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Epidemiological, phenotypic and genotypic characteristics difference of hypervirulent and carbapenem-resistant *Klebsiella pneumoniae* with different capsular serotypes

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ABSTRACT

Background: KL1, KL2 CR-hvKP and KL64, KL47 hv-CRKP all exhibit overlapping multidrug resistance and hypervirulence phenotypes, but the differences in epidemiological, phenotypic and genotypic characteristics between them remains unclear.
Methods: In this study, we collected non-repeated hv-CRKP/CR-hvKP isolates in a tertiary hospital in Shanghai, China from January 2019 to December 2022. Furthermore, we selected four typical hypervirulent and carbapenem-resistant *Klebsiella pneumoniae*, including ST23-KL1/ST86-KL2 CR-hvKP (WYKP3 and WYKP194) and ST11-KL64/ST11-KL47 hv-CRKP (WYKP589 and WYKP188), and tried to clarify and compare their differences in virulence and drug resistance characteristics and plasmid distribution.
Results: Our study found that ST23-KL1 and ST86-KL2 CR-hvKP exhibited less plasmid diversity than that of ST11-KL64 and ST11-KL47 hv-CRKP. Compared with ST11-KL64 and ST11-KL47 hv-CRKP, ST23-KL1/ST86-KL2 CR-hvKP harbored significantly fewer antimicrobial resistance genes but more virulence genes, which contributed to the higher virulence of these strains and exhibited resistance to fewer antibiotics. ST11-KL64 hv-CRKP has emerged as the most prevalent hypervirulent and carbapenem-resistant *Klebsiella pneumoniae* probably due to its clonal transmission within hospitals as well as the transmission of virulence plasmids with the help of conjugative resistance plasmids.
Conclusions: Due to the different evolutionary mechanisms of hypervirulent and carbapenem-resistant *Klebsiella pneumoniae* with different capsular serotypes, the epidemiological, phenotypic and genotypic characteristics of KL1, KL2 CR-hvKP and KL64, KL47 hv-CRKP are different.

1. Introduction

Klebsiella pneumoniae (KP) is an opportunistic pathogen that can cause a wide range of infections, including pneumonia, liver abscess, urinary tract infections, bacteremia and meningitis.[1] In general, KP can be divided into classical *K. pneumoniae* (cKP) and hypervirulent *K. pneumoniae* (hvKP).[2,3] cKP usually causes nosocomial infection, with low virulence potential, easy to carry drug-resistant plasmids, conferring carbapenem resistance. In China, the most common MLST type of carbapenem-resistant *K. pneumoniae* (CRKP) is ST11.[4–6] HvKP usually causes community-acquired infections and is associated with invasive infections. Meanwhile, hvKP is prone to carry virulence plasmid, which is the main cause of hypermucoviscous and hypervirulent phenotypes.[2,3] For a long time, multidrug resistance and hypervirulence were considered to be two non-overlapping phenotypes, but in recent years, the continuous evolution of hypervirulent or carbapenem-resistant plasmids has led to the emergence of hypervirulent and carbapenem-resistant *K. pneumoniae* (hv-CRKP/CR-hvKP).[7–9] These hv-CRKP/CR-hvKP

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strains often resulted in severe and fatal infections in both hospital settings and the community.[7,10–12]

HvKP and cKP possess their own unique clonal lineages. For example, hvKP is usually associated with KL1 and KL2, while CRKP is usually associated with KL64 and KL47.[13,14] The formation mechanism of hv-CRKP/CR-hvKP can be divided into the following three evolutionary paths: (i) Carbapenem-resistance plasmids were acquired by K1/K2 hypervirulent *K. pneumoniae* (hvKP), known as CR-hvKP.[15] (ii) Carbapenem-resistant *K. pneumoniae* (CRKP) acquired virulence plasmids, recognized as hv-CRKP.[7] (iii) *K. pneumoniae* directly acquired hybrid plasmids encoding carbapenem resistance and hypervirulence, known as hv-CRKP or CR-hvKP.[16–18] The bla_{KPC}-positive plasmids are usually conjugative and can be transmitted horizontally among different strains.[3,19] However, virulence plasmids are generally considered non-conjugative owing to incomplete conjugative transfer-related modules for plasmid conjugation.[20] It seems easier for hvKP to acquire bla_{KPC}-positive plasmids than CRKP acquired virulence plasmids. Furthermore, CR-hvKP should be more widely spread than hv-CRKP. But in fact, hv-CRKP producing KPC carbapenemase was more prevalent than CR-hvKP.[21] Nosocomial outbreaks caused by hv-CRKP were frequently reported,[7,22,23] while those caused by CR-hvKP were rare. In China, the most prevalent type of hv-CRKP was ST11-KL64.[5]

Though the hospital popularity of ST11-KL64 hv-CRKP has been demonstrated, the factors affecting the emergence and prevalence of ST11-KL64 hv-CRKP in hospitals remain unclear. Our study investigated the prevalence and homology of CR-hvKP and hv-CRKP strains collected in a tertiary hospital in Shanghai, China from January 2019 to December 2022. The clinical hazard of these organisms was also discussed. Although KL1, KL2 CR-hvKP and KL64, KL47 hv-CRKP all exhibit overlapping multidrug resistance and hypervirulence phenotypes, the differences in virulence and drug resistance characteristics and plasmid distribution between KL1, KL2 CR-hvKP and KL64, KL47 hv-CRKP remains unclear. In this study, we selected four typical hypervirulence and carbapenem-resistant *K. pneumoniae* from 44 unique (one isolate per patient) hypervirulent and carbapenem-resistant *K. pneumoniae* isolates, including ST23-KL1/ST86-KL2 CR-hvKP (WYKP3 and WYKP194) and ST11-KL64/ST11-KL47 hv-CRKP (WYKP589 and WYKP188), and tried to clarify and compare their differences in virulence and drug resistance characteristics and plasmid distribution.

2. Methods

2.1. Bacterial strains and definitions

To explore characterization difference of typical KL1, KL2, KL47 and KL64 hypervirulent and carbapenem-resistant *Klebsiella pneumoniae*, we collected non-repeated hv-CRKP/CR-hvKP isolates in a tertiary hospital in Shanghai, China from January 2019 to December 2022. We removed duplicate strains from the same patient and only retain the first isolate hv-CRKP/CR-hvKP. Carbapenem resistance *K. pneumoniae*(CRKP) was defined as those resistant to imipenem or meropenem according to the guidelines of the Clinical Laboratory Standards Institute (CLSI, 2022).[24] Hypervirulent *K. pneumoniae*(hvKP) was defined as having a hypermucoviscous phenotype (a positive string test result) and carrying *K. pneumoniae* virulence plasmid-associated loci (*rmpA/rmpA2* and *iutA-iucABCD*).

2.2. Bacterial identification and antimicrobial susceptibility testing

Bacterial identification was performed using VITEK-2 compact automated microbiology analyzer (BioMérieux, France) and MALDI-TOF mass spectrometry (Bruker Daltonics, Billerica, MA, Germany). Antimicrobial susceptibility testing was performed using the broth microdilution method of the Clinical and Laboratory Standards Institute (CLSI). The results were interpreted according to 2022 CLSI breakpoints for all antimicrobial agents except tigecycline,[24] which was interpreted using the interpretative criteria of the Food and Drug Administration (FDA). *Escherichia coli* ATCC 25922 and *Klebsiella pneumoniae* ATCC 700603 were used as quality controls for bacterial identification and antimicrobial susceptibility testing.

2.3. Definition of hypermucoviscous phenotype

The hypermucoviscous phenotype was determined by the string test. A single bacterial colony was inoculated on 5 % sheep blood agar plates and cultured overnight at 37 °C. The bacterial colony was gently pulled up with the inoculation loop and repeated for 3 times. If viscous strings were formed three times and their lengths were longer than 5 mm, the string test was positive.[25]

The hypermucoviscosity was determined using a sedimentation assay.[26] Briefly, bacteria were sub-cultured to an OD600 of 0.2 in LB broth at 37 °C with shaking. The cultures were normalized to OD of 1.0 ml^{-1} and centrifuged at 1,000g for 5 min. The absorbance of supernatants was measured at OD600. Each assay was repeated three times.

2.4. Galleria mellonella larvae infection model

G. mellonella larvae infection model was performed to further assess the virulence potential of the strains. The infection protocol was performed as described in previous study, with minor modifications.[27] KL1 CR-hvKP(WYKP3), KL2 CR-hvKP(WYKP194), KL47 hv-CRKP (WYKP188) and KL64 hv-CRKP(WYKP589) were randomly selected for *G. mellonella* larvae infection model. Phosphate-buffered saline(PBS) was used to adjust the mid-log phase culture to the concentrations of 1×10^6 CFU/mL. *G. mellonella* larvae were purchased from Tianjin Huiyude Biotechnology Co., Ltd. (Tianjin, China) weighing about 250–300 mg. A group of ten *G. mellonella* larvae was infected with 10 μl of PBS and other groups were injected with 10 μl of bacterial suspension. The inoculated *G. mellonella* larvae were placed in an incubator at 37 °C for 72 h, and the survival of the larvae in each group was recorded every 4 h. The positive control was the ST23 K1 hvKP strain NTUH-K2044, and the negative control was phosphate-buffered saline (PBS). Each assay was repeated three times. Survival curves were generated according to the survival conditions of the larvae using the GraphPad Prism 8 software.

2.5. Serum resistance assay

The serum resistance assay was performed to determine in vitro virulence. The serum resistance assay was performed as described in previous study, with minor modifications.[26] The mid-log phase bacterial suspensions containing 1×10^6 CFU/ml were mixed with normal human serum at a 1:9 ratios. The mixture was then incubated at 37 °C and 300 rpm for 3 h, and colony counts were performed at 0, 1, 2, and 3 h using the serial dilution method. Each experiment was performed in triplicates.

2.6. Biofilm formation assay

Biofilm formation assay was performed as described in previous study, with minor modifications.[28] Briefly, 200 μl of a mid-log phase bacterial suspension (2×10^7 CFU/ml) was added to 96-well microtiter plates and incubated overnight at 37 °C. Next, the cultures were washed with phosphate-buffered saline and dried. The microtiter plate was stained with 200 μl of 0.2 % crystal violet solution for 30 min, then washed and dried. The biofilm formation was quantified by measuring the absorbance (OD595) after solubilized with 200 μl of 95 % ethanol. Each assay was repeated three times.

2.7. Quantitative siderophores production assay

The chrome azurol S (CAS) assay was performed as previously

described to determine the siderophore production of bacterial supernatants.[29] Briefly, the stationary-phase iron-chelated cultures (10 μl) were dropped on CAS and King's B (2:1) plates. After incubation for 48h at 37 °C, siderophore production was determined by the presence of the orange halos around the bacterial colonies. Each assay was repeated three times.

2.8. Whole genome sequencing and bioinformatics analysis

The genomic DNA of these strains was extracted using a QIAamp DNA mini kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. Fragmented genomic DNA was sequenced using the 150-bp paired-end Illumina NovaSeq platform(Illumina, San Diego, CA, United States). Furthermore, the genomic DNA of four representative isolates with different capsular serotypes(WYKP3, WYKP194, WYKP188 and WYKP594) was sequenced on the long-read Oxford Nanopore MinION platform (Nanopore Technologies, Oxford, United Kingdom) following a 10-Kbp library protocol. A hybrid assembly was generated by using Unicycler version 0.4.8 with short and long reads.[30] Finally, the genome sequence was acquired after the rectification by using pilon version 1.22. Gene annotation was performed using the Prokka version 1.12.[31]

Multilocus sequence typing (MLST), capsule serotypes and virulence genes were obtained by BIGSdb-Pasteur(https://bigsdb.pasteur.fr/cgi-bin/bigsdb/bigsdb.pl?db=pubmlst_klebsiella_seqdef&page=sequenceQuery). The acquired antibiotic resistance genes were identified using ResFinder (http://genepi.food.dtu.dk/resfinder) with the default threshold. Plasmid replicons and conjugative transfer-related modules of plasmids were predicted using VRProfile2.[32] Virulence score was identified in the Pathogenwatch platform using Kleborate (https://pathogen.watch/). Core genome MLST(cgMLST) analysis was performed using SeqSphere + version 9.0.8 (Ridom GmbH, Muenster, Germany).[33] The alignment of plasmids was visualized using the BLAST Ring Image Generator (BRIG) version 0.95.[34]

2.9. Phylogenetic analysis

We used single-nucleotide polymorphisms(SNPs) to investigate the transmission of *K. pneumoniae* with different capsular serotypes based on genome sequences mining. The phylogenetic analysis included 44 *K. pneumoniae* genomes from this study and 120 *K. pneumoniae* genomes with different capsular serotypes obtained from National Center for Biotechnology Information (NCBI). These 164 genome assemblies were subjected to removing recombination regions using Gubbins (https://github.com/nickjcroucher/gubbins) and thereafter to calling core SNPs with Snippy (https://github.com/nickjcroucher/gubbins) using strain WYKP58 (accession no. JBHZGP000000000) as the reference. We subsequently visualized and annotated a phylogenetic tree using Interactive Tree Of Life (iTOL) (https://itol.embl.de/).

2.10. Determining plasmid mobility

Plasmid mobility can be divided into conjugative, mobilizable, and non-mobilizable.[35] Plasmids are considered to be conjugative in the presence of four essential modules (oriT, relaxase, T4CP, and T4SS). Plasmids are considered mobilizable when oriT is present, but relaxase, type IV coupling protein [T4CP], and type IV secretion system [T4SS] are not present simultaneously. Plasmids are considered non-mobilizable when oriT is not present.

2.11. Statistical analysis

Data are presented as the mean ± standard deviation. Student's t-test or analysis of variance (ANOVA) was used to calculate significance. $P < 0.05$ was considered statistically significant. All statistical analyses were implemented in GraphPad Prism 7 (GraphPad Software, San Diego, CA).

3. Results

3.1. Clinical characteristics of 44 patients infected with hv-CRKP/CR-hvKP isolates

Forty-four isolates of hv-CRKP/CR-hvKP were screened out through bacterial identification, antimicrobial susceptibility testing, string test and virulence-associated gene detection. Clinical characteristics of 44 patients infected with hv-CRKP/CR-hvKP isolates are summarized in Table S1. The 44 hv-CRKP/CR-hvKP strains were isolated from 44 individual patients (32 male and 12 female) whose mean age was 79.3 ± 11.8 years. They came from 11 wards, more than half from the central ICU (25 cases). Among the 44 hv-CRKP isolates, a variety of clinical specimens were involved, including sputum (25/44, 56.8 %), urine (13/44, 29.6 %), and other specimens (6/44, 13.6 %). Underlying conditions included stroke (70.5 %, 31/44), hypertension (59.1 %, 26/44), coronary heart disease (27.3 %, 12/44), etc. Invasive procedures included tracheal intubation (63.6 %, 28/44), urinary catheter (27.3 %, 12/44), etc. Twenty-one of the 44 patients died(47.7 %, 21/44), and twenty-three patients improved and were discharged.

3.2. Antimicrobial resistance phenotype of the hv-CRKP/CR-hvKP isolates

The detailed antimicrobial resistance profile of the hv-CRKP/CR-hvKP isolates to 18 antibiotics was listed in Table 1. These 44 isolates of hv-CRKP/CR-hvKP exhibited multiple drug resistance(MDR) phenotype showing resistant to three or more antibiotic classes. All isolates were resistant to ampicillin, ampicillin/sulbactam, cefoperazone/sulbactam, piperacillin/tazobactam, cefazolin, cefuroxime, ceftazidime, ceftriaxone, imipenem, meropenem. The antimicrobial resistance rate of ceftazidime/Avibactam was the lowest (2.3 %), followed by tigecycline (4.5 %), trimethoprim-sulfamethoxazole (47.7 %), amikacin (75 %), gentamicin (77.3 %) and levofloxacin (93.2 %), cefoxitin (97.7 %), cefepime (97.7 %).

3.3. The distribution of antibiotic resistance genes, virulence genes, MLST and capsular serotypes

To gain a deeper understanding of molecular epidemiological characteristics of these strains, 44 hv-CRKP/CR-hvKP strains underwent whole-genome sequencing. MLST analysis revealed that the 44 hv-CRKP/CR-hvKP belonged to 5 different ST, ST11 was the most predominant ST (90.8 %, 40/44), followed by ST23 (2.3 %, 1/44), ST86

Table 1
Antibiotic Susceptibilities of 44 hv-CRKP and CR-hvKP.

Antibiotics	Sensitivity (%)	Intermediate (%)	Resistance (%)
Ampicillin	0	0	100
Ampicillin/sulbactam	0	0	100
Cefoperazone/sulbactam	0	0	100
Piperacillin/tazobactam	0	0	100
Cefazolin	0	0	100
Cefuroxime	0	0	100
Ceftazidime	0	0	100
Ceftriaxone	0	0	100
Cefepime	0	2.3	97.7
Cefoxitin	2.3	0	97.7
Imipenem	0	0	100
Meropenem	0	0	100
Amikacin	25	0	75
Gentamicin	22.7	0	77.3
Levofloxacin	6.8	0	93.2
Trimethoprim-sulfamethoxazole	52.3	0	47.7
Ceftazidime/Avibactam	97.7	0	2.3
Tigecycline	95.5	0	4.5

(2.3 %, 1/44), ST15 (2.3 %, 1/44) and ST828 (2.3 %, 1/44)(Fig. 1). Capsular serotypes analysis revealed four different K loci, KL64 was the most predominant serotype (77.3 %, 34/44), followed by KL47 (13.5 %, 6/44), KL1 (2.3 %, 1/44), KL2 (2.3 %, 1/44), KL5 (2.3 %, 1/44) and KL112 (2.3 %, 1/44). These 40 ST11 strains contained the KL64 capsular serotype(34 strains) and KL47 capsular serotype(6 strains). This suggests that ST11-KL64 hv-CRKP has emerged as the most prevalent hv-CRKP and may contribute to hospital outbreaks of infection.

The presence of carbapenemase genes is concerning, as it can cause resistance to many types of antibiotics, including carbapenems. All hv-CRKP/CR-hvKP strains carried the bla_{KPC} gene, bla_{KPC-2} was dominant (97.7 %, 43/44), and bla_{KPC-33} was rare (2.3 %, 1/44). Different ST or capsular serotype of hv-CRKP/CR-hvKP strains had different antibiotic resistance gene profiles, and ST23-KL1 and ST86-KL2 CR-hvKP strains had fewer antibiotic resistance genes than ST11 strains. Additionally, a significant proportion of strains carried aminoglycoside resistance genes and beta-lactamase genes.

The hypervirulence of hv-CRKP/CR-hvKP is due to its unique characteristics, including serum resistance, adherence, capsule, secretion systems, lipopolysaccharide, and siderophores. All isolates in the study carried virulence genes *iutA-iucABCD* and *rmpA/rmpA2*, which are critical genetic biomarkers contributing to the high pathogenicity of hvKP. The virulence gene *iroB* was only detected in ST23-KL1, ST86-KL2 and ST828-KL5 CR-hvKP strains, which contributed to the higher virulence of these strains. All hv-CRKP/CR-hvKP strains had a virulence score of 4, with the exception of ST23-KL1 CR-hvKP, which scored 5. The combination of these virulence genes varied among different ST type or capsular serotype, leading to different hypervirulence phenotypes.

This result suggests that strains with more resistance may have less virulence potential.

3.4. Phylogenetic analysis of hv-CRKP isolates

To gain a deeper understanding of the homology of these hv-CRKP/CR-hvKP isolates, we conducted whole-genome sequencing and core

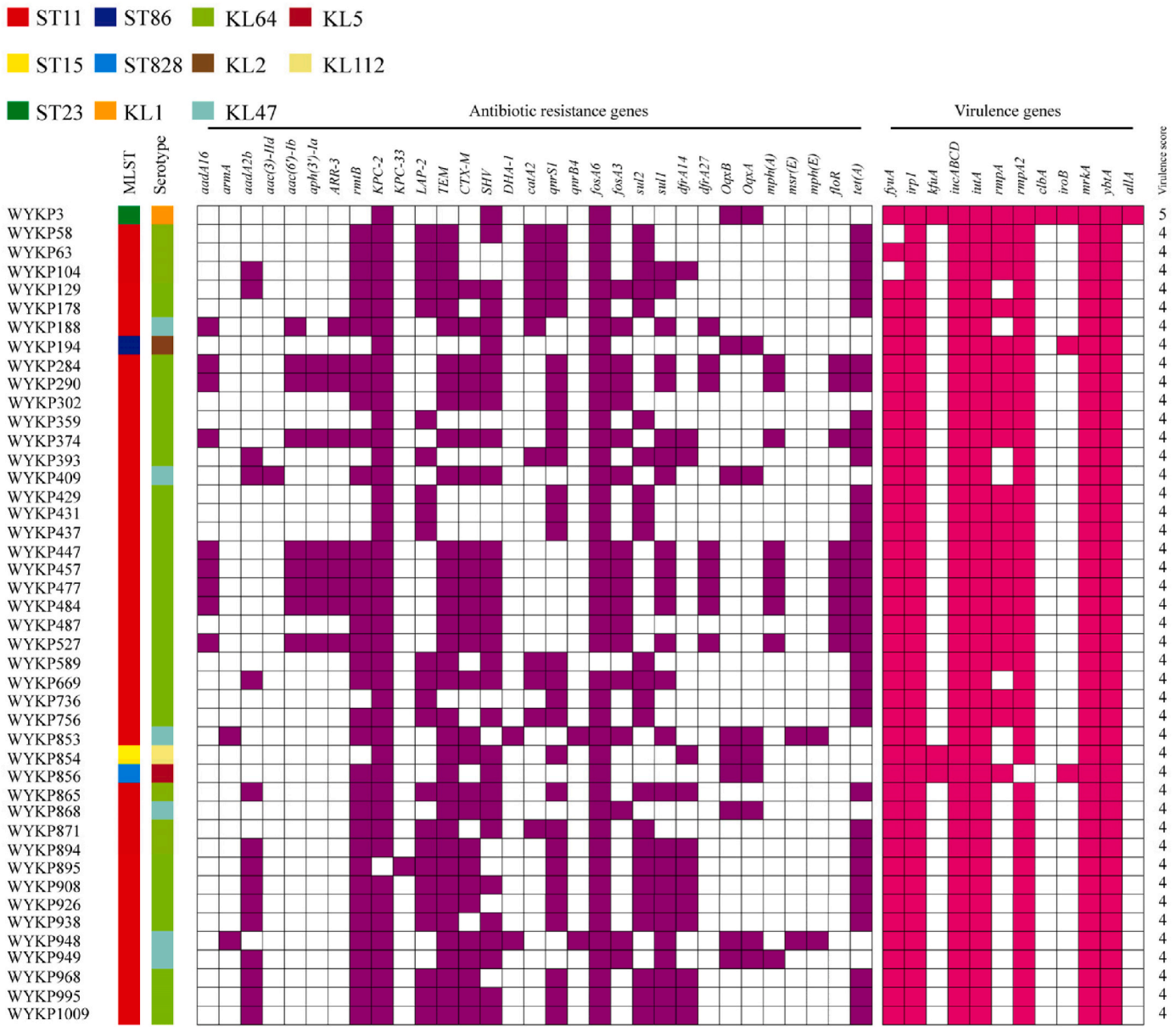

Fig. 1. Virulence genes, antibiotic resistance genes, capsular serotype and MLST of 44 hv-CRKP. The different MLST and capsular serotype were color-coded and illustrated at the tips. The occurrence of antibiotic resistance genes and virulence genes were also color-coded.

genome MLST(cgMLST) analysis (Fig. 2). The results showed that the ST11 hv-CRKP strains dominated and formed five single cluster. These ST11 hv-CRKP strains could be divided into two capsular serotypes, KL64 and KL47. These ST11-KL64 hv-CRKP strains contained three single clusters: cluster1(15 strains), cluster2(10 strains), and cluster3(9 strains). While ST11-KL47 hv-CRKP strains had two single clusters, cluster4(3 strains) and cluster5(2 strains).

This suggests that ST11-KL64 hv-CRKP has emerged as the most prevalent hypervirulent and carbapenem-resistant *K. pneumoniae*, which may lead to its clonal expansion within the hospital.

To investigate the transmission of *K. pneumoniae* with different capsular serotypes, we conducted single-nucleotide polymorphisms (SNPs) based phylogenetic tree. The phylogenetic analysis included 44 *K. pneumoniae* genomes from this study and 120 *K. pneumoniae* genomes with different capsular serotypes obtained from NCBI (Table S2). A total of 120 *K. pneumoniae* (KL1, KL2, KL47 and KL64) from different countries and different isolation times were selected as reference strains.

As shown in Fig. 3, the resulting phylogenetic tree had three main branches. ST23-KL1 and ST86-KL2 CR-hvKP occupy two main branches respectively, while ST11-KL64, ST11-KL47 hv-CRKP occupy the same main branches. Clusters of SNPs based phylogenetic trees were similar to those of Minimum-spanning trees of cgMLST. The results showed that ST11 hv-CRKP strains formed 5 single clusters, among which ST11-KL64 hv-CRKP strains formed 3 single clusters and ST11-KL47 hv-CRKP strains formed 2 single clusters. Although in the same main branches, *K. pneumoniae* with the same capsular serotype have obvious genetic distance if they come from different countries or have different isolation times. For the 44 strains in this study, *K. pneumoniae* with the same capsule serotype had closer genetic distance if the strains were isolated in the same year.

3.5. Virulence and antimicrobial resistance phenotypes of ST11-KL64, ST11-KL47 hv-CRKP and ST23-KL1, ST86-KL2 CR-hvKP

In this study, we selected four typical hypervirulence and carbapenem-resistant *K. pneumoniae* from 44 unique (one isolate per patient) hypervirulent and carbapenem-resistant *K. pneumoniae* isolates, including ST23-KL1/ST86-KL2 CR-hvKP (WYKP3 and WYKP194) and ST11-KL64/ST11-KL47 hv-CRKP (WYKP589 and WYKP188), and tried to clarify and compare their virulent and drug resistant features. The clinical hazard of these strains was also discussed.

K. pneumoniae strain WYKP3 (ST23-KL1 CR-hvKP), WYKP194 (ST86-KL2 CR-hvKP) and WYKP589 (ST11-KL64 hv-CRKP) were isolated from elderly patients with serious underlying diseases and eventually died. *K. pneumoniae* strain WYKP188 (ST11-KL47 hv-CRKP) was also isolated from elderly patients with serious underlying diseases but discharged with a good prognosis.

The sedimentation assay showed that compared with ST23-KL1 (WYKP3) and ST86-KL2 (WYKP194) strains, the mucoviscosity was lower in ST11-KL64 (WYKP589) and ST11-KL47 (WYKP188). Meanwhile, the mucoviscosity of strain WYKP589 was higher than that of strain WYKP188 (Fig. 4A). We further evaluated the virulence potential of these strains using biofilm formation and serum resistance assay. Compared with ST23-KL1 (WYKP3) and ST86-KL2 (WYKP194) strains, biofilm formation and serum resistance of the strains was reduced in ST11-KL64 (WYKP589) and ST11-KL47 (WYKP188) (Fig. 4B and D). Quantitative siderophores production assay showed that WYKP3 produced the most siderophore, followed by WYKP194, WYKP589 and WYKP188 (Fig. 4C). Moreover, we estimated the virulence potential of these strains by infecting *G. mellonella* larvae with an inoculum of 1×10^6 CFU. *G. mellonella* larvae infection model indicated that ST23-KL1 (WYKP3) and ST86-KL2 (WYKP194) strains showed comparable virulence resulting in 0 % survival, while the survival rates for the ST11-

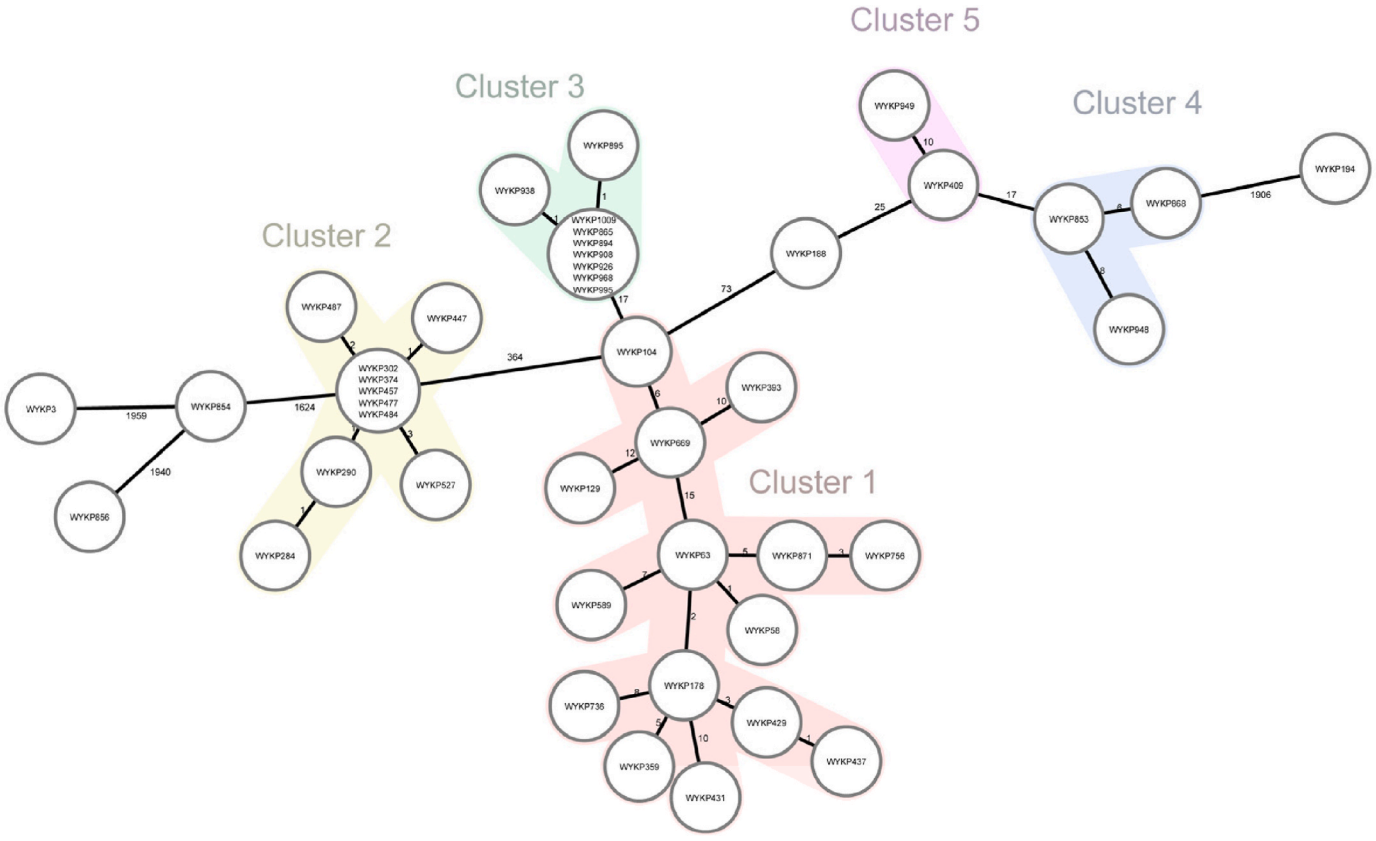

Fig. 2. Minimum-spanning tree of cgMLST profiles among 44 hv-CRKP. The minimum-spanning tree was generated based on cgMLST analysis with 2358 conserved genome-wide genes. A cluster was defined at a distance of ≤15 alleles.

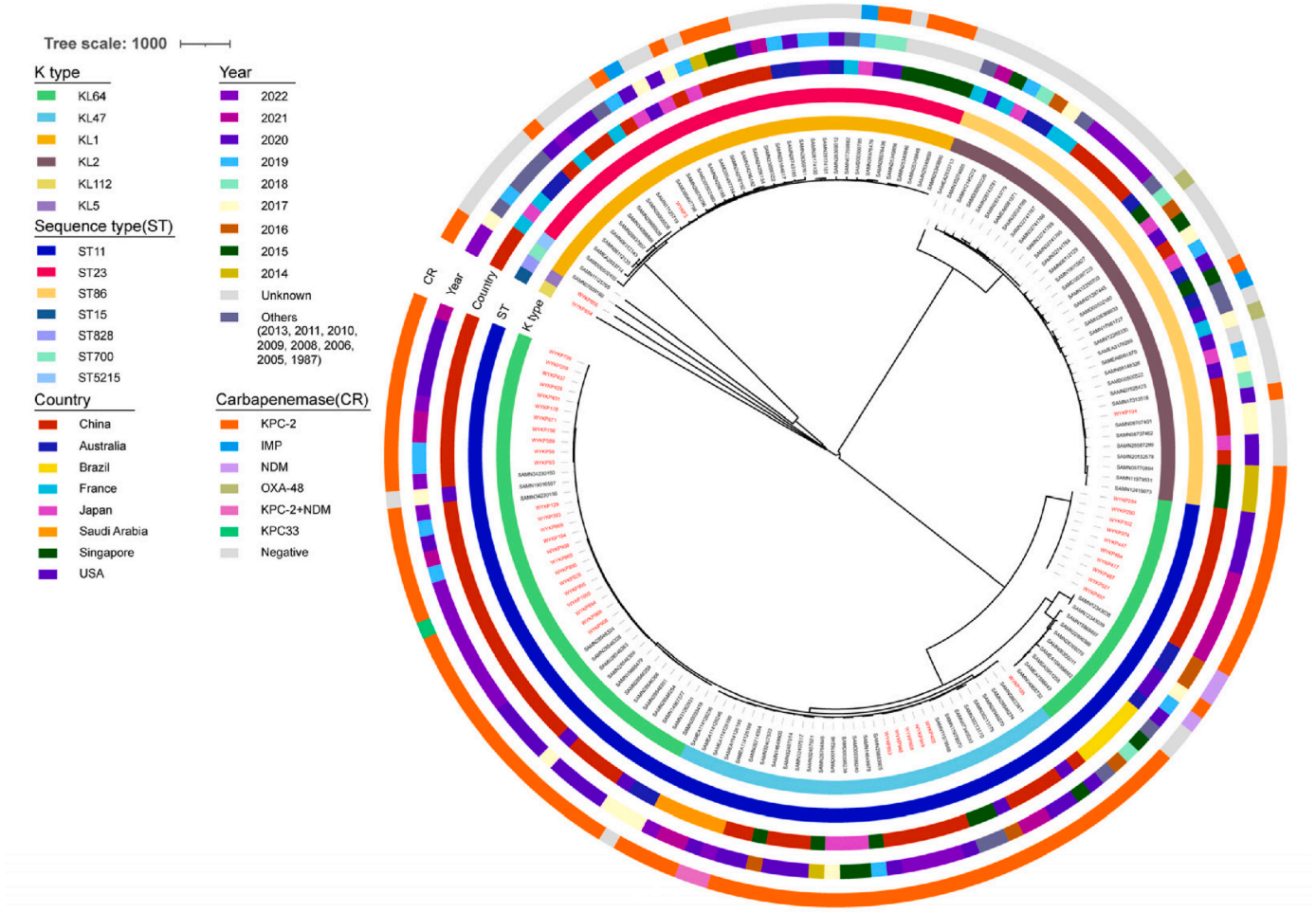

Fig. 3. Phylogenetic analysis of 44 hv-CRKP strains and 120 reference strains. Maximum likelihood phylogenetic tree constructed by core genes of 164 *K. pneumoniae* isolates. The circles from the inner to the outer represent K type, MLST, country, year, and carbapenemase, respectively. The 44 local *K. pneumoniae* strains were marked in red.

KL64 (WYKP589) and ST11-KL47 (WYKP188) strains are 20 % and 40 %, respectively (Fig. 4E).

The results of the antimicrobial susceptibility testing indicated that ST11-KL64, ST11-KL47 hv-CRKP, and ST23-KL1, ST86-KL2 CR-hvKP were all resistant to carbapenems (Table 2). However, it is noteworthy that the resistance levels of ST23-KL1 and ST86-KL2 CR-hvKP (MIC = 8) to imipenem and meropenem are much lower than those of ST11-KL64 and ST11-KL47 hv-CRKP (MIC = 128). ST23-KL1 and ST86-KL2 CR-hvKP were still sensitive to aminoglycosides (Amikacin, Gentamicin) and quinolones (Levofloxacin), while ST11-KL64 and ST11-KL47 hv-CRKP were resistant to these two classes of drugs.

In a word, ST11-KL64 and ST11-KL47 hv-CRKP were more resistant but less virulent than ST23-KL1 and ST86-KL2 CR-hvKP.

3.6. Comparative analysis of virulence and resistance plasmids in ST11-KL64, ST11-KL47 hv-CRKP and ST23-KL1, ST86-KL2 CR-hvKP

ST23-KL1, ST86-KL2 CR-hvKP carried only two plasmids (including the virulence plasmid and KPC resistance plasmid), while ST11-KL64, ST11-KL47 hv-CRKP carried five plasmids (including the virulence plasmid, KPC resistance plasmid and other resistance plasmids) (Table 3). The total size of plasmids of ST11-KL64 and ST11-KL47 hv-CRKP was larger than that of ST23-KL1 and ST86-KL2 CR-hvKP to carry more resistance genes (Table 3). Our data clearly showed that ST23-KL1, ST86-KL2 CR-hvKP exhibited less plasmid diversity than that of ST11-KL64, ST11-KL47 hv-CRKP.

The mobility of virulence plasmids (pWYKP3-1, pWYKP194-1, pWYKP589-1 and pWYKP188-2) carried by ST23-KL1, ST86-KL2 CR-hvKP and ST11-KL64, ST11-KL47 hv-CRKP were all mobilizable (Table 3). The mobility of KPC resistance plasmids (pWYKP3-2, pWYKP194-2 and pWYKP589-2) carried by ST23-KL1, ST86-KL2 CR-hvKP and ST11-KL64 hv-CRKP were all non-mobilizable, while the KPC resistance plasmid (pWYKP188-1) carried by ST11-KL47 hv-CRKP was conjugative (Table 3). Other resistance plasmids (pWYKP589-3, pWYKP188-3) carried by ST11-KL64 and ST11-KL47 hv-CRKP were also conjugative.

Comparing the virulence plasmids of four different isolates, we found that compared with plasmids pWYKP3-1 and pWYKP194-1, plasmids pWYKP589-1 and pWYKP188-2 lost virulence genes that mediated salmochelin (*iro*) [Fig. 5B, Table 3]. We also compared bla_{KPC-2} resistance plasmids from four different isolates and found that compared with plasmids pWYKP589-2 and pWYKP188-1, plasmids pWYKP3-2 and pWYKP194-2 lacked aminoglycoside resistance genes and quinolone resistance genes (Fig. 5A–Table 3).

4. Discussion

In recent years, more and more *K. pneumoniae* are characterized by both hypervirulence and multidrug resistance.[7,8] Zhou K et al. showed that ST11-K47 hv-CRKP was gradually replaced by ST11-KL64 hv-CRKP after 2016 in China, and ST11-KL64 hv-CRKP led to higher mortality in infected patients.[36] Many studies showed that the ST11-KL64 hv-CRKP

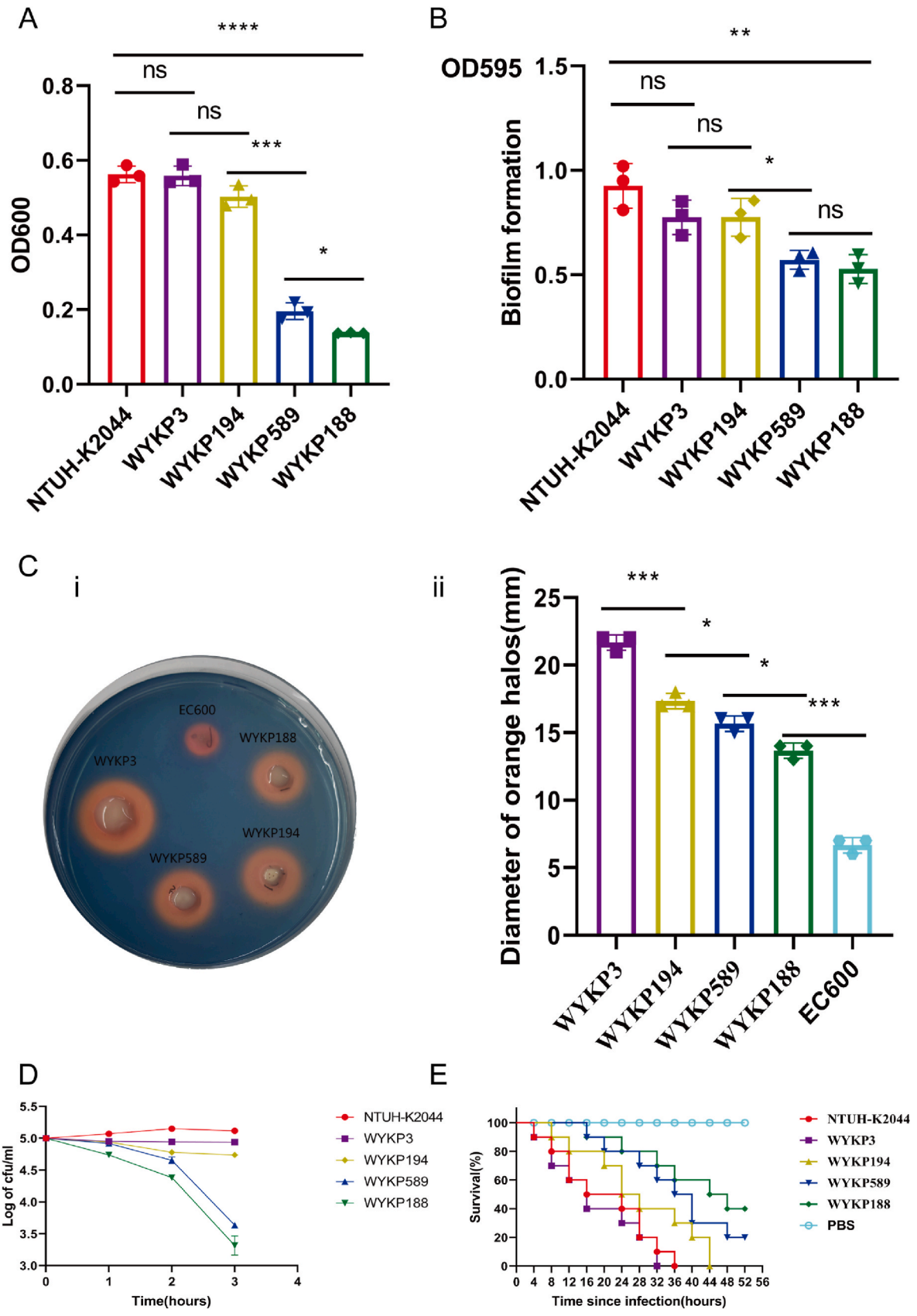

Fig. 4. The virulence phenotypes and levels of ST11-KL64, ST11-KL47 hv-CRKP and ST23-KL1, ST86-KL2 CR-hvKP. (A) Mucoviscosity. (B) Biofilm formation. (C) Siderophores production determined by CAS agar plate. (D) Serum resistance. (E) The survival curves of infected *G. mellonella*. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, ns: not significant.

Table 2
Antibiotic Susceptibilities of ST11-KL64, ST11-KL47 hv-CRKP and ST23-KL1, ST86-KL2 CR-hvKP.

Antibiotics	MIC (μg/mL)/antibiotic susceptibility			
	WYKP3	WYKP194	WYKP589	WYKP188
Ampicillin	≥32/R	≥32/R	≥32/R	≥32/R
Ampicillin/sulbactam	≥32/R	≥32/R	≥32/R	≥32/R
Cefoperazone/sulbactam	≥64/R	≥64/R	≥64/R	≥64/R
Piperacillin/tazobactam	≥128/R	≥128/R	≥128/R	≥128/R
Cefazolin	≥64/R	≥64/R	≥64/R	≥64/R
Cefuroxime	≥64/R	≥64/R	≥64/R	≥64/R
Ceftazidime	32/R	32/R	≥64/R	≥64/R
Ceftriaxone	≥64/R	≥64/R	≥64/R	≥64/R
Cefepime	8/SDD	≥32/R	≥32/R	≥32/R
Cefoxitin	8/S	≥64/R	≥64/R	≥64/R
Imipenem	8/R	8/R	128/R	128/R
Meropenem	8/R	8/R	128/R	128/R
Amikacin	≤2/S	≤2/S	≥64/R	≥64/R
Gentamicin	≤1/S	≤1/S	≥16/R	≥16/R
Levofloxacin	≤0.12/S	1/I	≥8/R	≥8/R
Trimethoprim-sulfamethoxazole	≤1/S	≤1/S	≤1/S	≥16/R
Ceftazidime/Avibactam	≤0.25/S	≤0.25/S	≤0.25/S	≤0.25/S
Tigecycline	0.5/S	0.5/S	1/S	1/S

Abbreviations: MIC, minimal inhibitory concentration; S, Sensitivity; I, Intermediate; R, Resistance; SDD, susceptible dose-dependent.

is the most prevalent clone among all its hypervirulent and carbapenem-resistant *K. pneumoniae*.[5,37,38] However, the factors affecting the emergence and prevalence of ST11-KL64 hv-CRKP in hospitals remain unclear. This study investigated the prevalence and homology of CR-hvKP and hv-CRKP isolated from a tertiary hospital in Shanghai. Additionally, the differences in virulence, drug resistance characteristics and plasmid distribution of different capsular serotypes of hv-CRKP/CR-hvKP are still unclear. In this study, four typical CR-hvKP/hv-CRKP were selected, including ST23-KL1/ST86-KL2 CR-hvKP (WYKP3 and WYKP194) and ST11-KL64/ST11-KL47 hv-CRKP (WYKP589 and WYKP188), and tried to clarify and compare their differences in virulence characteristics, drug resistance characteristics and plasmid distribution.

In this study, all 44 patients infected with hypervirulent and carbapenem-resistant *K. pneumoniae* underwent invasive procedures, of which tracheal intubation was the most common(63.6 %). Some studies have reported ventilator-associated pneumonia caused by hv-CRKP, suggesting that invasive procedures, especially tracheal intubation, may increase the risk of CR-hvKP infection.[7,39] Meanwhile, all 44 patients infected with hv-CRKP had underlying conditions, and stroke was the most common one, which was similar to the results of previous studies.[25,40] This phenomenon may be due to the low immunity of stroke patients, resulting in pathogens lurking in the body for a long time, and can only rely on the bactericidal effect of antibiotics for a long time, which will lead to multi-drug resistance and hypervirulence in the long run. Among the 44 patients, 21 patients died and 23 patients were improved and discharged, with a mortality rate of 47.7 % (21/44), suggesting that these 44 isolates could cause substantial mortality. The mortality rate observed in this study exceeded that reported in previous studies[23,41] but lower than that reported in others.[7,25,42]

Compared with ST11-KL64/ST11-KL47 hv-CRKP, ST23-KL1/ST86-KL2 CR-hvKP harbor significantly fewer antimicrobial resistance genes and exhibited resistance to a less number of antibiotics. The ST23-KL1/ST86-KL2 CR-hvKP strains would be killed by aminoglycosides or quinolones, due to the absence of corresponding antimicrobial resistance genes. The reason for this may be that ST23-KL1/ST86-KL2 hvKP impede the acquisition of additional plasmids due to their hypermucoviscous or lack the ability to maintain the plasmids obtained.[12] However, the ST11-KL64/ST11-KL47 hv-CRKP may be due to the lower mucosity, which results in easy access to multiple plasmids with lower fitness cost.[43,44] Once these CRKP lineages obtained the pLVPK-like virulence plasmids with the help of conjugative plasmids to generate hv-CRKP isolates, they can easily disseminate and cause a hospital outbreak. Notably, though the bla_{KPC-2} gene could be detected in all four representative isolates, the resistance levels of ST23-KL1 and ST86-KL2

Table 3
Genomic characteristics of the plasmids in WYKP3, WYKP194, WYKP589, and WYKP188.

Plasmid name	Genome size (bp)	Plasmid replicons	Mobial ability	Resistance genes	Virulence genes
pWYKP3-1	221446	IncHI1B, repB	Mobilizable (oriT, T4CP)	n	*iroB/iroC/iroD/iroN/rmpA/iucABCD/iutA/rmpA2*
pWYKP3-2	95973	n	Non-Mobilizable (T4SS, Relaxase)	bla_{KPC-2}	n
pWYKP194-1	219759	IncHI1B, repB	Mobilizable (oriT, T4CP)	n	*iucABCD/iutA/rmpA2/rmpA/iroN/iroD/iroC/iroB*
pWYKP194-2	94884	n	Non-Mobilizable (T4SS, Relaxase)	bla_{KPC-2}	n
pWYKP589-1	217869	IncHI1B, repB	Mobilizable (oriT, T4CP)	n	*rmpA2/rmpA/iucABCD/iutA/iroN*
pWYKP589-2	110599	IncFII, IncR	Non-mobilizable (T4SS)	bla_{SHV-12}/bla_{KPC-2}/*rmtB*/bla_{TEM-1B}	n
pWYKP589-3	83551	n	Conjugative (oriT, T4CP, T4SS, Relaxase)	*CatA2/sul2/tet(A)*/bla_{LAP-2}/*qnrS1*	n
pWYKP589-4	11970	CoIRNAI	Non-mobilizable (Relaxase)	n	n
pWYKP589-5	5596	CoIRNAI	Mobilizable (oriT, Relaxase)	n	n
pWYKP188-1	169249	IncFII, IncR	Conjugative (oriT, T4CP, T4SS, Relaxase)	bla_{KPC-2}/*rmtB*/bla_{TEM-1B}/$bla_{CTX-M-65}$/*fosA3/catA2*	n
pWYKP188-2	145681	IncHI1B, repB	Mobilizable (oriT, T4CP)	n	*rmpA2/iutA/iucABCD*
pWYKP188-3	96416	IncI1-I	Conjugative (oriT, T4CP, T4SS, Relaxase)	*aac(6′)-Ib-cr/ARR-3/dfrA27/aadA16/qacE/sul1*/$bla_{CTX-M-14b}$	n
pWYKP188-4	11970	ColRNAI	Non-Mobilizable (T4CP, Relaxase)	n	n
pWYKP188-5	5596	ColRNAI	Mobilizable (oriT, Relaxase)	n	n

Note: n, nonexistent information.

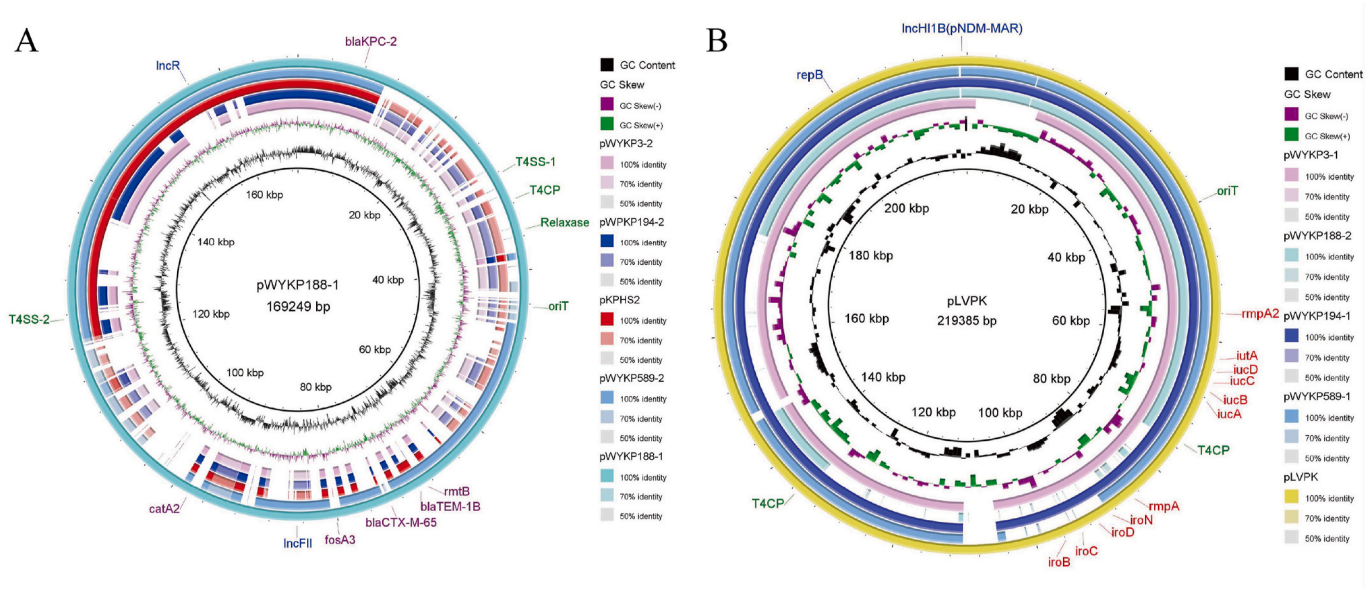

Fig. 5. Genomic features of virulence and resistance plasmids in ST11-KL64 hv-CRKP and KL1, KL2 CR-hvKP. (A) Alignment of plasmids pWYKP3-2, pWYKP194-2, pWYKP589-2, pWYKP188-1 and pKPHS2 (CP003224.1). pWYKP188-1 was used as the reference plasmid. (B) Alignment of plasmids pWYKP3-1, pWYKP194-1, pWYKP589-1, pWYKP188-2 and pLVPK (AY378100). pLVPK was used as the reference plasmid. The circular map of plasmids was generated with BRIG. Antibiotic resistance genes are shown in purple. Virulence genes are shown in red.

CR-hvKP (MIC = 8) to imipenem and meropenem are much lower than those of ST11-KL64 and ST11-KL47 hv-CRKP (MIC = 128). Previous studies have shown that KL1/KL2 hvKP with hypermucoviscosity did not exhibit corresponding high-level resistance to carbapenems after acquiring the bla_{KPC} plasmid,[45–47] which was similar to the results in this study. The hypermucoviscosity of strains may be related to the level of carbapenem resistance. Some studies showed that the loss of hypermucoviscosity of strains was accompanied by the increase of drug resistance, including carbapenems.[46,47] In summary, although KL1, KL2 CR-hvKP and KL64, KL47 hv-CRKP all exhibit multidrug resistance and carbapenem resistance phenotypes, KL1 and KL2 CR-hvKP were resistant to fewer drugs and showed lower levels of carbapenem resistance.

ST23-KL1/ST86-KL2 CR-hvKP harbored significantly fewer antimicrobial resistance genes but more virulence genes, which contributed to the higher virulence of these strains. According to the virulence phenotype results, ST23-KL1/ST86-KL2 CR-hvKP had a stronger virulence level than ST11-KL64/ST11-KL47 hv-CRKP, and ST11-KL64 hv-CRKP was slightly stronger than ST11-KL47 hv-CRKP. The reason for this phenomenon may be that ST23-KL1/ST86-KL2 CR-hvKP has a more complete virulence plasmid than ST11-KL64/ST11-KL47 hv-CRKP. However, some studies have shown that though ST11-KL64 hv-CRKP strains would lose several virulence genes for the fitness cost, the virulence phenotype was not significantly affected.[45,48] In this study, the results showed that ST11-KL64 hv-CRKP has emerged as the most prevalent hv-CRKP, which was similar to several studies.[5,37,38] Phylogenetic Analysis showed that these ST11-KL64 hv-CRKP strains contained three single clusters: cluster1(15 strains), cluster2(10 strains), and cluster3(9 strains). Our previous research also showed that the ST11 hv-CRKP is the most prevalent hypervirulent and carbapenem-resistant *K. pneumoniae* in our hospital.[25] This suggests that clonal spread may be one of the reasons why ST11-KL64 hv-CRKP is the most prevalent hypervirulent and carbapenem-resistant *K. pneumoniae*. Due to the perfect balance between drug resistance and virulence of ST11-KL64 hv-CRKP, with high resistance and virulence, and strong resistance to killing, this is also one of the reasons why ST11-KL64 hv-CRKP is the most prevalent hypervirulent and carbapenem-resistant *K. pneumoniae*.

Our data clearly show that ST23-KL1, ST86-KL2 CR-hvKP exhibited less plasmid diversity than that of ST11-KL64, ST11-KL47 hv-CRKP. We predict that CRKP are more likely to acquire virulence plasmids than hvKP in acquiring resistance plasmids. It is possible that the hypermucoviscosity of the strains hinder the frequent exchange resistance plasmids with other bacteria. Wyres et al. showed that hvKP may be subject to some sort of limitation for gene acquisition and shows considerably lower gene content diversity than CRKP clones.[14] Some studies showed that when the hypermucoviscosity of bacterial strains decreased or was lost, the horizontal transmission of plasmids between strains became easier.[26,47,49] In this study, the virulence plasmids (pWYKP3-1, pWYKP194-1, pWYKP589-1, and pWYKP188-2) carried by ST23-KL1, ST86-KL2 CR-hvKP and ST11-KL64, ST11-KL47 hv-CRKP were classified as mobilizable plasmids, which contained two essential modules (oriT and T4CP). Although the mobilizable virulence plasmid (containing oriT) cannot be transmitted between bacteria independently, it has been reported that it can be transferred into CRKP with the help of conjugative resistance plasmid, resulting in the formation of hv-CRKP.[45,46] The bla_{KPC}-positive plasmids are usually conjugative and can be transmitted horizontally among different strains.[50,51] Although KL1/KL2 hvKP strains seemed easier to acquire the bla_{KPC} plasmid, the co-existence of virulence plasmid and conjugative resistance plasmid facilitated the formation of ST11-KL64/ST11-KL47 hv-CPKP. This may also be one of the reasons why ST11-KL64 hv-CRKP is the most prevalent hypervirulent and carbapenem-resistant *K. pneumoniae*.

The most limitation of our study was that there was only one isolate in each KL type used in this study, which lacked a little representativeness. More *K. pneumoniae* with different capsular serotypes will be selected for comparison in the future to make our conclusion more convince.

In summary, our study found that ST23-KL1, ST86-KL2 CR-hvKP exhibited less plasmid diversity than that of ST11-KL64, ST11-KL47 hv-CRKP. Compared with ST11-KL64 and ST11-KL47 hv-CRKP, ST23-KL1/ST86-KL2 CR-hvKP harbored significantly fewer antimicrobial resistance genes but more virulence genes, which contributed to the higher virulence of these strains and exhibited resistance to fewer antibiotics. ST11-KL64 hv-CRKP has emerged as the most prevalent hypervirulent and carbapenem-resistant *K. pneumoniae* probably due to its clonal transmission within hospitals as well as the transmission of virulence plasmids with the help of conjugative resistance plasmids.

CRediT authorship contribution statement

Cong Zhou: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Wencai Ke:** Validation, Supervision, Resources, Methodology, Investigation, Formal analysis. **Hui Zhang:** Validation, Software, Investigation, Data curation. **Maosuo Xu:** Software, Methodology, Investigation, Data curation. **Baoyu Yuan:** Validation, Software, Methodology. **Yong Lin:** Supervision, Resources, Conceptualization. **Fang Shen:** Visualization, Validation, Supervision, Resources, Project administration, Investigation, Conceptualization.

Ethics approval

This study was approved by the Medical Ethics Council of Shanghai Fifth People's Hospital (Approval No. 85, 2023).

Sequence information

The genome data of the 44 hypervirulent and carbapenem-resistant *K. pneumoniae* were submitted to the National Center for Biotechnology Information (NCBI) under BioProject accession number PRJNA1165149 and PRJNA962850.

Disclosure

The authors report no conflicts of interest in this work.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmii.2025.02.010.

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