ORIGINAL RESEARCH

Virulence and genomic features of a $bla_{CTX-M-3}$ and $bla_{CTX-M-14}$ coharboring hypermucoviscous *Klebsiella pneumoniae* of serotype K2 and ST65

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Background: Capsular serotype K2 *Klebsiella pneumoniae* of sequence type (ST) 65 has been recognized as a hypervirulent clone. Simultaneous presence of different bla_{CTX-M} genes has never been reported in this clone. In the present study, the genetic characteristics and virulence phenotype of a CTX-M-3 and CTX-M-14 coproducing ST65 *K. pneumoniae* human isolate, KP_06, that caused an intracranial infection, are evaluated.
Methods: The potential virulence of KP_06 was assayed by in vitro and in vivo methods. The molecular biology and whole-genome sequencing technology were used to analyze the genomic features associated with the virulence of this strain.
Results: The KP_06 exhibited typical features of hypervirulent *K. pneumoniae* (hvKP), showing hypermucoviscosity phenotype and belonging to K2 and ST65. Apart from virulence genes linked to hvKP, including *rmpA*, *rmpA2*, and *clb* cluster and genes encoding siderophores, it was found to harbor a ~170 kb pLVPK-like virulence plasmid. In contrast to most hvKP, KP_06 was resistant to cephalosporins and the coexistence of $bla_{CTX-M-3}$ and $bla_{CTX-M-14}$ was detected. Further experiments demonstrated that this strain was classified as a nonbiofilm producer and serum sensitivity (grade 1) and killed only 30% of *Galleria mellonella* inoculated with 1×10^6 colony-forming unit of the specimen within 48 hours, suggesting relatively low virulence. Comparative genomic analysis of KP_06 with five K2 hypermucoviscous *K. pneumoniae* (HMKP) revealed seven unique orthologies with varied function in this strain. Intriguingly, the virulence genes identified in KP-06 were unexpectedly more diverse than those observed in five other K2 HMKP strains.
Conclusion: Our data support the notion that neither virulence-associated genes (clusters) nor the pLVPK-like virulence plasmid is sufficient for the hypervirulence of *K. pneumoniae*. Future studies aiming to explore the virulence of *K. pneumoniae* should take genome-based profile together with experimental work. The detailed mechanism involving in the impaired virulence of KP_06 remains to be further explored.
Keywords: *Klebsiella pneumoniae*, virulence factor, serotype K2, ST65, bla_{CTX-M}, comparative genome

Introduction

Since first identified in 1986 in Taiwan, hypervirulent *Klebsiella pneumoniae* (hvKP), which caused severe invasive infections such as liver abscesses, endophthalmitis, meningitis, osteomyelitis, and necrotizing fasciitis in otherwise healthy individuals,[1] has been increasingly reported and become the focus of concern recent years.

Traditionally, a positive string test, which is defined as hypermucoviscosity (HM) phenotype appeared to be a surrogate marker for hvKP.[2] However, new evidence

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has suggested that HM and hypervirulence are two distinct phenotypes of *K. pneumoniae* that should not be used synonymously.[3] Most recently, new genetic biomarkers for hvKP such as *iucA* located on the virulence plasmid encoding for aerobactin have been explored.[4] Hence, the precise definition of hvKP remains controversial and hypervirulence-associated determinants of *K. pneumoniae* required further study.

Isolates with serotypes K1 and K2 have been demonstrated as particularly virulent.[5] Compared to serotype K1 strains mainly associated with sequence type (ST) 23, *K. pneumoniae* strains with serotype K2 exhibited more diverse genetic types, among which ST65, ST86, and ST380 are predominant and considered to be hypervirulent clones.[6] The serotype K1 or K2 *K. pneumoniae* strains have not generally been associated with acquired antimicrobial resistance (AMR), but the last few years increasing reports of resistant strains, including those resistant to third-generation cephalosporins and even carbapenems, were observed.[7–9] Up to now, the AMR genes including $bla_{CTX-M-3}$,[7] $bla_{SHV-148}$,[10] bla_{KPC-2},[11] and bla_{NDM}[12] have been found in K2 serotype *K. pneumoniae* strains, whereas the simultaneous presence of different bla_{CTX-M} genes in the hypermucoviscous *K. pneumoniae* (HMKP) of serotype K2 has never been reported.

As the application of whole-genome sequencing (WGS) in clinical microbiology extensively, the genomes of many human source *K. pneumoniae* have been sequenced. However, to the best of our knowledge, only five genomic sequences of serotype K2 *K. pneumoniae* with varied genetic backgrounds were publicly available up to now (RJF293, U25, 52.145, hvKP1, and NUHL24835).[12–16] In the present study, we assayed the potential virulence and characterized the genomic features of a $bla_{CTX-M-3}$ and $bla_{CTX-M-14}$ coharboring *K. pneumoniae* strain of K2 isolated from human cerebrospinal fluid (CSF). We also compared the genome sequences of KP_06 with five other K2 *K. pneumoniae* strains aiming to investigate the strain-specific genes. Our finding will make up our current knowledge on the evolutionary relationship between virulence and resistance in serotype K2 *K. pneumoniae*.

Methods
Isolates and antimicrobial susceptibility testing
The VITEK-2 compact system (bioMérieux, Craponne, France) was used to establish the strain identification and antimicrobial susceptibility testing. The results were interpreted in accordance with the guideline document M100-S28 established by Clinical and Laboratory Standards Institute.[17] The species identification of the isolate was then confirmed by

matrix-assisted laser desorption/ionization mass spectrometry (Bruker Optics Inc, Billerica, MA, USA). *Escherichia coli* American Type Culture Collection (ATCC) 25922 and *K. pneumoniae* 700603 were used as quality control.

In the in vitro and in vivo virulence assessments, an HM ST23:K1 *K. pneumoniae* strain (KP_07), which isolated from pus of a patient with liver abscess with wild-type susceptibility profile to antibiotics, was used as the positive control. A KPC-2-producing ST11 *K. pneumoniae* strain, KP_36, without aerobactin-encoding genes, *rmpA* and *rmpA2*, was used as the control for low virulence as previously described.[18]

Detection of resistance mechanisms and virulence-associated factors
The ESBL genes (bla_{CTX-M} and bla_{SHV}) and carbapenemase genes (bla_{KPC}, bla_{NDM}, bla_{VIM}, and bla_{OXA-48}) were amplified by PCR. The amplicons of β-lactamase genes were purified and sequenced in an ABI 3730 DNA sequencer (Thermo Fisher Scientific, Waltham, MA, USA). The sequences obtained were compared with those in the NCBI database using the Blast software (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

The HM phenotype was determined by "string test". Briefly, when using a bacteriology loop to stretch bacterial colony cultured on an agar plate overnight at 37°C, the generation of a viscous string of >5 mm in length was defined as positive.[19] The presence of serotype-specific genes encoding for K1, K2, K5, K20, K54, and K57 and 14 known *K. pneumoniae* virulence genes, including *magA*, *rmpA*, *rmpA2*, *kfu*, *allS*, *fimH*, *wabG*, *ybtS*, *mrkD*, *uge*, *entB*, *iutA*, *iucA*, and *iroN*, were assessed as previously described.[20,21]

S1 nuclease pulsed-field gel electrophoresis (S1-PFGE) and southern blot hybridization
The number of plasmids and the size of the pLVPK-like virulence plasmid carried by *K. pneumoniae* strains were determined by S1-PFGE and southern blot hybridization. In brief, whole DNA of *K. pneumoniae* was subjected to S1 nuclease (Takara Bio Inc., Tokyo, Japan) digestion. Digested fragments were subjected to PFGE. Then, the gels were blotted onto nylon membranes (EMD Millipore, Billerica, MA, USA) according to standard techniques. The membranes were hybridized with digoxigenin-labeled *rmpA2* probe.

Growth curve assays
Growth curve was generated by diluting equal numbers of colony-forming unit (CFU) of each isolate (approximately 1×10^6 CFU/mL) in Luria-Bertani (LB) broth and incubated

in 96-well microplates. Then, the growth was measured by $OD_{600\,nm}$ using a microplate reader (Synergy HT; Biotek Instruments Inc., Winooski, VT, USA) equipped with the Gen5 Microplate software (Biotek Instruments). Each curve was performed in triplicate.

Biofilm formation assays

Biofilm formation was assessed by the method of Araújo et al.[22] A total of 1 µL of bacterial suspensions obtained from overnight culture was inoculated into 100 µL of fresh LB broth in individual wells of a 96-well flat-bottomed polystyrene plate. After 24 hours of static incubation at 37°C, the biomass was measured by crystal violet staining and acetic acid elution. The absorbance for the eluted dye was determined at $OD_{570\,nm}$ using the microplate reader (Synergy HT). The results were interpreted according to the criteria established by Saxena et al.[23] Each assay was performed at least three times at three occasions.

Susceptibility to serum killing

The bacterial susceptibility to serum was performed as described previously.[12] Briefly, bacteria grown in LB broth were collected during the mid-log phase, then diluted to 1×10^6 CFU/mL. A total of 25 µL of the bacterial suspension were mixed with 75 µL of pooled healthy human serum in microtiter plate and then incubated in 37°C with gently shake. Serial dilutions were plated on Mueller-Hinton (MH) agar at 0, 1, 2, and 3 hours to obtain the colony counts of viable bacteria. The results were graded from 1 to 6 as described by Mei et al,[12] with grade 1 considered to be the most serum susceptibility. Each strain was tested at least three times.

Infection of *Galleria mellonella* larvae

Bacteria from overnight cultures were washed with PBS and adjusted to concentrations 1×10^4, 1×10^5, 1×10^6, and 1×10^7 CFU/mL in PBS. Ten randomly selected Wax moth larvae (*G. mellonella*) weighing between 250 and 350 mg were for each isolate of each concentration. The insects were injected 10 µL of bacterial suspension via rear left proleg and then incubated in dark at 37°C. The numbers of dead insects were recorded every 12 hours for 48 hours. PBS injection controls and controls receiving no injection were used to evaluated trauma and attrition, respectively.

WGS and data analysis

The KP_06 strain was subjected to WGS. Genomic DNA was prepared using the QIAamp DNA Mini Kit (Qiagen NV,

Venlo, the Netherlands), and the whole genomic sequencing was conducted using the Illumina HiSeq 2500-PE125 platform. The low-quality reads were filtered and then assembled into scaffolds using SOAP de novo. The ST of KP_06 was identified using the Center for Genomic Epidemiology (CGE) service (https://cge.cbs.dtu.dk/services/MLST/),[24] and the serotype was determined by querying the *wzi* allele using Pasteur database (http://bigsdb.pasteur.fr/klebsiella/klebsiella.html). AMR genes were identified from the genome sequences against the Resfinder (https://cge.cbs.dtu.dk/services/ResFinder/)[25] and the Comprehensive Antibiotic Resistance Database (CARD) Version 2.0.1 (https://card.mcmaster.ca/).[26] The putative virulence factors were predicted by the Virulence Factor Database (VFDB) protein sequences of core data set (Version April 28, 2018, 2,595 genes, http://www.mgc.ac.cn/cgi-bin/VFs/v5/main.cgi)[27] and virulence genes deposited in *K. pneumoniae* multilocus sequence typing (MLST) database of Institut Pasteur (http://bigsdb.pasteur.fr/klebsiella/klebsiella.html) with an *e*-value cutoff of e^{-10} and an identify threshold of 60%. For comparative genomic analysis, the five available genome sequences of K2 *K. pneumoniae* (RJF293, U25, 52.145, hvKP1, and NUHL24835) were retrieved from the GenBank and the pairwise single-nucleotide polymorphisms' (SNPs) analysis was carried out using the CSI Phylogeny 1.4 pipeline available at https://cge.cbs.dtu.dk/services/CSIPhylogeny/. Multiple genome alignment was performed using Mauve and BRIG programs. The Shared orthologies between the six K2 *K. pneumoniae* strains were identified by custom python script then illustrated in network graph by using Cytoscape Version 3.5.1. Finally, the function of ortholog clusters was classified according to clusters of orthologous groups (COGs).

This Whole-Genome Shotgun project has been deposited at GenBank under the accession number QBUJ00000000. The accession numbers for the genomes of strains RJF293, U25, 52.145, hvKP1, and NUHL24835 are CP014008, CP012043, FO834906, AOIZ00000000, and CP014004, respectively.

Ethics approval and informed consent

This study was conducted in accordance with the Declaration of Helsinki, and the ethical approval was granted from the Ethics Committees and Review Board of the First Affiliated Hospital, College of Medicine, Zhejiang University. Written informed consent of this case was obtained from the patient for publication.

Results

Patient characteristics

A 66-year-old woman was admitted to our hospital with numbness in her left lower limb for 3 days. She was accompanied by type 2 diabetes mellitus for 5 years and had a history of hypertension for 18 years. After MRI of the cervical spine and skull base, significant herniation of the cerebellar tonsils was observed, which calling for surgery intervention. Ceftriaxone 2.0 g once was given intravenous before the surgery for prophylaxis, and vancomycin 0.5 g Q8H was given after surgery for prophylaxis. The first day after surgery, the patient developed occipital headache and fever. The CSF examination is positive, with cloudy appearance, substantially increased polymorphonuclear (PMN) cells (3,200/mm^3; 92% segmented neutrophils and 8% lymphocytes) and significantly elevated protein level (2.66 g/L). A presume diagnosis of meningitis was made. Empiric treatment of intravenous imipenem (0.5 g Q8H) and vancomycin (0.5 g Q6H) was initiated immediately. Culture of the CSF grew *K. pneumoniae*, designated as KP_06. Two days later, the temperature elevation resolved and repeated lumbar puncture yielded slight cloudy CSF with improved parameters: PMN 180/mm^3 and protein 1.2 g/L. The CSF culture remained negative for *K. pneumoniae*, and thereafter, until the patient was discharged 73 days later.

Microbiological characteristics of KP_06

The KP_06 strain showed resistance to cephalosporins (ceftriaxone, ceftazidime, and cefepime), β-lactam/β-lactamase inhibitor combination (ampicillin–sulbactam and piperacillin–tazobactam) and aztreonam, while susceptible to fluoroquinolones, carbapenems, and tigecycline (Table S1). The coexistence of $bla_{CTX-M-3}$ and $bla_{CTX-M-14}$ in this strain was subsequently identified by PCR and sequencing.

A viscous string longer than 30 mm by the string test was observed in the KP_06 strain, suggesting HM phenotype. The strain was determined to be ST65 (*gapA–infB–mdh–pgi–phoE–rpoB–tonB*: 2–1–2–1–10–4–13) and capsular serotype K2 by PCR and sequencing. Several virulence genes including *fimH*, *wabG*, *ybtS*, *mrkD*, *uge*, and *entB* and the pLVPK-derived loci (*rmpA*, *rmpA2*, *iucA*, *iutA*, and *iroN*) were detected in KP_06. A pLVPK-like virulence plasmid of approximately 170 kb was further confirmed by hybridization of S1-digested DNA with *rmpA2* probe (Figure S1).

In vitro and in vivo virulence assessment of KP_06

The growth rates between KP_06 and other two strains (KP_07: ST23:K1 strain and KP_36: KPC-2-producing ST11 *K. pneumoniae* strain) were not differently significant (Figure S2).

Figure 1A depicts the ability of biofilm formation of the strains. The level of biofilm formation of KP_06 was similar to that of KP_36, while significantly lower than that of KP_07 (OD$_{570 nm}$, 0.29±0.01 vs 0.83±0.10, $P<0.001$). According to the criteria established by Saxena et al,[23] the cutoff OD value (OD$_C$) in our study was determined as OD$_{570 nm}$ = 0.29, hence the KP_06 and KP_36 were identified as nonbiofilm producers (OD$_{570 nm}$ ≤ 0.29), whereas the KP_07 was moderate producer (0.58 ≤ OD$_{570 nm}$ = 0.83≤1.16).

Serum killing sensitive grades 1 and 2 were found in KP_06 and KP_36 strains, respectively, whereas KP_07 showed serum resistance (grade 6) (Figure 1B). Consistent with the results of biofilm formation and serum killing assays, the KP_06 strain produced very low mortality rate in *G. mellonella*, leading to about 70% survival of *G. mellonella* at 48 hours after being challenged with 10^6 CFU of bacteria. The KP_36 showed to be more virulent than KP_06, resulting in 50% survival at 48 hours after being challenged with 10^6 CFU of bacteria. The classic ST23:K1 strain KP_07 resulted in 0% survival at 36 hours, displaying the most virulent strain (Figure 1C). Data about the effects of other inoculums of the strains are available in Figure S3.

Comparative genomic analysis

The genome sequence of KP_06 was 5,655,975 bp in size and had a GC content of 56.9% in 65 contigs, with N50 spanning 259,669 bp. The general genomic features of KP_06 are listed in Table 1. Although KP_06 and five other publicly available K2 *K. pneumoniae* strains (RJF293, U25, 52.145, hvKP1, and NUHL24835) displayed HM phenotype, they belonged to varied STs (Table 2). Pairwise SNP analysis for these six strains based on their raw sequencing reads showed that their core genome differed much from each other (Table S2), which consistent with the results of comparative chromosome analysis visualized by the circular maps (Figure 2A). Further comparative genomic analysis identified 1,378 shared orthologies among the six K2 *K. pneumoniae* strains, whereas the KP_06 contained seven unique orthologies (Figure 2B). We also analyzed the functional classification of ortholog clusters using the COGs' database. The results obtained are summarized in Table 3. The most abundant category in the shared orthologies was amino acid transport and metabolism
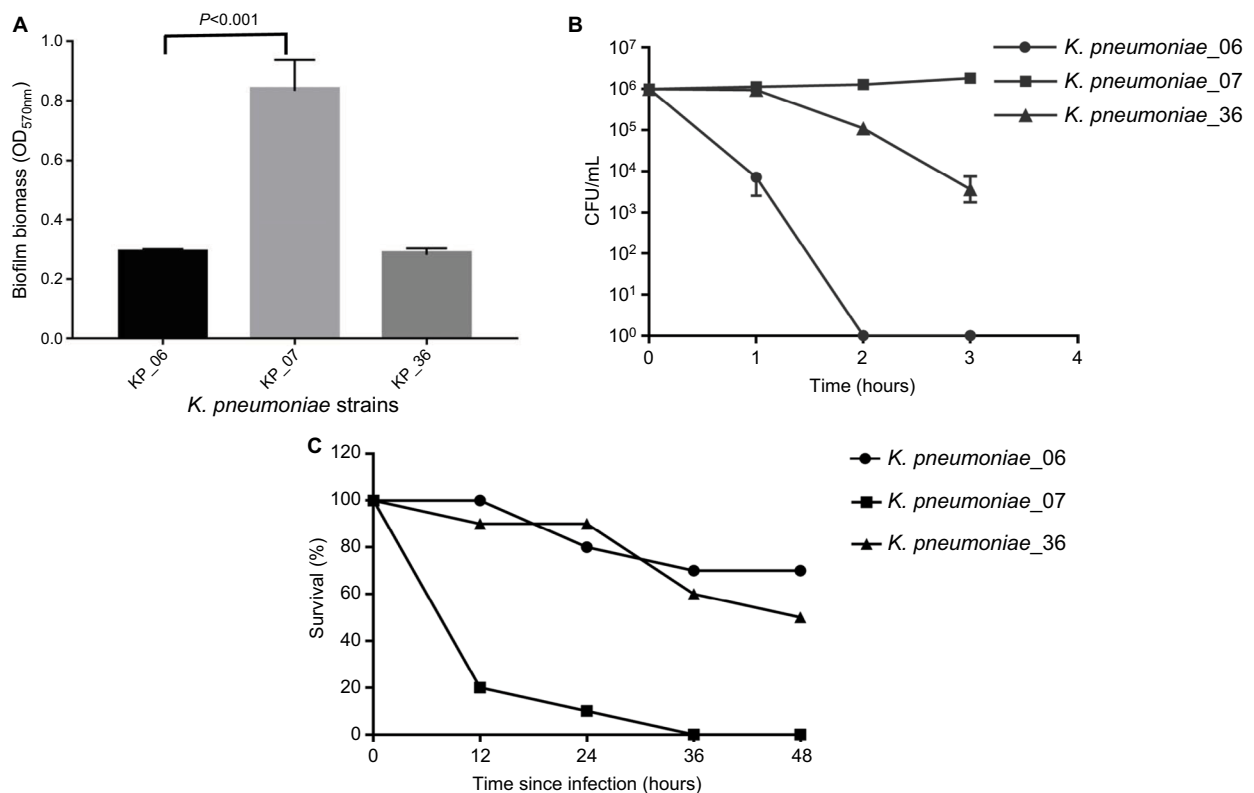
Figure 1 The detection of virulence potential of the KP_06.

Notes: (**A**) Biofilm biomass expressed as crystal violet OD ($OD_{570\,nm}$). Data are expressed as mean ± SD (error bars) of at least three independent experiments for each strain. (**B**) Survival of each strain was assessed by enumerating viable counts at 0, 1, and 3 hours of incubation in the pooled human serum at 37°C. Data are presented as mean ± SD (n=3 for each strain). (**C**) The effect of 1×10⁶ CFU of each *K. pneumoniae* strain on the survival of *G. mellonella*, whereas other doses of each *K. pneumoniae* strain induce dose-dependent lethality are shown in Figure S2. KP_07 is a ST23:K1 strain that harbored a ~220 kbp virulence plasmid and is used as positive control. KP_36 is a bla_{KPC-2}-producing ST11 strain that did not harbor virulence plasmid and is used as a negative control in these studies.

Abbreviations: CFU, colony-forming unit; *G. mellonella*, *Galleria mellonella*; *K. pneumoniae*, *Klebsiella pneumoniae*; ST, sequence type.

Table 1 General genomic features of the KP_06 isolate

Elements and characteristics	Value
Genome size (bp)	5,655,975
N50 (bp)	259,669
DNA coding region (%)	86.7
DNA GC content (%)	56.9
Contig number	65
Total genes	5,390
sRNA genes	46
tRNA genes	81
Plasmids	2
Protein-coding genes	5,338
Genes assigned to COGs	4,443
Genes assigned to Pfam domains	3,875

Abbreviation: CO, clusters of orthologous groups.

(172), followed by carbohydrate transport and metabolism (140), and inorganic ion transport and metabolism (124). The unique orthologies of KP_06 were identified as involved in (V) defense mechanisms, (Z) cytoskeleton, (M) cell wall/membrane/envelope biogenesis, (H) coenzyme transport and metabolism, (D) cell cycle control, cell division, and chromosome partitioning, (I) lipid transport and metabolism, (G) carbohydrate transport and metabolism, and (S) function unknown.

## Detection of virulence and antibiotic-resistant genes profiles by WGS

Multiple genes have been presumed associated with increased virulence in *K. pneumoniae* strains, hence WGS was used to identify virulence genes previously described as linked to hvKP against publicly available databases. All the six K2 strains carried the type 1 and type 3 fimbrial gene clusters (*mrk* and *fim*), the genes encoding for yersiniabactin (*ybt* and *irp* complex) and the genes involved in the synthesis of enterobactin (*ent* and *fep*). The detailed virulence genes detected are listed in Table S3. Although in vitro and in vivo virulence assays showed that the virulence of KP_06 was low, this strain unexpectedly carried more abundant of putative virulence genes (118 virulence genes) compared with five other K2 strains. It also harbored genes including

Table 2 General distribution of virulence and resistance genes of KP_06 and five other serotype K2 strains that were compared

Features of virulence and resistance	KP_06	RJF293	U25	KP52.145	hvKP1	NUHL24835
HM phenotype	Positive	Positive	Positive	Positive	Positive	Positive
Capsule serotype	K2	K2	K2	K2	K2	K2
Sequence types	ST65	ST374	ST14	ST66	ST86	ST14
HM phenotype regulator genes						
rmpA	+	+	+	+	–	–
rmpA2	+	+	+	+	–	–
Siderophore systems						
Enterobactin (*entABCEF*)	+	+	+	+	+	+
Aerobactin (*iucABCD* cluster)	+	+	+	+	–	–
Aerobactin receptor (*iutA*)	+	+	+	+	+	+
Yersiniabactin (*ybt* and *irp* complex)	+	+	+	+	+	+
Salmochelin (*iroBCD*)	+	+	+	+	–	–
Salmochelin receptor (*iroN*)	+	+	+	+	+	+
Fimbrial genes						
Type 3 fimbrial genes (*mrk* cluster)	+	+	+	+	+	+
Type 1 fimbrial genes (*fim* cluster)	+	+	+	+	+	+
Genotoxin						
Colibactin (*clbA* to *clbR* cluster)	+	+	–	–	–	–
Ferric uptake						
kfuABC cluster	–	+	–	+	+	+
Antibiotic-resistant genes						
β-Lactamases	bla_{SHV-11} bla_{TEM-1} $bla_{CTX-M-3}$ $bla_{CTX-M-14}$	$bla_{SHV-148}$	bla_{SHV-28} bla_{TEM-1} $bla_{CTX-M-15}$ bla_{OXA-9}	–	bla_{SHV-1}	bla_{SHV-28} $bla_{CTX-M-15}$ bla_{NDM-5}
Aminoglycoside-resistant genes	–	–	*aadA1* *aac(3)-IId* *aph(6)-Id*	–	*aph(3')-Ia*	*aadA2*
Fluoroquinolone-resistant genes	*qnrS1*	–	–	–	–	–
Other resistance genes	*fosA*	*fosA*	*fosA* *sul1*	*fosA*	*fosA*	*fosA*

Notes: "+" indicates the presence of the corresponding gene. "–" indicates the absence of the corresponding gene.
Abbreviation: HM, hypermucoviscosity.
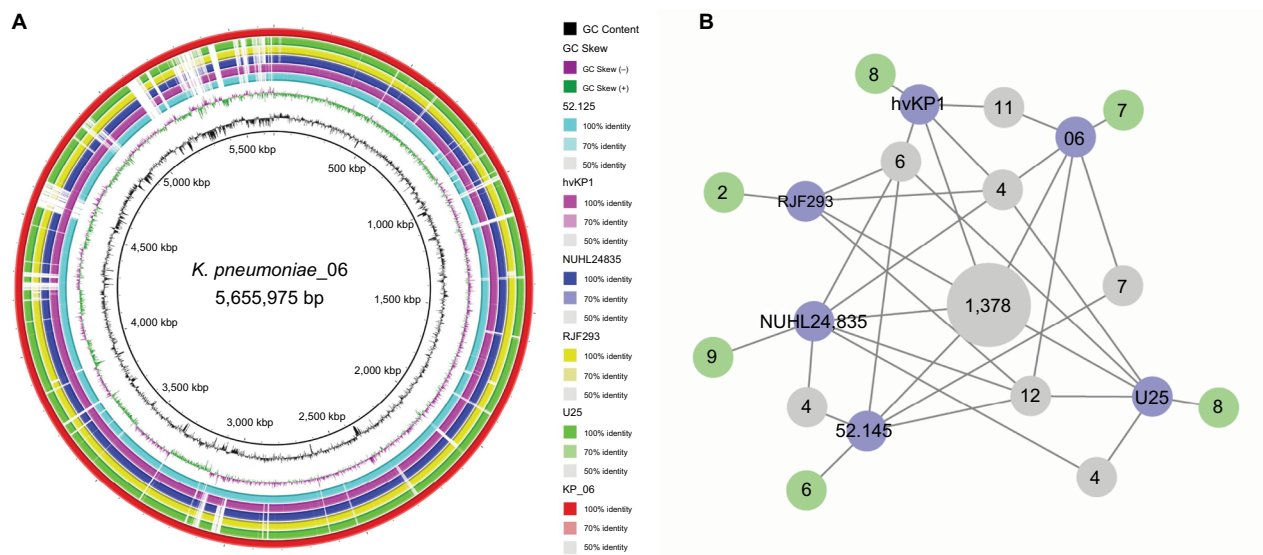
Figure 2 Genomic sequence comparative analysis of KP_06 and five HM K2-serotype *K. pneumoniae* strains.
Notes: (**A**) Whole-genome sequences' comparison of the strains. The circles from inside to outside indicate GC content of KP_06, GC skew of KP_06, and the K2-serotype *K. pneumoniae* strains 52.125, hvKP1, NUHL24835, RJF293, U25, and KP_06. The white and colored regions of the rings indicate absent and present, respectively. (**B**) Network graph shows shared orthologies and unique orthologies among the strains. Blue nodes: the name of *K. pneumoniae* strains; green nodes: the number of unique orthologies of the corresponding strain; the gray nodes: the number of shared orthologies among strains.
Abbreviations: HM, hypermucoviscosity; *K. pneumoniae*, *Klebsiella pneumoniae*.

Table 3 The distribution of COGs' functional catalogs of the orthologies for the six *K. pneumoniae* strains

COGs' categories	Shared orthologies	Unique orthologies					
		KP_06	RJF293	U25	52.145	hvKP1	NUHL24835
Nucleotide transport and metabolism (F)	49	0	0	0	0	0	0
Function unknown (S)	52	2	0	0	0	I	0
Signal transduction mechanisms (T)	65	0	I	0	0	0	0
Cell motility (N)	10	0	0	0	0	0	0
Transcription (K)	53	0	0	I	0	0	0
Amino acid transport and metabolism (E)	172	0	0	0	0	I	I
Defense mechanisms (V)	21	I	0	I	I	I	I
Cytoskeleton (Z)	0	I	0	0	0	0	0
Secondary metabolites biosynthesis, transport and catabolism (Q)	44	0	I	0	0	I	0
Cell wall/membrane/envelope biogenesis (M)	112	I	0	0	0	I	2
Energy production and conversion (C)	112	0	0	0	0	0	0
Replication, recombination and repair (L)	69	0	0	I	0	0	2
Post-translational modification, protein turnover, and chaperones (O)	58	0	0	0	0	I	I
Translation, ribosomal structure, and biogenesis (J)	100	0	0	0	0	I	I
Extracellular structures (W)	6	0	0	0	0	0	0
Mobilome: prophages, transposons (X)	5	0	0	2	2	0	I
Inorganic ion transport and metabolism (P)	124	0	0	0	I	I	0
Chromatin structure and dynamics (B)	I	0	0	0	0	0	0
Coenzyme transport and metabolism (H)	94	I	0	I	I	0	0
Cell cycle control, cell division, and chromosome partitioning (D)	24	I	0	0	0	0	0
General function prediction only (R)	116	0	0	2	0	0	0
Lipid transport and metabolism (I)	53	I	0	0	0	0	0
Carbohydrate transport and metabolism (G)	140	I	0	0	0	0	0
Intracellular trafficking, secretion, and vesicular transport (U)	30	0	0	0	I	I	I
Not assigned (–)	2	0	0	0	0	0	0

Abbreviations: COGs, clusters of orthologous groups; *K. pneumoniae*, *Klebsiella pneumoniae*.

iucABCD-iutA, *iroBCDN*, *rmpA*, and *rmpA2*, which usually located in the pLVPK-like virulence plasmid. This observation was consistent with the results of hybridization analysis, wherein the *rmpA2*-encoding virulence plasmid was present in KP_06 and KP_07 but not in the KP_36 strain (Figure S1). Additionally, the colibactin synthesis locus *clb* was detected in KP_06 and RJF293.

Although it is common sense that AMR genes were rarely found in HMKP strains, β-lactam resistance genes were identified in all of the analyzed K2 strains except KP52.145. Besides the simultaneous presence of different bla_{CTX-M} ($bla_{CTX-M-3}$ and $bla_{CTX-M-14}$) in KP_06, the presence of other β-lactam-resistant genes bla_{SHV-11} and bla_{TEM-1}, fluoroquinolone-resistant gene *qnrS1*, and fosfomycin-resistant gene *fosA* was also detected (Table 2).

Discussion

During the past decades, *K. pneumoniae* especially hvKP was recognized as the leading causative pathogen for adult bacterial meningitis in several Asian regions such as Taiwan[28] and South Korea.[29] However, to the best of our knowledge, there was only one case reporting suspected hvKP meningitis in mainland China up to now and the detailed virulence and genomic features of the causing pathogen were not investigated.[30] In the present study, the phenotypic and genomic features of a K2 HMKP causing meningitis were characterized.

The patient developing postneurosurgical meningitis was accompanied by diabetes mellitus and hypertension, both of which were well-documented predisposing factors for hvKP infections.[31,32] The KP_06 strain subsequently isolated from CSF exhibited HM nature and carried critical virulence determinants such as HM phenotype regulators (*rmpA*/*rmpA2*) and siderophore encoding systems (aerobactin, enterobactin, salmochelin, and yersiniabactin). The association between mortality and mucoid phenotype in K2 *K. pneumoniae* strain had been established in infected mouse model by Yu et al.[5] Thereafter, the terms of HMKP and hvKP have often been used synonymously.[3] As the in-depth research of virulence

mechanisms of this new variant, siderophores especially aerobactin are considered integral to bacterial virulence due to its ability allowing bacteria to scavenge for iron from host transport proteins.[33] Hence, recent studies viewed genetic factors associated with HM together with iron acquisition as unequivocal marker for hvKP identification.[34,35] However, in the current case, even harboring these typical features of hvKP, the KP_06 was assigned to a lowly virulent strain by comprehensive virulence assays. Therefore, future studies focus on hvKP calling for a combination of phenotype, genomic data, and experimental virulence. The identification of biomarkers with high accuracy for hvKP would be very imperative.

K. pneumoniae isolates with high degrees of virulence and drug resistance have emerged.[7,8,11,12] Our analysis showed that ESBL genes were detected in four K2 strains (KP_06, RJF293, U25, and NUHL24835), which indicated that the presence of AMR genes in serotype K2 *K. pneumoniae* might be underestimated previously. The successful transformation of plasmids carrying bla_{CMY-2}, bla_{KPC-2}, or bla_{DHA-1} genes into hvKP has been documented.[8,36,37] Otherwise, a carbapenem-resistant *K. pneumoniae* strain that acquired part of an hvKP virulence plasmid causing a lethal nosocomial outbreak has been reported recently.[21] Given the KP_06 belonged to the K2 and ST65 hypervirulent clone, we speculated it might be hvKP that acquired plasmids harboring different bla_{CTX-M} genes.

As described by several studies,[18] we chose a wild-susceptible hypermucoviscous ST23:K1 *K. pneumoniae* strain KP_07 as the control of hypervirulence in virulence assays. The KP_06 strain displayed much weaker capability of biofilm formation, serum resistance, and lethality compared to KP_07. Lavigne et al[38] demonstrated that the presence of bla_{KPC} was accompanied by impaired virulence of the isolates in *Caenorhabditis elegans* model. Choi et al[39] also illustrated that the acquisition of colistin resistance leaded to the loss of HM phenotype, resulting in a decreased virulence in hvKP. These findings suggested that acquiring antibiotic resistance might impair the virulence of the clinical strains. However, comparative genomic analysis showed the virulence genes were more abundance in KP_06 than in other K2 hvKP (RJF293, 52.145, and NUHL24835). In addition, the presence of pLVPK-like virulence plasmid in this strain was confirmed. These results implied that neither possessing a virulence plasmid nor having virulence-associated genes (clusters) is equal to a higher level of virulence. Recently, Palacios et al[40] reported the mutation of two *marR* (multiple antibiotic resistance regulator) homologous genes, *kvrA* and

kvrB, significantly impaired the virulence of K1 and K2 serotype hvKP strains through reducing the expression of capsule synthesis associated genes. Moreover, a strong correlation between the quantitative siderophore production and in vivo virulence of *K. pneumoniae* has also been identified by Russo et al.[4] Hence, it was reasonable to speculate that the decreased virulence of KP_06 might attribute to the low expression level of some critical virulence-associated determinants, but the exact mechanisms remain further exploration.

Although the in vitro and in vivo assays showed a decreased virulence of KP_06, the isolate caused a serious intracranial infection in a postoperative patient. One explanation was the disruption of skin and mucous barriers due to cervical spine surgery facilitated the invasion of bacteria, even if relatively low virulence isolate. Additionally, the actual virulence of bacteria in patients can be affected by the host environment at the site of infection. For instance, high glucose concentrations enhanced capsular polysaccharide biosynthesis of *K. pneumoniae*,[41] which may happen in patients with diabetes mellitus. The biofilm formation significantly induced by the subinhibitory concentration of imipenem in *Acinetobacter baumannii* has also been reported.[42] Further experiments to evaluate the virulence of KP_06 under stress conditions, such as antibiotics, are needed.

Conclusion

We investigated the virulence and genetic features of an HM *K. pneumoniae* strain (KP_06) collected from human CSF. Although displaying typical hypervirulent characteristics, this coharboring $bla_{CTX-M-3}$ and $bla_{CTX-M-14}$ K2 and ST65 *K. pneumoniae* was demonstrated to be a lowly virulent strain by in vitro and in vivo methods. However, the detailed mechanisms concerned with an impaired virulence of this strain remained further exploration. Although it is thought that the prevalence of *K. pneumoniae* strain with high degree of drug resistance and virulence will pose great threat to public health, our study still makes a valuable addition to the growing knowledge of the evolutionary relationship between resistance and virulence in *K. pneumoniae*.

Consent for publication

Written informed consent for publication of the individual details was obtained from the patient.

Acknowledgments

thank the team of curators from the Institute Pasteur MLST (Paris, France) for importing novel alleles and profiles at http://bigsdb.pasteur.fr/klebsiella/klebsiella.html. This work was supported by the National Natural Science Foundation (no 81301459), Natural Science Foundation of Zhejiang Province (no LQ13H190001), Medical Science and Technology Project of Zhejiang Province (no 2014KYB096), and National Key Program for Infectious Diseases of China (no 2017ZX10103008).

Disclosure

The authors report no conflicts of interest in this work.

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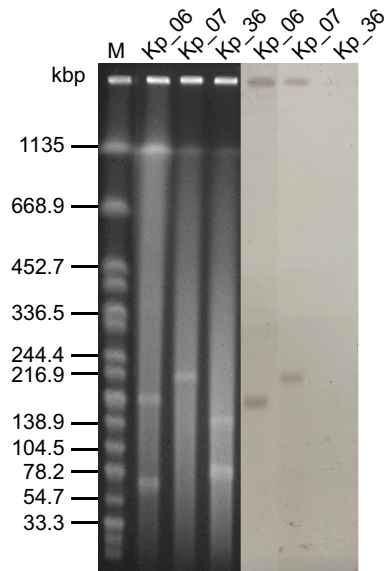
Supplementary materials

Figure S1 S1-PFGE and southern hybridization analysis of the *K. pneumoniae* strains.
Notes: S1 nuclease digestion of total DNA of *K. pneumoniae* isolates was followed by PFGE. Plasmid bands are shown as linearized fragments on the gel. The presence of the virulence plasmid was confirmed by hybridizing with the rmpA2 probe. Lane M, reference standard strain *Salmonella* serotype Braenderup H9812 restricted with XbaI.
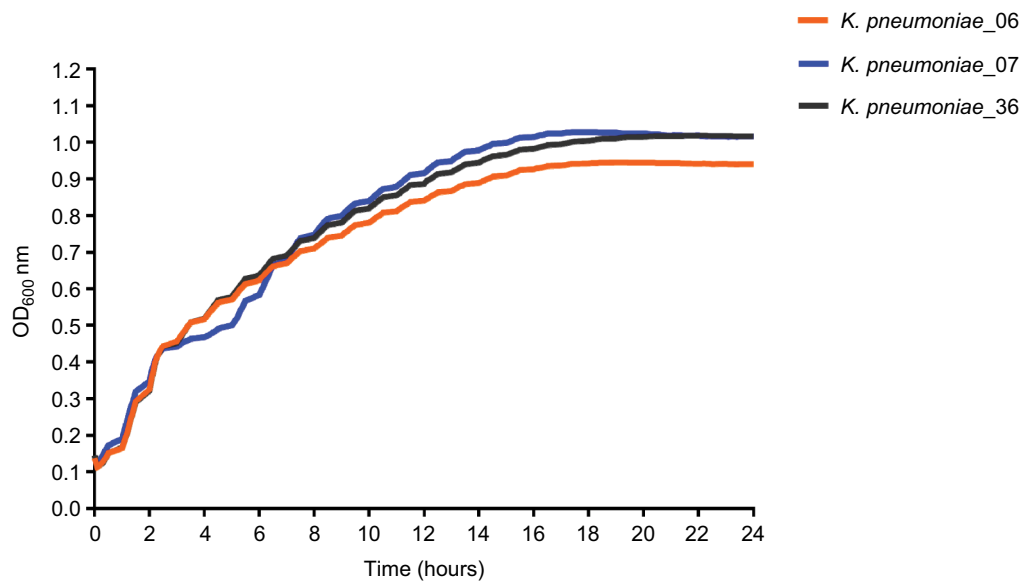**Abbreviations:** *K. pneumoniae*, *Klebsiella pneumoniae*; M, marker; PFGE, pulsed-field gel electrophoresis.

Figure S2 The growth curves of the *K. pneumoniae* strains.
Abbreviation: *K. pneumoniae*, *Klebsiella pneumoniae*.

A *K. pneumoniae*_06
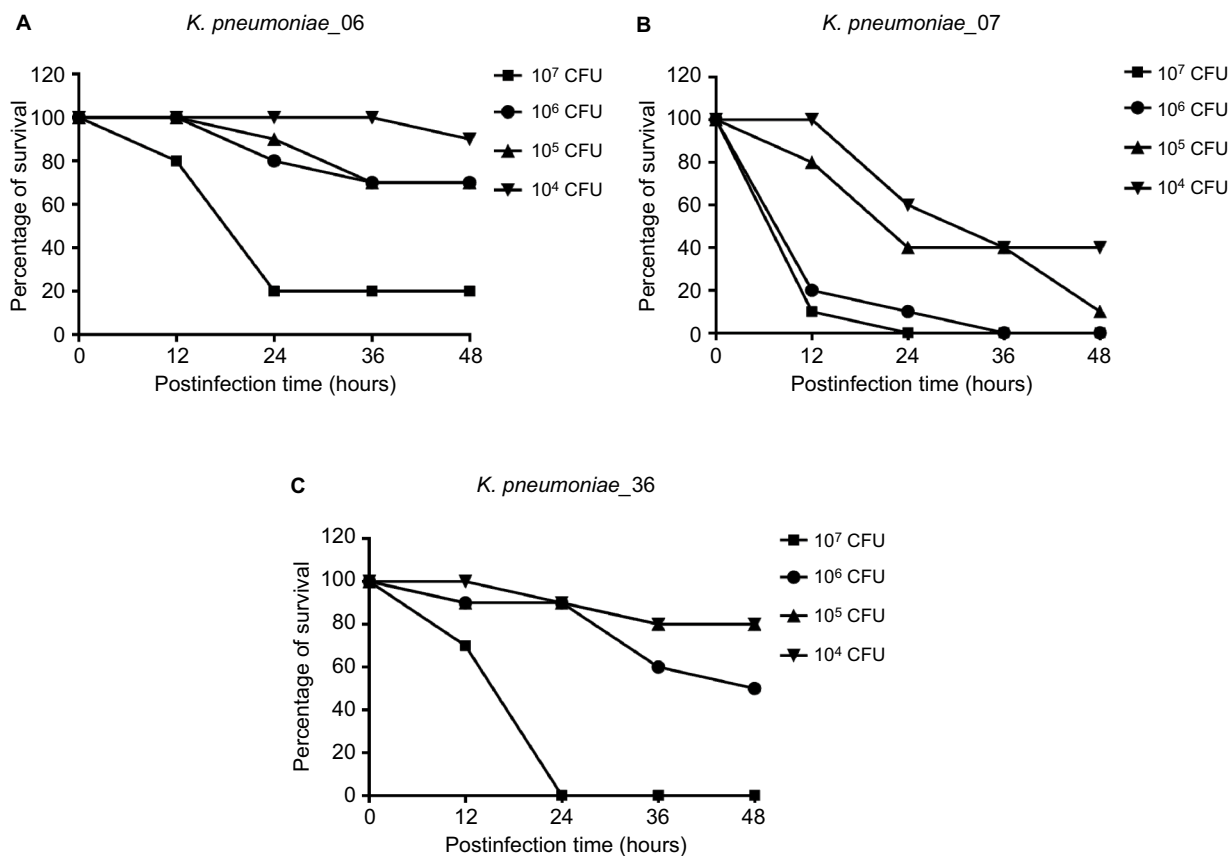
B *K. pneumoniae*_07

C *K. pneumoniae*_36

Figure S3 Each dose of *K. pneumoniae* strains' infection of *G. mellonella* induces dose-dependent lethality.
Abbreviations: CFU, colony-forming unit; *G. mellonella*, *Galleria mellonella*; *K. pneumoniae*, *Klebsiella pneumoniae*.

Table S1 Antibiotics susceptibility profile of the KP_06 strain.

Antibiotics	MIC (µg/mL)	Interpretation
Ampicillin	≥32	R
Amikacin	≤2	S
Aztreonam	16	R
Ciprofloxacin	1	S
Ceftriaxone	≥64	R
Gentamicin, CN10	≤1	S
Levofloxacin	1	S
Ceftazidime	≥64	R
Cefepime	≥32	R
Ampicillin–sulbactam	≥32	R
Piperacillin–tazobactam	8	R
Ertapenem	≤0.12	S
Imipenem	0.5	S
Cefotetan	≤4	S
Trimethoprim–sulfamethoxazole	≤20	S
Tigecycline	1	S

Abbreviations: MIC, minimum inhibitory concentration; R, resistant; S, susceptible.

Table S2 Pairwise SNP comparison between KP_06 isolate and five other HM serotype K2 strains

Strain ID	KP_06	52.145	NUHL24835	RJF293	U25	hvKP1
KP_06	0	20,386	21,551	21,578	21,550	21,096
52.145	20,386	0	21,316	21,281	21,316	20,919
NUHL24835	21,551	21,316	0	19,447	597	20,382
RJF293	21,578	21,281	19,447	0	19,465	20,434
U25	21,550	21,316	597	19,465	0	20,374
hvKP1	21,096	20,919	20,382	20,434	20,374	0
NTUH-K2044	20,286	19,950	19,218	19,196	19,113	19,720

Notes: The complete genomic sequence of NTUH-K2044 was used as the reference. The numbers depicted differences in SNPs exhibited by each strain pair.
Abbreviations: HM, hypermucoviscosity; hvKP1, hypervirulent Klebsiella pneumoniae; SNPs, single-nucleotide polymorphisms.

Table S3 Putative virulence genes detected in the genome of KP_06 strain and five other serotype K2 strains

Virulence genes	*K. pneumoniae* strains					
	KP_06	RJF293	U25	52.145	hvKP1	NUHL24835
Chromosome						
clbA	+	+	−	−	−	−
clbB	+	+	−	−	−	−
clbC	+	+	−	−	−	−
clbD	+	+	−	−	−	−
clbE	+	+	−	−	−	−
clbF	+	+	−	−	−	−
clbG	+	+	−	−	−	−
clbH	+	+	−	−	−	−
clbI	+	+	−	−	−	−
clbJ	+	+	−	−	−	−
clbK	+	+	−	−	−	−
clbL	+	+	−	−	−	−
clbM	+	+	−	−	−	−
clbN	+	+	−	−	−	−
clbO	+	+	−	−	−	−
clbP	+	+	−	−	−	−
clbQ	+	+	−	−	−	−
clbR	+	+	−	−	−	−
kfuA	−	+	−	+	+	+
kfuB	−	+	−	+	+	+
kfuC	−	+	−	+	+	+
kvgA	+	−	−	+	−	−
kvgS	+	−	+	+	−	−
mceC	+	+	+	+	−	−
mrkA	+	+	+	+	+	+
mrkB	+	+	+	+	+	+
mrkC	+	+	+	+	+	+
mrkD	+	+	+	+	+	+
mrkF	+	+	+	+	+	+
mrkH	+	+	+	+	+	+
mrkI	+	+	+	+	+	+
mrkJ	+	+	+	+	+	+
clpP	+	+	+	+	−	−
clpE	−	−	+	−	−	−
algU	+	+	+	+	+	+
ureB	+	+	+	+	+	+
ureG	+	+	+	+	+	+
rfaE	+	+	+	+	+	+

(*Continued*)

Table S3 (*Continued*)

Virulence genes	K. pneumoniae strains					
	KP_06	**RJF293**	**U25**	**52.145**	**hvKP1**	**NUHL24835**
rfaD	+	+	+	+	+	+
fyuA	+	+	+	+	+	+
ybtE	+	+	+	+	+	+
ybtT	+	+	+	+	+	+
ybtU	+	+	+	+	+	+
irp1	+	+	+	+	+	+
irp2	+	+	+	+	+	+
ybtA	+	+	+	+	+	+
ybtP	+	+	+	+	+	+
ybtQ	+	+	+	+	+	+
ybtX	+	+	+	+	+	+
ybtS	+	+	+	+	+	+
mgtC	+	+	+	+	+	+
fimB	+	+	+	+	+	+
fimE	+	+	+	+	+	+
fimA	+	+	+	+	+	+
fimI	+	+	+	+	+	+
fimC	+	+	+	−	+	+
fimD	+	+	+	−	+	+
fimF	+	+	+	−	+	+
fimG	+	+	+	−	+	+
fimH	+	+	+	−	+	+
focA	+	+	+	+	+	+
foci	−	−	−	+	−	−
focC	+	+	+	−	+	+
focD	+	+	+	−	+	+
chuS	+	+	+	+	+	+
chuU	+	+	+	+	+	+
fepA	+	+	+	+	+	+
fepB	+	+	+	+	+	+
fepC	+	+	+	+	+	+
fepD	+	+	+	+	+	+
fepG	+	+	+	+	+	+
entF	+	+	+	+	+	+
entC	+	+	+	+	+	+
entE	+	+	+	+	+	+
entB	+	+	+	+	+	+
entA	+	+	+	+	+	+
lcl	+	+	+	+	+	+
ompA	+	+	+	+	+	+
sfaA	+	+	+	+	+	+
htpB	+	+	+	+	+	+
sodB	+	+	+	+	+	+
lspE	+	−	−	−	−	−
gspE	+	+	+	+	+	+
gspG	+	+	+	+	+	+
manB	+	+	+	+	+	+
yagY/ecpB	+	+	+	+	+	+
ykgK/ecpR	+	+	+	+	+	+
yagZ/ecpA	+	+	+	+	+	+
yagX/ecpC	+	+	+	+	+	+

(*Continued*)

Table S3 (*Continued*)

Virulence genes	*K. pneumoniae* strains					
	KP_06	**RJF293**	**U25**	**52.145**	**hvKP1**	**NUHL24835**
yagW/ecpD	+	+	+	+	+	+
yagV/ecpE	+	+	+	+	+	+
acpXL	+	−	+	−	−	−
sfaD	−	−	−	+	−	−
sfaE	+	+	+	−	+	+
sfaF	+	+	+	−	+	+
shuS	+	+	+	+	+	+
shuU	+	+	+	+	+	+
kdsB	+	+	+	+	+	+
msbA	+	+	+	+	+	+
galU	+	+	+	+	+	+
rffG	+	+	+	+	+	+
lpxD	+	+	+	+	+	+
lpxB	+	+	+	+	+	+
lpxA	+	+	+	+	+	+
rfaF	+	+	+	+	+	+
lpxC	+	+	+	+	+	+
gmhA/lpcA	+	+	+	+	+	+
kdsA	+	+	+	+	+	+
pilW	+	−	+	+	−	−
luxS	+	+	+	+	+	+
pavB/pfbB	−	−	+	−	−	−
Fes	+	+	+	+	+	+
entS	+	+	+	+	+	+
IlpA	+	+	+	+	+	+
Plasmid						
rmpA	+	+	+	+	−	−
rmpA2	+	+	+	+	−	−
iroN	+	+	+	+	+	+
iroD	+	+	+	+	−	−
iroC	+	+	+	+	−	−
iroB	+	+	+	+	−	−
iucA	+	+	+	+	−	−
iucB	+	+	+	+	−	−
iucC	+	+	+	+	−	−
iucD	+	+	+	+	−	−
iutA	+	+	+	+	+	+

Notes: "+" indicates the presence of the corresponding gene. "−" indicates the absence of the corresponding gene.
Abbreviations: hvKP1, hypervirulent *K. pneumoniae*; *K. pneumoniae*, *Klebsiella pneumoniae*.