	>64	>16	>32	>32	2	>8	<=0.5	<=0.5
KP6	8	>32	>8	>2	>64	>16	>32	>32	2	>8	<=0.5	<=0.5
KP7	>32	>32	<=1	>2	>64	>16	>32	>32	>8	>8	<=0.5	<=0.5
KP8	>32	>32	>8	>2	>64	>16	>32	>32	>8	>8	<=0.5	<=0.5
KP9	>32	>32	>8	>2	>64	>16	>32	>32	>8	>8	<=0.5	<=0.5
KP10	>32	>32	>8	>2	>64	>16	>32	>32	>8	>8	<=0.5	<=0.5
KP11	<=2	>32	4	>2	>64	>16	>32	>32	>8	>8	<=0.5	<=0.5
KP12	>32	>32	>8	>2	>64	>16	>32	>32	>8	>8	<=0.5	<=0.5
KP13	>32	>32	>8	>2	>64	>16	>32	>32	>8	>8	<=0.5	<=0.5
KP14	>32	>32	>8	>2	>64	>16	>32	>32	>8	>8	2	<=0.5
KP15	>32	>32	>8	>2	>64	>16	>32	>32	2	>8	<=0.5	<=0.5
KP16	<=2	16	>8	2	>64	>16	>32	>32	>8	>8	<=0.5	<=0.5
KP17	>32	>32	<=1	>2	>64	>16	>32	>32	>8	>8	<=0.5	<=0.5
KP18	8	>32	>8	>2	>64	>16	>32	>32	2	>8	<=0.5	<=0.5
KP19	>32	>32	8	>2	>64	>16	>32	>32	>8	>8	<=0.5	<=0.5

**Abbreviations:** MIC, Minimum inhibitory concentration; AMK, Amikacin; CIP, Ciprofloxacin; ATM, Aztreonam; SXT, Sulfamethoxazole; TZP, Piperacillin/Tazobactam; FEP, Cefepime; CTX, Cefotaxime; CAZ, Ceftazidime; TGC, Tigecycline; IPM, Imipenem; MEM, Meropenem; POL, Polymyxin B.

(218592bp, 100630bp, and 85038bp). Plasmid 1 contained the virulence genes *rmpA*, *rmpA2*, and *iucABCD*, was quite similar to the virulence plasmid pLVPK (AY378100), and had a coverage of 91% and consistency of 99.05% (Figure 5A). Plasmid 3 carried *bla<sub>KPC-2</sub>*, had 99.98% homology with NTUH-K2044-CR plasmid unnamed2 (MZ475710.1) and 100% with pKPC-CR-hvKP-C789 (CP034417.1), phvKP12-KPC (CP103319.1) and pJX2-2 (CP064248.1), and query coverages of 80–100% (Figure 5B).

## Discussion

This study retrospectively investigated 19 hv-CRKP-VAP cases from March 2019 to September 2021 enrolled at the EICU of a Chinese teaching hospital and analyzed the outbreak characteristics of ST11-KL64 and ST15-KL112 hv-CRKP strain. Following the analysis, 13 risk variables associated with CRE infection were identified, including infection acquired from ICU, invasive procedures, mechanical ventilation, medical devices, CRE exposure, carbapenem use,  $\beta$ -lactam use, cephalosporin use, glycopeptide use, quinolone use, demographic patient characteristics, underlying disease or condition, and exposure to hospital care.<sup>35</sup> To our knowledge, outbreaks of HAIs due to CRKP are very commonly reported, especially in VAP due to intubation. PFGE assays revealed that the homology of the eight *kpc-2*-producing ST11-KL64 CR-hvKP strains was >90%. These strains were isolated from elderly patients hospitalized in the EICU between March 2019 to June 2020, most of whom had neurological diseases. All patients underwent invasive procedures and had a morbidity and mortality rate of 73.7%, suggesting that the consequences of hv-CRKP-induced VAP are severe. Recently, infections and outbreaks caused by this pathogen have been reported in many regions.<sup>24,36,37</sup> This pathogen requires more attention from clinicians and infection control physicians. Since the first report of *K. pneumoniae* carrying OXA-232 in Shanghai, in 2017, its epidemics have been reported in China.<sup>38–40</sup> In this study, the four strains of OXA-232-producing ST15-KL112 hv-CRKP had >92% homology. Different from the *kpc-2*-producing CRE strains (MIC  $\geq$  16ug/mL), these strains showed intermediary sensitivity to imipenem with MIC values of 2ug/mL, possibly because of more available drug options. 50% of patients with OXA-232-producing strains improved after treatment compared with



**Figure 1** 19 hv-CRKP isolates' antimicrobial resistance genes, virulence-related genes, and string test results. The blue box represents the presence of genes or a positive string test result.

the KPC-2-producing CRE strain. A deadly VAP epidemic induced by ST11 hv-CRKP was documented in a 2017 article and occurred in a Chinese hospital.<sup>18</sup> The stains were highly transmissible, multidrug-resistant, and hypervirulent at the same time. Genomic analyses revealed that the virulence plasmid acquired by the hv-CRKP strains was the classic ST11 CRKP stains. Simply, hv-CRKP strains have high virulence and multidrug resistance, and therefore possess a great challenge to clinical treatment and are a severe threat to public health. Fortunately, no homologous strains in VAP patients were detected after July 2020 following intervention with stringent infection control measures.

hv-CRKP consists of two evolutionary mechanisms, one in which CRKP strains acquire virulence genes or virulence plasmids, and the other in which hvKP strains acquire chromosomal or plasmid-encoded drug resistance genes.<sup>16</sup> The strains in this investigation fall in the former type. There are three main mechanisms responsible for *K. pneumoniae*'s resistance to carbapenem: enzyme production, efflux pumps, and pore protein mutations.<sup>41</sup> In China, KPC-2 is the most common carbapenemase for CRKP, and ST11 is the most common sequence type.<sup>10,24,36</sup> This study comprises the majority of the KPC-2-producing ST11 *K. pneumoniae* strains. The hvKP strains are frequently used to describe the

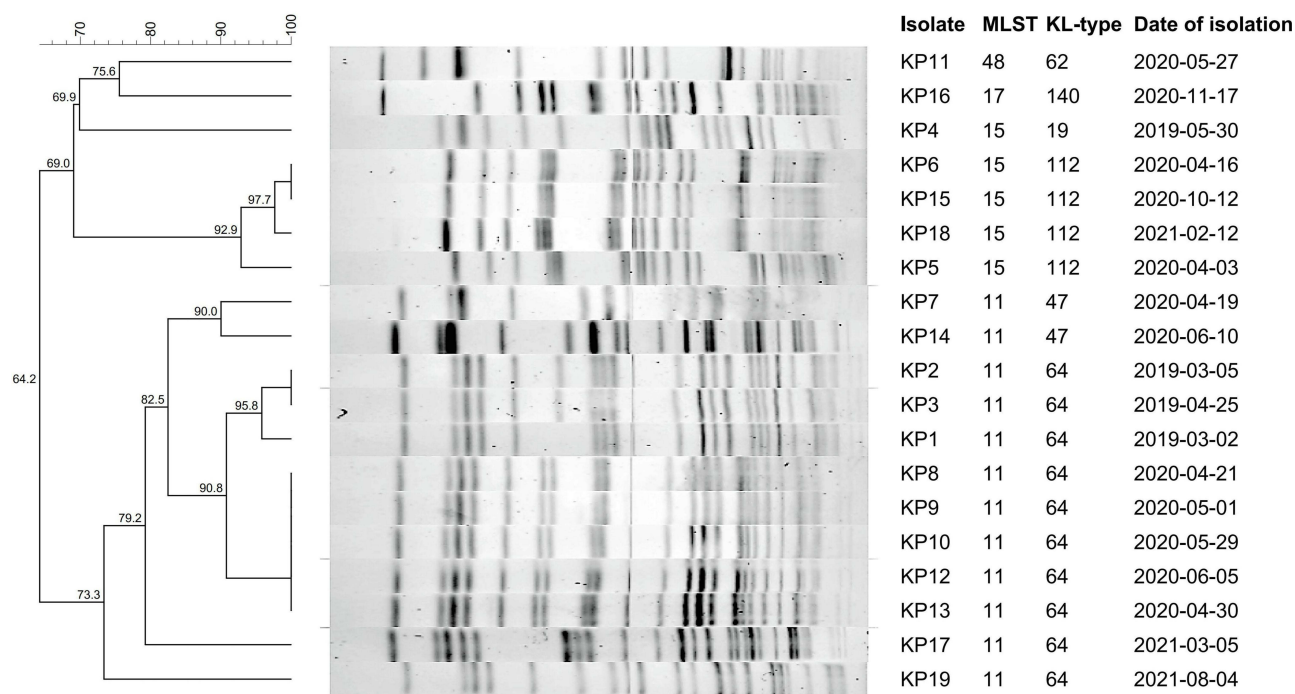


Figure 2 PFGE results, MLST type, capsular serotype, and date of isolate for 19 hv-CRKP isolates. Similarity >85% was considered closely related.

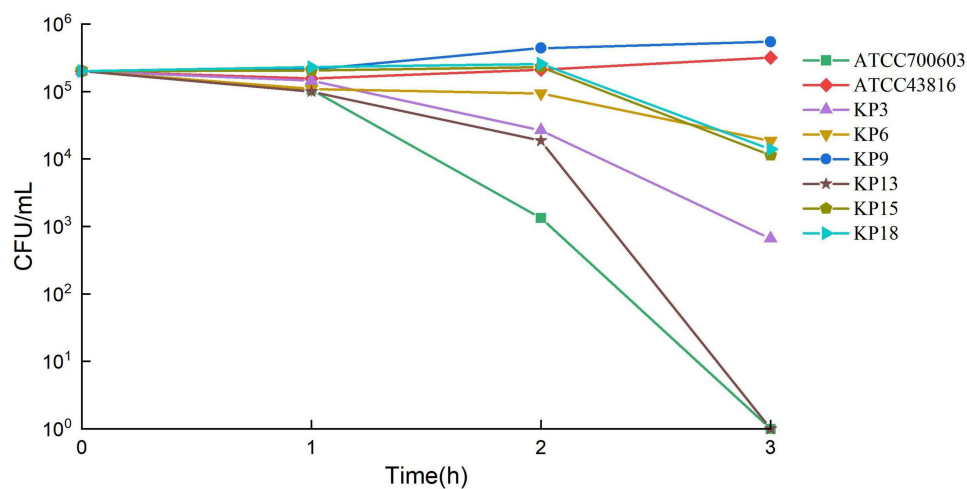
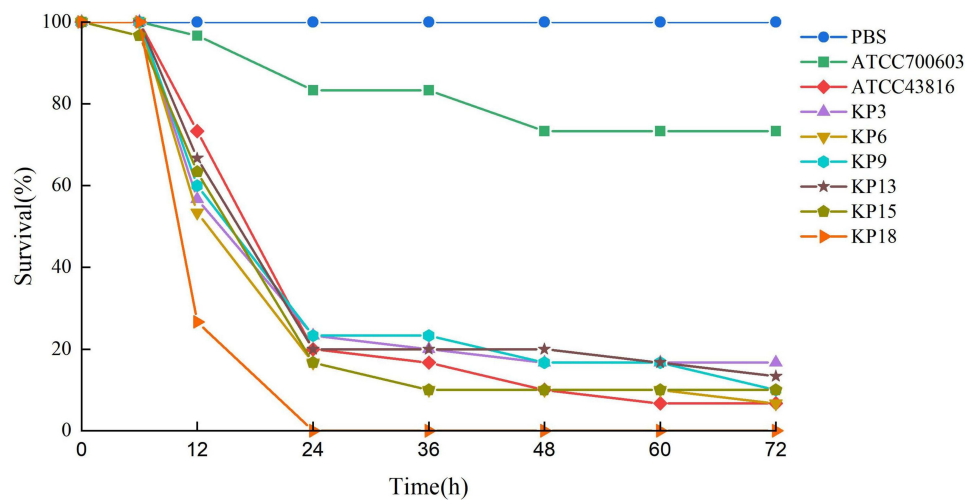
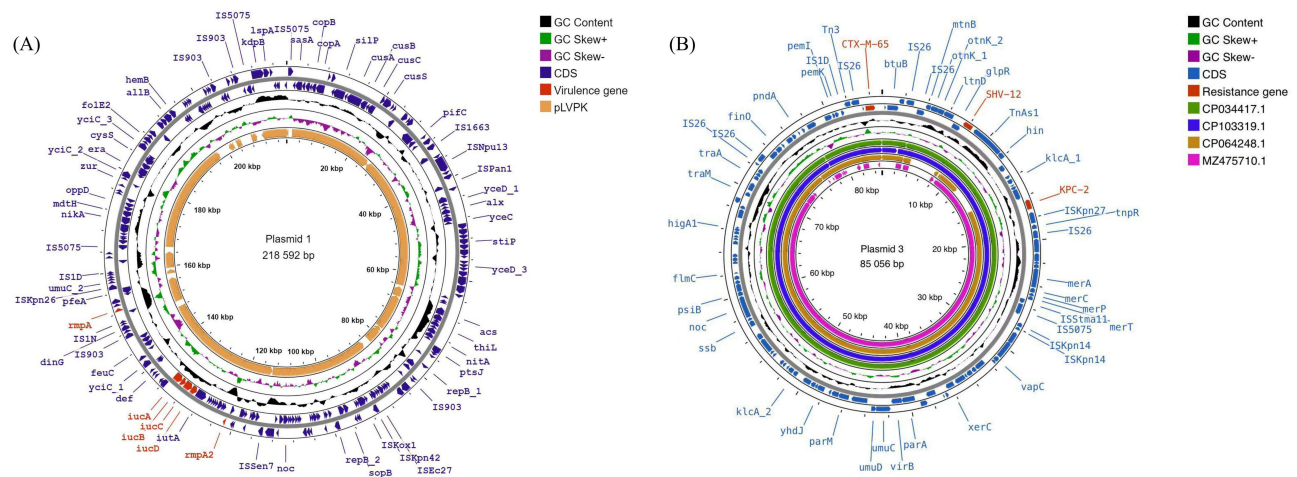


Figure 3 Serum killing assay of 6 selected isolates. ATCC43816 and ATCC700603 were hypervirulence and low virulence controls, respectively.

positive strains of the “string test”. However, it has also been reported that these lack hypervirulent phenotypes. Therefore, the string test cannot be the only indicator of the hvKP strain.<sup>42</sup> A study suggested that *rmpA*, *rmpA2*, *iucA*, *iroB*, and *peg-344* are the most accurate molecular markers to define hvKP because these genes are all present on virulence plasmids.<sup>43</sup> Capsular polysaccharide, siderophore system, lipopolysaccharide, outer membrane protein, and efflux pump are currently recognized hvKP pathogenic components, however, the capsule has been identified as a major virulence component.<sup>44</sup> The *rmpA* and *rmpA2* genes increase muco-viscosity and pathogenicity and are linked to high capsule productivity.<sup>42</sup> Here, all strains had a hyper-viscous phenotype and most of them contained *rmpA* or *rmpA2* except KP4 and KP7, indicating that other mechanisms may also affect the hyper-mucoviscosity.<sup>45</sup> Aerobactin is encoded by the *iucABCD* gene, and its corresponding receptor is expressed by the *iutA*. *iucA* encodes a IucA ortholog that is



**Figure 4** Survival rate of *G. mellonella* larvae infected with  $1 \times 10^6$  CFU/mL of each selected isolate. ATCC43816 and ATCC700603 were hypervirulence and low virulence controls, respectively. PBS was the experimental control.



**Figure 5** Two plasmid circle maps were generated with the GCview website. (A) Plasmid 1 carried *rmpA*, *rmpA2*, and *iucABCD* and was highly similar to pLVPK. The white regions indicate absence. (B) Plasmid 3 carried *bla<sub>KPC-2</sub>* and shared a high identity with CP034417.1, CP103319.1, CP064248.1, and MZ475710.1 by comparison.

thought to bind ahLys onto the primary carboxylates of citrate to produce aerobactin siderophore.<sup>44,46</sup> Aerobactin is considered the critical siderophore system of hvKP, expressed in >90% of its strains but only in 6% of classic *K. pneumoniae*.<sup>44</sup> No isolate from this study expressed *aerobactin*, further indicating that these strains were produced by CRKP obtaining virulence genes or virulence plasmids. The *bla<sub>KPC-2</sub>* and *iucABCD* were also identified on plasmids by whole genome sequencing of the KP3 strain.

In vitro serum killing assays and in vivo *G. mellonella* larvae infection assays were conducted to assess the virulence of these strains. Six strains were selected randomly for the virulence test and all displayed high virulence in *G. mellonella* larvae. However, the serum survival rate of KP3, KP6, and KP13 were low. Such inconsistency in the in vitro and in vivo virulence results are not unique. Shen P also reported a similar situation.<sup>47</sup> The underlying mechanisms behind these differences have yet to be determined. According to a study, humans and *G. mellonella* have different levels of *K. pneumoniae* virulence.<sup>48</sup> For the virulence investigation, ST11-KL64 and ST15-KL112 hv-CRKP were chosen. The results revealed that ST11-KL64 included both *rmpA* and *rmpA2*, while ST15-KL112 only contained *rmpA2*, but the findings from serum killing assays and *G. mellonella* larvae infection assays were identical. The *G. mellonella* larvae infection model, however, has been proposed as a preliminary discriminant between

*K. pneumoniae* strains with comparable virulence. According to Tang, the LD<sub>80</sub> of K1 and K57 was 1.0×10<sup>6</sup> CFU/mL while the LD<sub>80</sub> of K2, K5, and K20 was 1.0×10<sup>7</sup> CFU/mL after 96 hours of inoculation, indicating that K1 and K57 were more virulent than K2, K5, and K20.<sup>49</sup> Insua also distinguished between K1 and K2 pathogenicity using *G. mellonella* larvae.<sup>50</sup> Although the *G. mellonella* infection model has many benefits, it has been reported that the murine model is better at differentiating between hypervirulent and standard *K. pneumoniae*.<sup>51</sup> Nevertheless, one study showed that *G. mellonella* larvae are a crucial infection model in assessing the infection process, antioxidant response, and extent of cell damage of the *K. pneumoniae* pathogen. Furthermore, *G. mellonella* larvae may be appropriate models for antibiotic susceptibility investigations as well as host interactions with resistant *K. pneumoniae*.<sup>52</sup>

This study illustrates that hv-CRKP has caused an outbreak in hospitals, and clarified the clinical and molecular characteristics of VAP caused by ST11 and ST15 hv-CRKP. It offers suggestions for clinical prevention, control, diagnosis, and therapy. However, this study has some limitations. Retrospective analysis prevents us from gathering more detailed clinical information and taking environmental samples to understand the route of the outbreak more accurately. Unfortunately, only a few strains were used in this single-center investigation and only the entire genome of one strain was read, sequencing the genomes of all the strains would allow for a deeper understanding of the genetic traits. In addition, we did not use the murine model to assess virulence, and using only *G. mellonella* larvae may not reveal the real hypervirulent natures of the studied strains.

## Conclusions

Our findings show the prevalence of hv-CRKP in Chinese hospitals, with ST11-blaKPC-2 being the most common. After acquiring a virulence plasmid, the strain can infect patients across many systems, which significantly raises the mortality rate. The transmission mechanism of virulence plasmids, as well as variances in drug resistance and virulence among different subtypes, are concerns that should be addressed in the future. As a result, it provides substantial data support for infection control.

## Data Sharing Statement

The following datasets are included in this study: NCBI GenBank NO.: CP116491, CP116492, CP116493, and CP116494.

## Ethical Approval and Consent to Participate

The medical ethics committee of The Affiliated Hospital of Xuzhou Medical University granted this study ethical approval (XYFY2020-KL084).

## 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

There are no relevant conflicts of interest reported by any of the authors.

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