

# Phenotypic and Genomic Characterization of Virulence Heterogeneity in Multidrug-Resistant ST11 *Klebsiella pneumoniae* During Inter-Host Transmission and Evolution

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Chao Liu<sup>1,2</sup>  
Pengcheng Du<sup>3</sup>  
Jiankang Zhao<sup>2</sup>  
Binbin Li<sup>2</sup>  
Chunlei Wang<sup>2</sup>  
Lingxiao Sun<sup>2,4</sup>  
Binghuai Lu<sup>2</sup>  
Yimin Wang<sup>2</sup>  
Yingmei Liu<sup>2</sup>  
Bin Cao<sup>1,2,4-6</sup>

<sup>1</sup>Peking Union Medical College, Institute of Respiratory Medicine, Chinese Academy of Medical Sciences, Beijing, People's Republic of China; <sup>2</sup>Department of Pulmonary and Critical Care Medicine, Laboratory of Clinical Microbiology and Infectious Diseases, China-Japan Friendship Hospital, Beijing, People's Republic of China; <sup>3</sup>Institute of Infectious Diseases, Beijing Ditan Hospital, Capital Medical University and Beijing Key Laboratory of Emerging Infectious Diseases, Beijing, People's Republic of China; <sup>4</sup>Clinical Center for Pulmonary Infections, Capital Medical University, Beijing, People's Republic of China; <sup>5</sup>National Clinical Research Center of Respiratory Diseases, Beijing, People's Republic of China; <sup>6</sup>Tsinghua University-Peking University Joint Center for Life Sciences, Beijing, People's Republic of China

**Background:** Multidrug-resistant (MDR) ST11 hypervirulent *Klebsiella pneumoniae* (hvKp) is emerging in China.

**Purpose:** The aim of this study was to track the transmission and evolution of hvKp.

**Materials and Methods:** A retrospective study focused on Kp infection was conducted. Clinical data were collected from electronic medical records. Whole-genome sequencing of Kp strains was performed. Single-nucleotide polymorphisms (SNPs) were analyzed and a transmission map was constructed. Sequence type, and antimicrobial and virulence-associated genes were characterized. Strains with some combination of the virulence genes, *p*, *rmpA*, *p*, *rmpA2*, *iucA*, *iroB*, and *peg-344*, were defined as hvKp. Kp virulence phenotypes were evaluated using the *Galleria mellonella* model.

**Results:** All 33 Kp strains were MDR-Kp and 13 (39.4%) were hvKp. Most hvKp strains (84.6%, 11/13) were hospital-acquired infections (HAIs). Two unique combinations of virulence-associated genes were detected among hvKp strains. Eleven cases were associated with *p*, *rmpA2+iucA* and two strains presented with *peg-344+p*, *rmpA+p*, *rmpA2+iucA*. Surprisingly, two community-acquired MDR-hvKp infection cases were identified. Eight hvKp strains (61.5%, 8/13) exhibited a hypervirulent phenotype in the *G. mellonella* model. Five MDR-hvKp strains with the hypervirulence phenotype originated from a single cluster. Additionally, nine clones were identified among the two clades, six of which were hvKp. Moreover, the hvKp in clade 1 carried the IncHI1B plasmid replicon, whereas none of the hvKp strains in clade 2 harbored IncHI1B. These data, showing that different hvKp clones distributed into separate clades, indicate that transmission and evolution occurred within the hospital.

**Conclusion:** During inter-host evolution and transmission, various virulence clusters of the epidemic clone, MDR-ST11, converged, conferring phenotypic virulence heterogeneity and spread within the hospital and possibly the community. Mobile/conjugative genetic elements associated with virulence-encoding gene clusters might emerge and have been transmitted within the hospital, suggesting that enhanced ongoing surveillance is essential.

**Keywords:** hypervirulent *Klebsiella pneumoniae*, multidrug resistance, whole-genome sequencing, hospital-acquired infection, community-acquired infection

## Introduction

*Klebsiella pneumoniae* (Kp) is a major emerging Gram-negative bacteria involved in hospital-acquired infection, particularly various fatal infections.<sup>1</sup> There are two distinct

Correspondence: Bin Cao  
Tel +86 10-84205288  
Email caobin\_ben@163.com

pathotypes of Kp: hypervirulent (hvKp) and classical (cKp),<sup>2–5</sup> where cKp is notorious for acquiring antibiotic-resistant genes, representing a threat to public health. Further, cKp is rapidly becoming resistant to the majority of available antibiotics, primarily driven by worldwide dissemination of a specific multidrug-resistant clone, defined as clone group (CG) 258, particularly the ST11 in China.<sup>6,7</sup> Kp ST11 is typically encountered in isolates producing Kp carbapenemase (KPC).<sup>6,8</sup> Further, the hvKp pathotype is frequently associated with aggressive, invasive community infections, such as bacteremia and pyogenic liver abscesses, in immunocompetent ambulatory younger adults with no underlying disease.<sup>9–11</sup> Previously, hvKp was defined by its hypermucoviscosity phenotype (string test, >5 mm);<sup>9</sup> however, recent evidence from in vitro and in vivo models shows that the hypermucoviscosity phenotype is not actually closely associated with virulence.<sup>4,12–15</sup> Further, compared with genetic traits, hypermucoviscosity is an inferior marker for differentiating hvKp and cKp,<sup>16</sup> A combination of five virulence-associated genes, *peg-344*, *iroB*, *iucA*, *p<sub>rmpA</sub>*, and *p<sub>rmpA2</sub>*, showed higher diagnostic accuracy for hvKp than phenotype or other virulence-associated genes alone.<sup>16</sup> Moreover, cKp strains are at considerably higher risk of acquiring virulence genes to become hypervirulent and drug resistant, relative to hvKp clones.<sup>17</sup> Importantly, a previous study reported that the epidemic clone in China (ST11) is becoming hypervirulent due to acquisition of various virulence genes or a pLVKP-like plasmid.<sup>18</sup>

Most previous studies have primarily focused on outbreaks of cKp. Although the multidrug resistance (MDR) and extended-spectrum- $\beta$ -lactamase (ESBL) producing hvKp, particularly those resistant to colistin and carbapenems, are emerging in China,<sup>19–21</sup> few reports have demonstrated that hvKp can be the source of hospital infection outbreaks.<sup>18</sup> Most importantly, reports of the dynamic genomic evolution of hvKp strains are rare.

Here, during surveillance, we discovered an increasing number of ST11 hypermucoviscosity and MDR Kp strains highly clustered in the hospital for approximately 1 year and associated with poor prognosis. Therefore, we conducted this study to characterize the genomic differences and phylogenetic relationships of Kp, to reconstruct its transmission route and study the genomic basis underlying the poor outcomes of patients infected with this pathogen. Surprisingly, during transmission and evolution, ST11 MDR-Kp evolved into two different pathotypes, resulting in multiple clones transmitted via different routes.

## Materials and Methods

### Patients Enrolled

A retrospective study was conducted from March 2017 to June 2018 at a hospital, comprising three sites: a headquarters, and west and north campuses. The respiratory department spanned two sites, the headquarters and the north campus, and the medical staff worked shifts at the different sites. Patient clinical characteristics were obtained from medical records, including basic demographic features, underlying disease, antibiotic agent exposure, mechanical ventilation, use of invasive devices, and outcome. Poor prognosis (death or withholding life-sustaining therapy) within 30 days was defined as the primary endpoint. Sequential organ failure assessment (SOFA) score and acute physiology and chronic health evaluation II (APACHE II) were conducted for patients positive for Kp by culture. The main inclusion criteria were: 1) age > 18 years; 2) Kp cultured twice or more and simultaneous clinical infection symptoms. Exclusion criteria were: 1) insufficient clinical data or bacterial strain samples; and 2) duplicate isolates from the same patient within two weeks. Hospital-acquired (HA) and community-acquired (CA) Kp infections were defined as previously described.<sup>9</sup> CA and HA infections were differentiated for all patients.

The protocol for this study was approved by the China–Japan Friendship Hospital Ethics Committee (2018-GZR-199), and the Guidelines for Human Experimentation (PRC) were followed throughout. Informed consent was not needed, due to the retrospective nature of the study. Additionally, data for all patients enrolled in this study were anonymized.

### Antimicrobial Resistance and Virulence-Associated Phenotype

All isolates were stored at  $-80^{\circ}\text{C}$  and analyzed by MALDI-TOF mass spectrometry. HvKp was defined as *peg-344*, *iroB*, *iucA*, *p<sub>rmpA</sub>*, or *p<sub>rmpA2</sub>* positive.<sup>16</sup> The primers used are described in [Supplementary Table 1](#).

Antimicrobial susceptibility testing was performed as previously described, and the results were interpreted according to the 2017 Clinical and Laboratory Standards Institute (CLSI) guidelines. The antibiotics used for testing included amikacin, gentamicin, tobramycin, ampicillin/sulbactam, aztreonam, cefazolin, cefepime, ceftriaxone, ceftazidime, ciprofloxacin, levofloxacin, piperacillin/tazobactam, and trimethoprim/sulfamethoxazole. An MDR strain was defined

as a strain resistant to three or more different antimicrobial categories, as described previously.<sup>5</sup>

The hypermucoviscous phenotype was detected by string test, as described previously.<sup>9</sup> The virulence phenotype was also evaluated using the wax moth, *Galleria mellonella*, model (insects of approximately 300 mg). Overnight cultures of Kp strains were adjusted using physiological saline to  $1 \times 10^6$  CFU/mL. Then, Kp isolates were injected into the *G. mellonella*, as described previously<sup>18</sup> and their survival rate recorded at 12, 24, 36, and 48 h, respectively. The survival rate of *G. mellonella* infected with the K1 hypervirulent Kp isolate was used as reference to define the hypervirulent phenotype.

Serum resistance assays of all isolates were conducted as previously described.<sup>22</sup> Briefly, bacterial suspensions were collected and then mixed with healthy human serum at 1:3. The mixture was agitated at 37°C and clone counts recorded at time 0 and after 30, 60, 120, and 180 min of culture for 24 h. Survival percentages at different time points were used to determine serum susceptibility/resistance. Each strain was tested three times.

## Whole-Genome Sequencing to Identify Genomic Features

All strains were sequenced using the Illumina HiSeq 2500 sequencing platform by constructing paired-end libraries to obtain 150 bp reads. Raw data were filtered to remove low-quality reads, then assembled using SPAdes v3.13. Sequencing type (ST) was identified using MLST 2.0 (Center for Genomic Epidemiology). Draft genome sequences were annotated using Prokka. Capsular types were analyzed using Kaptive. Antimicrobial resistance genes, virulence genes, and plasmid replicon types were annotated by comparison with relevant databases (ResFinder, Virulence Factor Database, plasmidFinder), using BLAST software. Antimicrobial resistance and virulence genes were identified using thresholds of 90% identity and minimum length coverage of 80%. Additionally, PCR was applied for MLST and *peg-344*, *iroB*, *iucA*, *p<sub>rmpA</sub>*, and *p<sub>rmpA2</sub>*, to validate our sequencing findings.

For phylogenetic analysis, we identified single-nucleotide polymorphisms (SNPs) by mapping sequencing reads to the genome sequence of Kp strain HS11286 (GenBank accession: NC\_016845.1) using bowtie2 software, followed by filtering the results using Samtools. High-quality SNPs (hqSNPs), supported by more than 5 reads, were retained and adjacent mutations within 5-bp

filtered, to avoid recombination. Finally, the concatenated sequences were used to perform phylogenetic analysis using FastTree v2.1.10 with the maximum likelihood method. We deposited the read data in the Sequencing Read Archive database (SRP186665 and SRP229572).

## Results

### Clinical Characteristics

Thirty-three cases were enrolled in the study. Thirteen strains (13/33, 39.4%) were defined as hvKp. The mean age of patients was  $65.09 \pm 20.02$  years and 17 (51.5%) were female. Thirty-one cases (93.9%) were identified as hospital-acquired infection (HAI) and two (6.1%) with community-acquired infection, triggered by hvKp. The main infection type was pneumonia ( $n = 26$ , 78.8%), followed by urinary tract infection ( $n = 6$ , 18.2%) and bacteremia ( $n = 1$ , 3.0%). Twenty-four patients (24/33, 72.7%) presented with severe infection (sepsis or septic shock) and seventeen patients (17/33, 51.5%) were immunosuppressed. All patients had been exposed to antibiotics and three (9.1%) were not intubated. For 22 (66.7%) patients, APACHE II scores were  $\geq 16$ , while 23 (69.7%) had SOFA scores  $\geq 6$ . Twenty-five (75.8%) patients had Charlson Comorbidity Index scores  $\geq 3$ . Twelve patients (36.4%) died within 30 days and life-sustaining therapy was withheld for 3 patients (9.1%) (Table 1).

### Phylogenetic Relationships and Distribution of the Isolates

In total, 1313 hqSNPs were identified and used to perform phylogenetic analysis (Supplementary Table 2). Although all strains were sequence type 11 (ST11), they clustered into two separate clades (Figures 1 and 2). The separation into two clades was supported by 749 hqSNPs, including 372 unique to clade 1 and 377 unique to clade 2. Clade 1 comprised six isolates distributed among the International Medical Service ( $n = 1$ ), Respiratory ( $n = 4$ ), and Neurology ( $n = 1$ ) departments. Clade 2 contained two clusters: clade 2a and clade 2b, comprising 11 and 16 isolates, respectively. In clade 2, the majority of strains were from the Respiratory department (Respiratory-H and Respiratory-N) (16/27) and SICU (5/27).

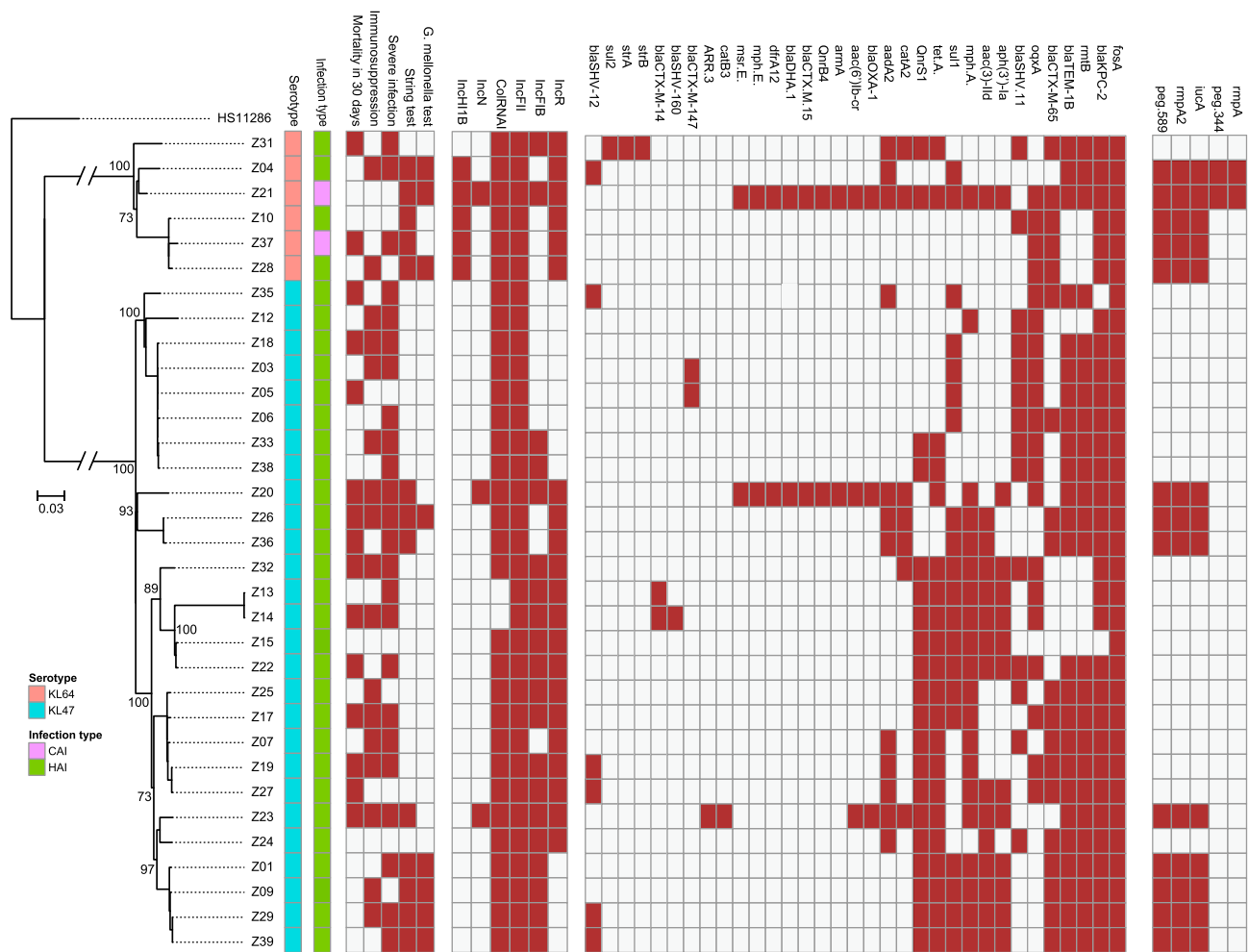
### Antimicrobial Resistance and Virulence Profiles

All strains exhibited multidrug resistant phenotypes (Supplementary Table 3), with diverse drug resistance

**Table 1** Clinical Characteristics of Patients with *Klebsiella pneumoniae* Infection

Patient	Age	Sex	Specimen	Collection Date	Department	Immunosuppression	Antibiotic Agent Exposure	Intubation	HAI	Type of Infection	APACHE II >16	SOFA ≥6	CCI	Outcome in 30 Days
Z1	78	M	Sputum	20171218	Respiratory-N	N	Y	Y	Y	VAP	Y	Y	6	Survive
Z3	60	M	Sputum	20180101	Respiratory-H	Y	Y	Y	Y	Pneumonia	Y	Y	3	Survive
Z4	48	M	Tissue	20180103	Respiratory-H	Y	Y	Y	Y	Pneumonia	Y	Y	4	Survive
Z5	73	F	Urine	20180104	Gastroenterology-H	N	Y	Y	Y	UTI	Y	N	3	Death**
Z6	37	M	BALF	20180104	Respiratory-H	N	Y	Y	Y	VAP	Y	Y	1	Survive
Z7	87	M	Sputum	20180108	SICU	Y	Y	Y	Y	VAP	Y	Y	4	Survive
Z9	57	F	Tissue	20180112	Respiratory-H	Y	Y	N	Y	Pneumonia	N	N	3	Survive
Z10	35	M	Sputum	20180115	Internal Medicine	N	Y	Y	Y	VAP	N	N	3	Survive
Z12	80	M	Sputum	20180121	Respiratory-N	Y	Y	Y	Y	Pneumonia	Y	Y	6	Survive
Z13	82	F	Sputum	20180122	Respiratory-N	N	Y	Y	Y	Pneumonia	N	Y	3	Survive
Z14	44	F	BALF	20180122	Respiratory-H	Y	Y	Y	Y	VAP	Y	Y	1	Death
Z15	87	F	Urine	20180201	Respiratory-N	N	Y	N	Y	CAUTI	Y	Y	4	Survive
Z17	42	F	Drainage liquid	20180208	SICU	Y	Y	Y	Y	Pneumonia	N	Y	4	Death
Z18	35	M	BALF	20180211	Respiratory-H	Y	Y	Y	Y	VAP	Y	Y	4	Death
Z19	80	M	Sputum	20180213	SICU	Y	Y	Y	Y	VAP	Y	Y	4	Death
Z20	28	M	Sputum	20180224	Respiratory-H	Y	Y	Y	Y	VAP	Y	Y	14	Death
Z21	84	F	Sputum	20180218	Respiratory-H	Y	Y	Y	Y	VAP	N	N	1	Death**
Z22	87	F	Urine	20180221	Respiratory-N	N	Y	Y	Y	CAUTI	N	N	1	Survive
Z23	81	M	Urine	20180222	Nephrology-N	N	Y	Y	Y	CAUTI	Y	Y	4	Death
Z24	86	F	Urine	20180224	General Medicine-N	N	Y	Y	Y	UTI	Y	Y	4	Death
Z25	83	F	Sputum	20180309	SICU	Y	Y	Y	Y	Pneumonia	Y	N	5	Survive
Z26	70	F	Sputum	20180310	Respiratory-N	Y	Y	Y	Y	VAP	Y	Y	5	Survive
Z27	68	M	Sputum	20180315	SICU	N	Y	Y	Y	VAP	Y	Y	5	Death
Z28	35	M	Sputum	20180317	Neurology-N	Y	Y	Y	Y	VAP	Y	Y	2	Death
Z29	76	M	BALF	20180417	Respiratory-H	Y	Y	Y	Y	Pneumonia	Y	N	2	Survive
Z31	58	F	BALF	20180427	Respiratory-N	N	Y	Y	Y	VAP	Y	N	6	Survive
Z32	28	F	Blood	20170618	Respiratory-N	Y	Y	Y	Y	Pneumonia	N	N	2	Death
Z33	58	M	Sputum	20180517	Respiratory-H	Y	Y	Y	Y	BSI	Y	Y	4	Death
Z35	78	F	BALF	20180529	Respiratory-H	N	Y	Y	Y	VAP	Y	N	3	Survive
Z36	78	M	Sputum	20180614	Respiratory-N	N	Y	Y	Y	VAP	Y	Y	2	Death
Z37	78	F	BALF	20180617	Respiratory-H	N	Y	Y	Y	Pneumonia	Y	Y	4	Death**
Z38	57	F	Urine	20180618	CCU	N	Y	Y	CAI	VAP	N	Y	6	Death
Z39	90	F	Sputum	20180110	Respiratory-N	N	Y	Y	Y	CAUTI	Y	Y	5	Survive
						N	Y	Y	Y	Pneumonia	N	Y	4	Survive

**Note:** \*\*Withheld life-sustainable treatment; The -H and -N department means headquarters and north campus, respectively.  
**Abbreviations:** CAI, community-acquired infection; HAI, hospital-acquired infection; BALF, bronchoalveolar lavage fluid; CAUTI, catheter-associated urinary tract infection; VAP, ventilator-associated pneumonia; BSI, bloodstream infection; UTI, urinary tract infection; SICU, surgery intensive care medicine; CCU, cardiac intensive care unit; Y, yes; N, no.



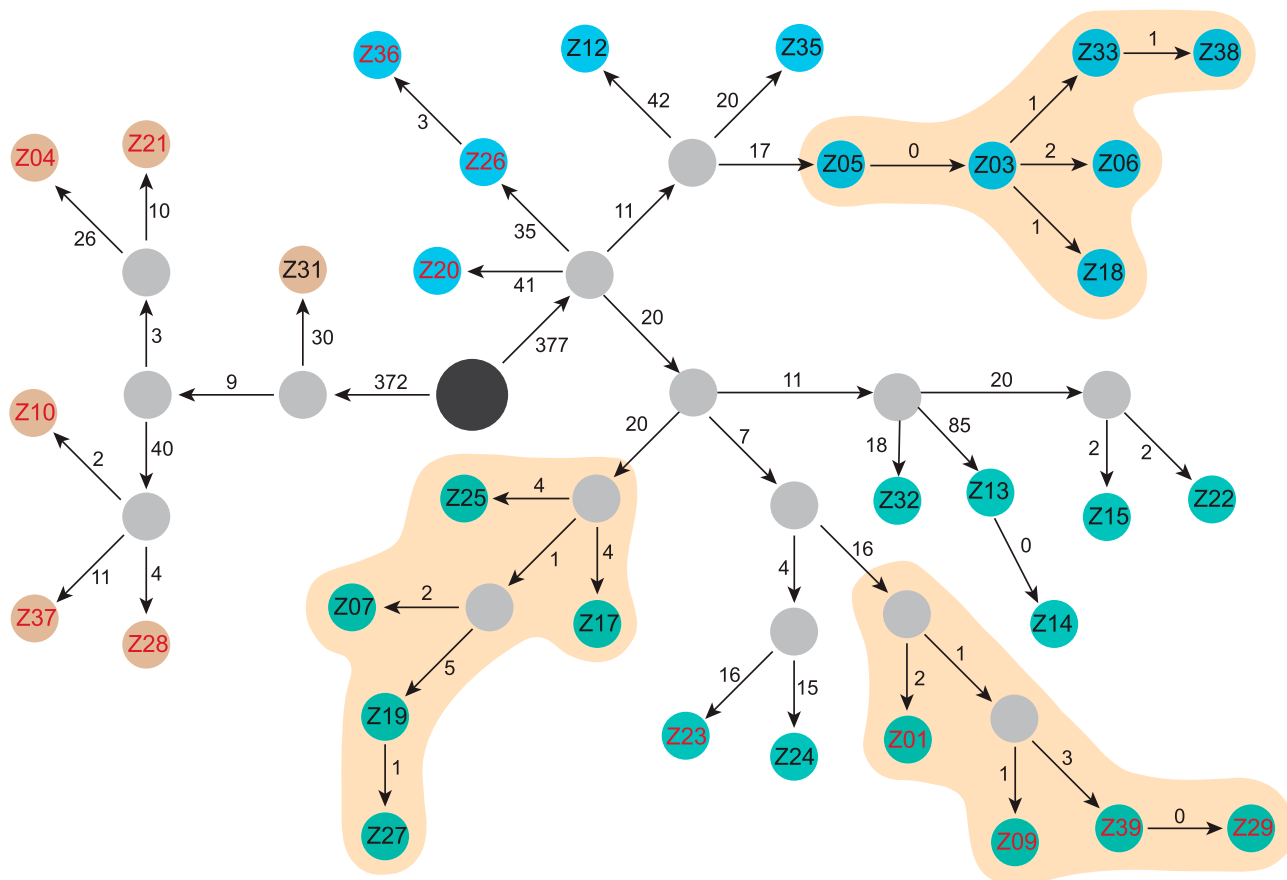
**Figure 1** Phylogenetic tree of MDR-Kp strains. Brown: positive. Green: hospital-acquired infection. Purple: community-acquired infection. Blue: KL47. Pink: KL64. Hypervirulent phenotype: *G. mellonella* mortality is not lower than the reference strain (K1-hvKp) at 48 h.

mechanisms detected (Figure 1). Of 33 strains, 31 carried *bla<sub>KPC-2</sub>*, as well as resistance to carbapenem. *RmtB* and *bla<sub>TEM</sub>* were co-detected in 25 strains, while 21 strains carried *tet(A)*. Surprisingly, *bla<sub>OXA-1</sub>* and *bla<sub>DHA-1</sub>* were co-identified in two strains and diverse *bla<sub>CTX-M</sub>* genotypes were present among these strains. Notably, although there was no evidence of pre-exposure to fosfomycin, all strains carried *fosA*.

Further, 13 strains were hypermucoviscous and all strains carried the *yersiniabactin* gene (Supplementary Table 4). Thirteen strains possessed *iucA*, and *p<sub>rmpA2</sub>* was also detected in 13 strains, two of which were also positive for *p<sub>rmpA</sub>* and *peg-344*, whereas none harbored *iroB*. *Peg-589*, a factor strongly associated with poor prognosis,<sup>16</sup> was also detected in the 13 *p<sub>rmpA2</sub>*-positive strains; therefore, these strains were defined as hvKp, due to the presence of five virulence genes, and two types of virulence profile were identified. Eleven cases were associated with *p<sub>rmpA2</sub>*, *iucA*,

and *peg-589*, while the other two strains carried *peg-344+peg-589+p<sub>rmpA</sub>+p<sub>rmpA2</sub>+iucA*. No isolate possessed *iroB+peg-344+iucA+p<sub>rmpA</sub>+p<sub>rmpA2</sub>*; however, surprisingly, 61.5% (8/13) hvKp strains exhibited a hypervirulent phenotype in the *G. mellonella* model (Supplementary Figure 1). Common serotypes (ie, K1, K2, K5, K20, and K54) were not detected among these strains; clade 1 strains were all serotype KL64 and those in clade 2 were all KL47. Additionally, no strain exhibited serum resistance phenotype.

All strains contained the IncFII plasmid replicon and 93.9% (31/33) carried the ColRNAI plasmid replicon. Further, 60.6% (20/33) and 63.6% (21/33) were positive for the IncFIB and IncR plasmid replicons, respectively. The aforementioned were the common replicon types in our strains. Five (15.2%, 5/33) strains harbored IncHI1B and three isolates contained IncN (Figure 1). More than three types of replicon were present in 27/33 (81.8%) isolates,



**Figure 2** Evolutionary and transmission map of isolated Kp strains. Brown: clade 1 strains. Green: clade 2a strains. Blue: clade 2b strains. Black circle, ancestral strain determined by SNP analysis. Gray, strains during transmission not captured in this study. Red numbers, hvKp defined by the combination of five key virulence genes. The numbers on the arrows are SNPs.

indicating that they carried multiple plasmids. Interestingly, five of six strains in clade 1 carried the IncHI1B plasmid replicon

. In contrast, all hvKp strains in clade 2 harbored IncFIB, but not the IncHI1B plasmid replicon, suggesting that a type of plasmid, or mobile genetic element, harboring virulence gene clusters may be present.

## Virulence Shift During Evolution and Transmission

Based on the combined SNP and epidemiological information, we determined potential transmission routes (Figure 2). For clade 1, based on SNP variants, we estimate that the hvKp ancestor may have separated from a common ancestor with Z31, and then evolved into two clones, which further evolved into Z04 and Z21. The other clone evolved into Z10, Z28, and Z37. Notably, hvKp strains in clade 1 acquired different virulence gene clusters; however, interestingly, only the Z04, Z21, and Z28 strains exhibited hypervirulence in the *G. mellonella*

model. Moreover, patients infected with the other clade 1 strains (Z31 and Z37) died within 30 days. Notably, phenotypic virulence heterogeneity was detected in the *G. mellonella* model, suggesting that it may be possible to omit the use of *G. mellonella* for identification of the virulence phenotype. Fortunately, the two clones were present in different wards and did not result in an outbreak.

The common ancestor of clade 2a evolved into three different clones, two of which did not acquire virulence genes or present a virulence phenotype. Combined with clinical information, these data indicate that the two clones were spread in the SICU and Respiratory-N departments, respectively. Interestingly, two SICU doctors were responsible for medical care of five patients. Overall, our data confirm that a genuine hypervirulent clone is emerging and has spread in the Respiratory department of our hospital. Among the hypervirulent clone cluster, five of six strains acquired *iucA* + *rmpA2* + *peg-589* and most of them exhibited hypervirulence in *G. mellonella*. Of patients infected with the hypervirulent clone, 80% (4/5) suffered from septic shock. Our

data, which indicate that Kp strains combining hypervirulent and MDR profiles are emerging, are alarming. Worse, patients infected with the Z21 and Z37 strains were defined as community-acquired infection, due to the presence of MDR-hvKp, representing a warning that the nosocomial MDR-hvKp strain could have spread into the community.

## Discussion

In this study, we mapped inter-host evolution and transmission of ST11, which has acquired unique hvKp virulence genes, including *iucA* and *rmpA2* (but not *iroB*, *rmpA*, or *peg-344*), conferring a heterogeneous hypervirulent phenotype. Specifically, these clones have spread within the hospital for years and may have transmitted to the community. In addition, we detected the interesting phenomenon that key virulence genes (*iucA* and *rmpA2*) that can frequently be located in the unique plasmid, pVir-CR-hvKp4 or pLVPK, emerged in different clones from clade 1 and clade 2a clusters, as well as a rare, sporadic strain in clade 2b. Moreover, strains in clade 2 carried the IncFIB, but not the IncHI1B, plasmid replicon, suggesting that the conjugative plasmid, or other mobile elements associated with virulence-encoding genes, may emerge within the hospital.

Multidrug-resistant Kp strains have become prominent in the last decade, particularly in China. In a Chinese survey of carbapenem-resistant Enterobacteriaceae, conducted from 2012 to 2016 at multiple centers, 66.7% were CR-Kp.<sup>6</sup> The rapid rise in the rates of MDR strains has been attributed to the dominant emergence and expansion of a specific clone of Kp, referred to as ST11.<sup>6,8</sup> In China, ST11 Kp is the predominant strain of cKp, and exhibits reduced susceptibility to most available antibiotics, mainly due to the insertion of KPC-2.<sup>6,23</sup> Recently, increasing numbers of outbreaks caused by ST11-MDR-Kp have been reported.<sup>24–26</sup> Importantly, the emergence of ST11 Kp has coincided with its increasing carriage of virulence-associated genes, which are crucial for hospital infections and outbreaks.<sup>18</sup> HvKp is most commonly isolated from patients with community-acquired infection, sensitive to various antibiotics. Importantly, hvKp transmitted within the hospital, where it has been exposed to various antibiotics, could emerge as MDR-hvKp. Our study confirms that MDR-hvKp, the so-called “super bug”, could be transmitted from hospital into the community, warning that ongoing surveillance should focus more closely on the community.

The emergence of MDR-hvKp threatens the viability of the current therapeutic approach,<sup>18,27</sup> and clear understanding of its evolution and transmission characteristics is essential. In our study, 53.8% (7/13) of patients infected with hvKp were immunodeficient. Within the immunodeficient patient who hospitalized long term, diverse phenotype and genotype of Kp might emerge and cause persistent infection.<sup>22</sup> This phenomenon has been observed in many pathogens. For example, a previous study demonstrated that mutation of the *mutS* gene in *Salmonella* resulted in a hypermutator phenotype and diversification during evolution in an immunosuppressive patient.<sup>28</sup> Additionally, *Pseudomonas aeruginosa* during long-term infections evolves their iron acquisition metabolism pathways from the promoter mutations of the *phu* system.<sup>29</sup> The SNP mutations in hvKp strains distributed in different Clades and sporadic strains were detected ([Supplementary Figures 2–4](#)); however, no significant mutations were detected in any of the hvKp strains. Enhanced surveillance of immunosuppressed patients may be warranted.

Previous studies of outbreaks have suggested that they are primarily associated with cKp, with few reports demonstrating that outbreaks were triggered by hvKp.<sup>18</sup> To date, only ST11 CR-hvKp has been reported as associated with HAI outbreaks. One reason may be the original capsule,<sup>17</sup> while another possible explanation is a strong association with plasmid compatibility.<sup>30</sup> Interestingly, a fatal outbreak induced by hvKp reported by Gu et al also originated from the same clone of ST11,<sup>18</sup> however, in this study, various ST11-MDR-hvKp clones were involved inter-host evolution and transmission and distributed among different clades. It is alarming that hvKp, as a source of hospital infection, is tending toward polymorphism, indicating that an increase in ongoing surveillance is urgently required. Yang et al<sup>30</sup> reported a conjugative virulence plasmid that can rapidly enhance dissemination of virulence-encoding elements among Gram-negative bacterial pathogens, particularly Kp. In our study, all hvKp strains in clade 2 carried the IncFIB, but not the IncHI1B, plasmid replicon and harbored the same virulence gene cluster (*iucA*+*rmpA*), similar to the pVir-CR-hvKp4 plasmid, suggesting that conjugative virulence-encoding plasmid, or other mobile elements, may be present.

During their evolution and transmission, some ST11 strains have acquired virulence-associated genes, but do not exhibit a genuine virulence phenotype in the *G. mellonella* model. Previous study reported that carriage of virulence plasmids is not always associated with hypermucoviscosity and hypervirulence phenotypes in Kp.<sup>31</sup>

Indeed, the hypermucoviscosity phenotype was not lost in this study. On one hand, it may be necessary to reassess the definition of hvKp and the animal model for identifying hvKp in further studies, particularly for strains isolated from China. On the other hand, there might exist a new virulence-associated gene that could confer hypervirulence phenotype, which should be confirmed by further study.<sup>2</sup>

Outbreaks of various pathogens have been reported, where major reservoirs included water, healthcare workers, endoscopes, and other medical equipment.<sup>32–34</sup> Infection control measures are essential for routine clinical practice. A previous study suggested that clinical departments previously infected with MDR-hvKp should be disinfected and left unoccupied for more than 2 weeks before new patients are admitted.<sup>18</sup> This may be a good strategy to prevent large outbreaks, particularly in critically ill, mechanically ventilated patients. Regarding the potential emerging conjugative virulence-associated plasmid, our hospital has improved awareness among healthcare staff using online and offline education, strengthened active hand hygiene, encouraged use of disposable gloves, and ensured sterilization of breathing apparatus, among other measures. Although screening of fecal carriage status was implemented previously, the frequency of this procedure should be increased in the ICU department.

The main limitations of our study were that it had a retrospective design and was conducted at a single center. Further, the number of strains detected was small. We strongly believe that further prospective studies involving international collaboration are essential to address these limitations. Additionally, the use of long-read sequencing techniques may assist deep exploration of the plasmid and genomic content of Kp.

## Conclusions

In summary, in this study, we observed that various virulence clusters of the epidemic clone, MDR-ST11, converged and subsequently spread as they evolved and were transmitted within the hospital, and possibly to the community. Heterogeneity of phenotypic virulence during inter-host transmission and evolution was also detected using the *G. mellonella* model. The detection of new types of plasmid, including virulence genes, may indicate that conjugative/mobile genetic elements have emerged and triggered spread of the organism within the hospital. Therefore, ongoing surveillance should be enhanced to determine whether this trend is also occurring elsewhere.

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## Disclosure

The authors report no conflicts of interest in this work.

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