A fatal outbreak of ST11 carbapenem-resistant hypervirulent *Klebsiella pneumoniae* in a Chinese hospital: a molecular epidemiological study

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Summary

Background **Hypervirulent *Klebsiella pneumoniae* strains often cause life-threatening community-acquired infections in young and healthy hosts, but are usually sensitive to antibiotics. In this study, we investigated a fatal outbreak of ventilator-associated pneumonia caused by a new emerging hypervirulent *K pneumoniae* strain.**

Methods **The outbreak occurred in the integrated intensive care unit of a new branch of the Second Affiliated Hospital of Zhejiang University (Hangzhou, China). We collected 21 carbapenem-resistant *K pneumoniae* strains from five patients and characterised these strains for their antimicrobial susceptibility, multilocus sequence types, and genetic relatedness using VITEK-2 compact system, multilocus sequence typing, and whole genome sequencing. We selected one representative isolate from each patient to establish the virulence potential using a human neutrophil assay and *Galleria mellonella* model and to establish the genetic basis of their hypervirulence phenotype.**

Findings **All five patients had undergone surgery for multiple trauma and subsequently received mechanical ventilation. The patients were aged 53–73 years and were admitted to the intensive care unit between late February and April, 2016. They all had severe pneumonia, carbapenem-resistant *K pneumoniae* infections, and poor responses to antibiotic treatment and died due to severe lung infection, multiorgan failure, or septic shock. All five representative carbapenem-resistant *K pneumoniae* strains belonged to the ST11 type, which is the most prevalent carbapenem-resistant *K pneumoniae* type in China, and originated from the same clone. The strains were positive on the string test, had survival of about 80% after 1 h incubation in human neutrophils, and killed 100% of wax moth larvae (*G mellonella*) inoculated with 1×10^6 colony-forming units of the specimens within 24 h, suggesting that they were hypervirulent *K pneumoniae*. Genomic analyses showed that the emergence of these ST11 carbapenem-resistant hypervirulent *K pneumoniae* strains was due to the acquisition of a roughly 170 kbp pLVPK-like virulence plasmid by classic ST11 carbapenem-resistant *K pneumoniae* strains. We also detected these strains in specimens collected in other regions of China.**

Interpretation **The ST11 carbapenem-resistant hypervirulent *K pneumoniae* strains pose a substantial threat to human health because they are simultaneously hypervirulent, multidrug resistant, and highly transmissible. Control measures should be implemented to prevent further dissemination of such organisms in the hospital setting and the community.**

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Introduction

Klebsiella pneumoniae is a major Gram-negative bacterial pathogen that can cause invasive hospital-acquired infections among immunocompromised patients.[1] Pyogenic liver abscesses caused by *K pneumoniae* have become a serious clinical challenge in Asia. In Taiwan, more than 3000 new cases of pyogenic liver abscesses occur each year.[2] Compared with the classic *K pneumoniae* strains that cause other types of opportunistic infections, pyogenic liver abscess-associated *K pneumoniae* strains often have substantially higher virulence and are therefore designated hypervirulent *K pneumoniae*. Hypervirulent *K pneumoniae* has the ability to cause life-threatening, community-acquired infections such as liver abscesses, pneumonia, meningitis, and endophthalmitis in young and healthy individuals and is therefore associated with high morbidity and mortality.[3] These strains can efficiently acquire iron and produce an increased amount of capsular substance compared with classic *K pneumoniae*, which confers a hypermucoviscous phenotype that is detectable as a positive result on the string test (a viscous string of >5 mm in length is produced when touched with an inoculation loop).[3] The hypervirulent phenotype of *K pneumoniae* is thought to be attributable to the carriage of a virulence plasmid harbouring two capsular polysaccharide (CPS) regulator genes (*rmpA* and *rmpA2*) and several siderophore gene clusters that contribute to the hypermucoviscous phenotype.[4,5] Correlation between carriage of the virulence plasmid and the hypervirulence phenotype has also been reported.[6] To date, most hypervirulent *K pneumoniae* strains have been K1 or K2 types and have remained antibiotic-sensitive.[7]

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Research in context

Evidence before this study

We searched PubMed with no language restrictions for reports that were published from Jan 1, 2000, to May 5, 2017, and contained the terms "Hypervirulent and *Klebsiella pneumoniae*", "Hypervirulent and *Klebsiella pneumoniae* and antimicrobial resistance", "*Klebsiella pneumoniae* and ST11 and antimicrobial resistance", "China and Hypervirulent *Klebsiella pneumoniae*", "Hypervirulent *Klebsiella pneumoniae* and virulence plasmid", "ST11 and *Klebsiella pneumoniae* and virulence plasmid ", "Hypervirulent *Klebsiella pneumoniae* and virulence ", and "*Klebsiella pneumoniae* and ventilator-associated pneumonia". We found only one publication that described the emergence of carbapenem-resistant K2/ST65 and K2/ST25 hypervirulent *Klebsiella pneumoniae* strains, as well as the first case of ST11 hypervirulent *K pneumoniae* infection in Beijing, China, in 2015, which was defined on the basis of string test result. However, no virulence assays, such as human neutrophil survival assay or those involving animal models, were used to confirm the hypervirulence phenotype of this strain, in which no virulence plasmid or virulence-encoding genes were detectable. Additionally, the study found no significant difference between the clinical symptoms of patients infected by this carbapenem-resistant hypervirulent *K pneumoniae* strain and those infected with classic *K pneumoniae* strains. To date, carbapenem resistance and hypervirulence phenotypes are associated predominantly with K1 and K2 type organisms carrying virulence plasmids, with only carbapenem-resistant K1 hypervirulent *K pneumoniae* strains having been reported to cause lethal infections. No report has described the phenotypic features and underlying resistance mechanisms of the hypervirulent ST11 *K pneumoniae* strains or clinical manifestations of diseases caused by such strains.

Added value of this study

Our results showed that carbapenem-resistant ST11 hypervirulent *K pneumoniae* (ST11 carbapenem-resistant hypervirulent *K pneumoniae*) strains have emerged and can cause fatal ventilator-associated pneumonia in patients in hospital. Such strains have disseminated across various regions of China, accounting for as much as 3% of clinical ST11 carbapenem-resistant *K pneumoniae* infections in the country. ST11 carbapenem-resistant hypervirulent *K pneumoniae* has a hypervirulence phenotype characterised by a positive string test result, extremely high survival on exposure to human neutrophils, and high virulence in a wax moth (*Galleria mellonella*) larva infection model. The emergence of ST11 carbapenem-resistant hypervirulent *K pneumoniae* strains was due to the acquisition of a roughly 170 kbp virulence plasmid carrying the *rmpA2* and aerobactin biosynthesis genes by classic ST11 carbapenem-resistant *K pneumoniae* strains.

Implications of all the available evidence

ST11 carbapenem-resistant hypervirulent *K pneumoniae* strains are expected to pose a substantial threat to human health because these strains are simultaneously hypervirulent, multidrug resistant, and highly transmissible. Because of the genetic similarity between ST11 and ST258 types of *K pneumoniae*, ST258 hypervirulent *K pneumoniae* strains might have emerged and become transmissible in the USA and in European countries. Future research should focus on the development of intervention measures to prevent further dissemination of such organisms in hospital settings.

In reports from 2013, the US Centers for Diseases Control and Prevention (CDC) described the emergence of carbapenem-resistant Enterobacteriaceae strains, which commonly cause untreatable or hard-to-treat infections among patients in hospitals, as an urgent public health threat. Carbapenem-resistant *K pneumoniae* strains account for roughly 70–90% of clinical carbapenem-resistant Enterobacteriaceae infections in the European Union and China.[8,9] The most common clinical carbapenem-resistant *K pneumoniae* strains are those of the clonal group (CG) 258, with ST258 and ST11 being the most prevalent multilocus sequence types in different parts of the world.[10] ST258 is a hybrid clone composed of 80% of ST11 genome and 20% of ST442 genome.[10,11] ST258 has disseminated worldwide since its emergence in the early 2000s and has become particularly prevalent in North America, Latin America, and several European countries.[10] However, in Asia, the dominant clone is ST11 carbapenem-resistant *K pneumoniae*, which accounts for up to 60% of carbapenem-resistant *K pneumoniae* in China.[9] Consequently, in recent years, ST11 has been regarded as the most transmissible clone contributing to the increasing prevalence of carbapenem-resistant *K pneumoniae* in China. In this study, we investigate a fatal ventilator-associated pneumonia outbreak in an intensive care unit (ICU) in a Chinese hospital with the aim of identifying the molecular basis for the hypervirulence of the emerging ST11 carbapenem-resistant *K pneumoniae* strains responsible for this outbreak.

Methods

Outbreak investigation

In late March and April, 2016, we identified several cases of severe pneumonia in the integrated ICU of a new branch of the Second Affiliated Hospital of Zhejiang University (Hangzhou, China). This new integrated ICU consists of three units with a total of 26 wards and 40 beds, and has been open since November, 2015. Before the outbreak described, no patients in this ICU had reported severe symptoms of pneumonia or had poor outcomes with antibiotic treatments. We therefore initiated an outbreak investigation that included five patients. The first patient in this outbreak, who was suspected of being the index

patient, had previously been admitted to a local hospital (Fuyang People's Hospital in the Fuyang district of Hangzhou) for multiple trauma caused by a car accident and was transferred to the ICU of our hospital on Feb 28, 2016. The other four patients were admitted in March and April. All five patients stayed in different wards of the ICU, but all had overlapping stays in the ICU during this outbreak. All clinical data were collected in accordance with the ORION checklist. Bacterial strains were isolated from sample specimens at the clinical laboratory of the hospital. We subjected the isolated strains to phenotypic and genotypic characterisation to identify the causative agent and understand the molecular basis of the high mortality of these causative agents. We obtained permission to report the cases from the patients' families.

Phenotypic characterisation

We used a VITEK-2 compact system (bioMérieux, Marcy-l'Étoile, France) to establish the strain identity and antimicrobial susceptibilities of the isolates and we interpreted these in accordance with the guideline document M100-S26 established by Clinical and Laboratory Standards Institute.[12] We confirmed the species identity of all isolates via matrix-assisted laser desorption/ionisation mass spectrometry (Bruker Daltonics, Billerica, MA, USA).

We identified capsular antigens by PCR targeting genes encoding for K1, K2, K5, K20, K54 and K57 antigens as previously described.[13] We screened for β lactamase genes by PCR as previously reported.[14]

We did conjugation experiments, pulsed-field gel electrophoresis (PFGE) multilocus sequence typing (MLST), S1-PFGE, and Southern hybridisation as reported previously.[14] We also did plasmid curing experiments, which were done as previously described,[15] to test whether the putative virulence plasmid contributed to the hypervirulence phenotype of these strains.

For the string test to identify the hypermucoviscous phenotype, all isolates were inoculated onto agar plates containing 5% sheep blood and incubated at 37°C overnight. The string test was deemed positive when a viscous string longer than 5 mm could be generated by touching and pulling a single colony upwards with a standard inoculation loop.[3]

As a test of virulence, we did the human neutrophil assay as described previously.[16] Briefly, we isolated neutrophils from the venous blood of healthy volunteers, who had provided written informed consent before participation in the studies. We measured the bactericidal activity of neutrophils by incubating 1×10^6 neutrophils with 1×10^6 colony-forming units (CFU) of opsonised *K pneumoniae* in RPMI/H medium at 37°C with intervals of 15 min, 30 min, and 60 min, with gentle rotation. 1% saponin was added to each tube, mixed, and then chilled on ice for 15 min before diluting and plating on Luria broth agar. Survival was expressed as the percentage of CFUs recorded after neutrophil treatment compared with the control. We analysed the data using a one-way ANOVA and Tukey's post-hoc test on GraphPad Prism version 6.0b).

We also tested virulence in wax moth (*Galleria mellonella*) larvae weighing about 300 mg (purchased from Tianjin Huiyude Biotech Company, Tianjin, China) and maintained on woodchips in the dark at 15°C until being used. Overnight cultures of *K pneumoniae* strains were washed with phosphate-buffered saline (PBS) and further adjusted with PBS to concentrations of 1×10^4 CFU/mL, 1×10^5 CFU/mL, 1×10^6 CFU/mL, and 1×10^7 CFU/mL. We infected the *G mellonella* with the bacteria as described previously and we recorded the survival rate of the *G mellonella*.[17,18] All experiments were done in triplicate.

Genomic characterisation

We sequenced genomes using the NextSeq 500 sequencing platform (Illumina, San Diego, CA, USA). We trimmed or filtered raw reads to remove low-quality sequences and adaptors and assembled them de novo with the SPAdes Genome Assembler version 3.9.1.[19] We annotated the draft genome sequences with the RAST tool version 2[20] and Prokka 1.12.[21] We compared genome sequences using the BLAST Ring Image Generator (BRIG) version 0.95.[22] We did the pangenome analysis with Roary: the pangenome pipeline (version 3.6.0) using the Prokka annotation.[23] We analysed single nucleotide polymorphisms (SNPs) with the CSI Phylogeny 1.4 pipeline available from the Center for Genomic Epidemiology with default settings and whole genome sequence raw reads for the analysis. We did capsular typing on the assembled sequences using Kaptive.[24] We deposited the genome sequences in GenBank under the accession numbers SAMN07259328 (*K pneumoniae* 1), SAMN07259327 (*K pneumoniae* 2), SAMN07259329 (*K pneumoniae* 3), SAMN07259326 (*K pneumoniae* 4), SAMN07259325 (*K pneumoniae* 5), SAMN07259330 (FJ8), SAMN07259331 (FJ9), SAMN07259332 (hypervirulent *K pneumoniae* 1088), and SAMN07259333 (carbapenem-resistant *K pneumoniae* SH1). We identified the antibiotic resistance genes and virulence loci with the assembled genome sequences using ResFinder 2.1[25] and the BIGSdb *Klebsiella* genome database.[26] We generated a heatmap of the virulence loci with Genesis software version 1.7.7.[27]

For the **CSI Phylogeny pipeline** see https://cge.cbs.dtu.dk/services/CSIPhylogeny/

To check for the presence of the virulence plasmid in clinical ST11 carbapenem-resistant *K pneumoniae* isolates collected from patients in major hospitals located in 25 provinces and municipalities in China (provided by our collaborators),[9] we screened the isolates with four sets of primers (appendix p 8) targeting the *rmpA*, *rmpA2*, *iucA*, and *iroN* genes.

See **Online** for appendix

Role of the funding source

The funder of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Results

Five patients aged 53–73 years were admitted to the ICU between late February and April, 2016. These five patients were admitted for various forms of trauma due to car accidents, falling objects, or atlantoaxial subluxation. They all underwent surgery, followed by antimicrobial treatment and mechanical ventilation (figure 1; appendix p 9). The five patients developed pneumonia and all showed the typical symptoms of pulmonary oedema, pleural effusion, excessive sputum, and shortness of breath. Multiple species of bacteria could be isolated from blood, sputum, and faecal samples of the patients, with carbapenem-resistant *K pneumoniae* being consistently isolated in all patients and present in different types of specimen before the patients died, suggesting that carbapenem-resistant *K pneumoniae* might be the causative agent of the outbreak. They responded poorly to antibiotic treatment. The duration of the infections lasted from 10 days to 4 months for these patients. All five patients died of severe lung infection, multiorgan failure, or septic shock after carbapenem-resistant *K pneumoniae* could be recovered from their blood or sputum samples. Detailed information about the patients is available in the appendix (pp 3–7, 14, 15).

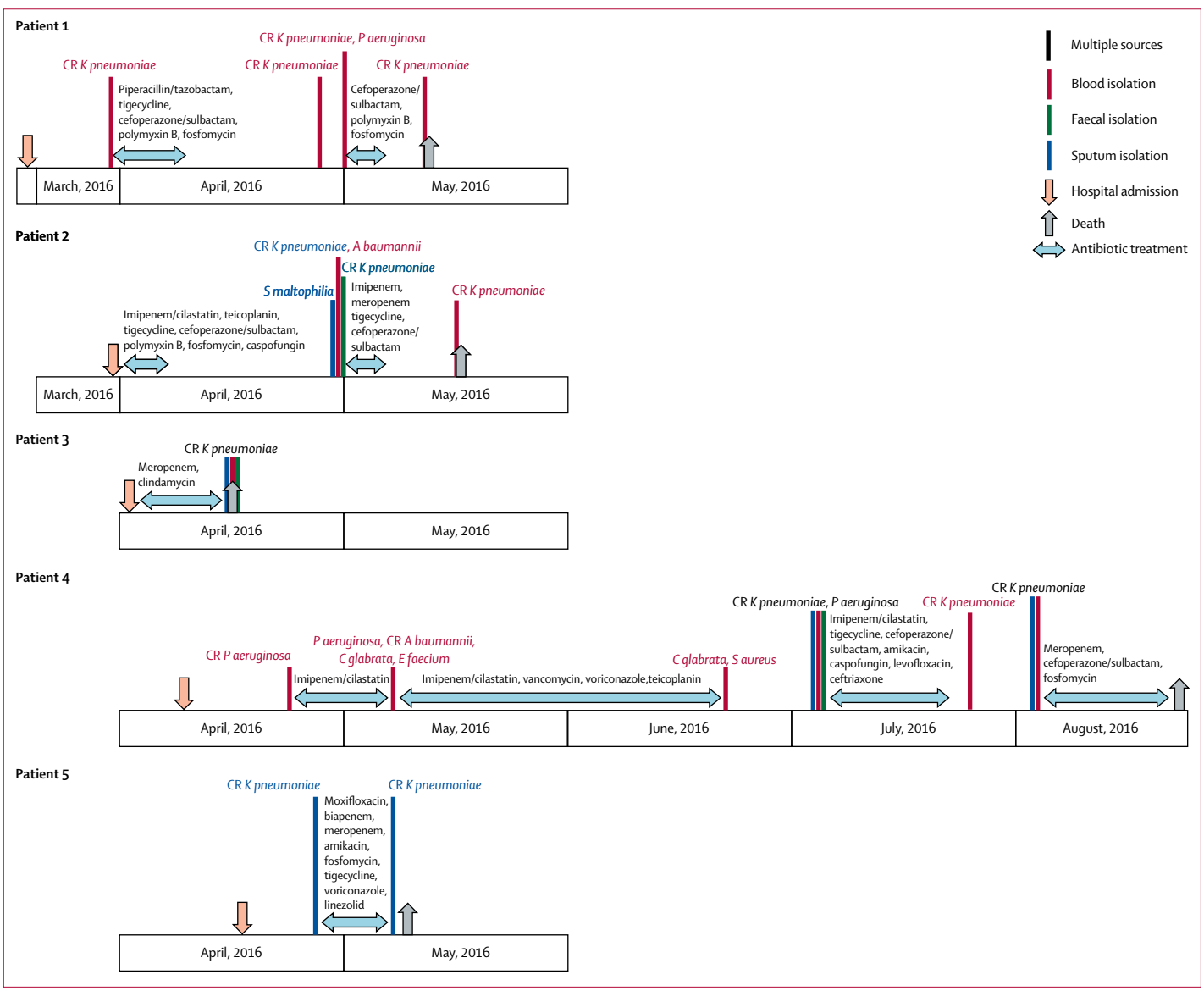

Figure 1: **Epidemiology of the *Klebsiella pneumoniae* outbreak cases**

Detailed case descriptions are provided in the appendix (pp 3–7, 9, 14–15). Coloured text and bars represent the source that the bacterial species was isolated from. CR=carbapenem-resistant. *K pneumoniae=Klebsiella pneumoniae. P aeruginosa= Pseudomonas aeruginosa. S maltophilia=Stenotrophomonas maltophilia. A baumannii= Acinetobacter baumannii. C glabrata=Candida glabrata. E faecium=Enterococcus faecium. S aureus=Staphylococcus aureus.*

21 non-repeated carbapenem-resistant *K pneumoniae* strains were recovered from various clinical specimens of the five patients. These strains had almost identical antibacterial susceptibility profiles, belonged to ST11 and shared highly similar PFGE patterns (appendix pp 16–18). Strains recovered from the same patient had identical PFGE profiles, except for patient 4, who had four strains that belonged to the major PFGE pattern and two strains (*K pneumoniae* 4.4 and 4.5) with different PFGE patterns and plasmid profiles, suggesting that patient 4 was simultaneously infected by two different clones of carbapenem-resistant *K pneumoniae* (appendix pp 16–20). We selected one representative carbapenem-resistant *K pneumoniae* strain from each patient (*K pneumoniae* 1–5; for patient 4, we selected the strain isolated from blood samples) for further genetic and phenotypic characterisation (table). The corresponding transconjugants are termed C1–C5, respectively. The carbapenem and cephalosporin resistance phenotypes of these five strains could be transferred to the *Escherichia coli* strain EC600. All five isolates carried the bla_{KPC-2}, $bla_{CTX-M-65}$, and bla_{TEM-1} genes, which were located in conjugative plasmids (table). The MLST using the draft genome data showed that these five strains, *K pneumoniae* 1–5, belonged to ST11 and serotype K47 (*wzi* 209). Pairwise SNP analysis for these five strains based on their raw sequencing reads showed that their core genome differed only by a few SNPs (n≤4), suggesting that these strains originated from a single clone (appendix p 10).

Compared with other ST11 carbapenem-resistant *K pneumoniae* infections, the carbapenem-resistant *K pneumoniae* strains in this outbreak caused much more severe pneumonia and higher mortality.[28] We speculated that these ST11 carbapenem-resistant *K pneumoniae* strains might be more virulent than previously reported ST11 carbapenem-resistant *K pneumoniae* strains, which prompted us to investigate the virulence potential of these isolates. The string test was positive for all five ST11 carbapenem-resistant *K pneumoniae* outbreak strains, which each produced strings longer than 20 mm (appendix p 21). We used a human neutrophil assay to test the virulence potential of two representative outbreak strains, *K pneumoniae* 4 and 5, two classic ST11 strains, FJ8 and FJ9, which produced a negative string test result, and two K1 hypervirulent *K pneumoniae* strains, 1088 and 91, which were reported previously.[29] The *K pneumoniae* 4 and 5 strains had survival of about 80% after incubation with the human neutrophils for 60 min, which was slightly higher than that of the K1 hypervirulent *K pneumoniae* strains 1088 and 91 (figure 2). However, the survival of strains FJ8 and FJ9, at 25% after 15 min, 10% after 30 min, and 3% after 60 min incubation respectively, was significantly lower than that of *K pneumoniae* 4 and 5 ($p<0·0001$ by one-way ANOVA; figure 2).

We infected *G mellonella* larvae with selected *K pneumoniae* strains: with an inoculum of 1×10^6 CFU, the classic ST11 strain FJ8 survival was 80% at 48 h after

	Source of isolate	MLST	Antimicrobial resistance genes present	Minimum inhibitory concentration (μg/mL)											
				Imipenem	Ertapenem	Cefepime	Ceftriaxone	Cefazolin	Aztreonam	Amoxicillin plus clavulanic acid	Amikacin	Ciprofloxacin	Gentamicin	Tobramycin	Tigecycline	Piperacillin plus tazobactam
K pneumoniae 1	Patient 1	ST11	Yes	>16	>8	>64	>64	>64	>64	>32	>64	>4	>16	>16	0·5	>128
K pneumoniae 2	Patient 2	ST11	Yes	>16	>8	>64	>64	>64	>64	>32	>64	>4	>16	>16	0·5	>128
K pneumoniae 3	Patient 3	ST11	Yes	>16	>8	>64	>64	>64	>64	>32	>64	>4	>16	>16	1	>128
K pneumoniae 4	Patient 4	ST11	Yes	>16	>8	>64	>64	>64	>64	>32	>64	>4	>16	>16	0·5	>128
K pneumoniae 5	Patient 5	ST11	Yes	>16	>8	>64	>64	>64	>64	>32	>64	>4	>16	>16	0·5	>128
Transconjugant C1	..	..	Yes	>16	>8	32	>64	>64	>64	>32	>64	≤0·25	>16	>16	≤0·5	>128
Transconjugant C2	..	..	Yes	>16	>8	32	>64	>64	>64	>32	>64	≤0·25	>16	>16	≤0·5	>128
Transconjugant C3	..	..	Yes	>16	>8	32	>64	>64	>64	>32	>64	≤0·25	>16	>16	≤0·5	>128
Transconjugant C4	..	..	Yes	>16	>8	32	>64	>64	>64	>32	>64	≤0·25	>16	>16	≤0·5	>128
Transconjugant C5	..	..	Yes	>16	>8	32	>64	>64	>64	>32	>64	≤0·25	>16	>16	≤0·5	>128
EC600	..	..	No	≤1	≤0·5	≤1	≤1	≤4	≤1	≤2	≤2	≤0·25	≤1	≤1	≤0·5	2

Antimicrobial genes were deemed to be present if the isolate had three antimicrobial resistance genes, including bla_{KPC-2}, $bla_{CTX-M-65}$, and bla_{TEM-1}. MLST=multilocus sequence type. EC600=*Escherichia coli* 600.

Table: **MLST type and antibiotic resistance characteristics of *Klebsiella pneumoniae* outbreak strains and their corresponding transconjugants**

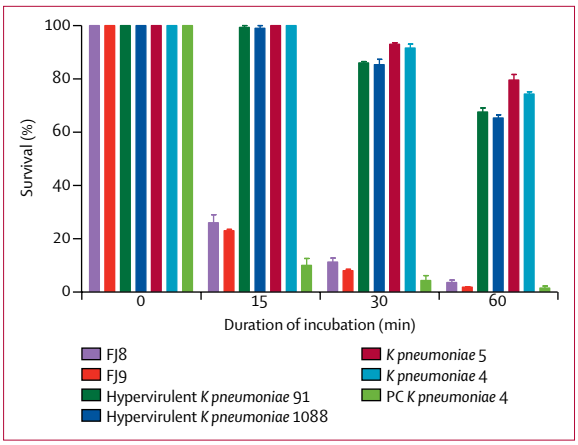

Figure 2: **Human neutrophil assays of *Klebsiella pneumoniae* strains**
FJ8 and FJ9 are classic ST11 *K pneumoniae* strains that did not a harbour virulence plasmid. Hypervirulent *K pneumoniae* 1088 and 91 are two ST23 K1 hypervirulent *K pneumoniae* strains reported in a previous study.[29] *K pneumoniae* 4 and 5 are two ST11 hypervirulent *K pneumoniae* strains tested in this study. Strain PC *K pneumoniae* 4 is a mutant of strain *K pneumoniae* 4, in which the virulence plasmid has been removed in plasmid curing experiments.

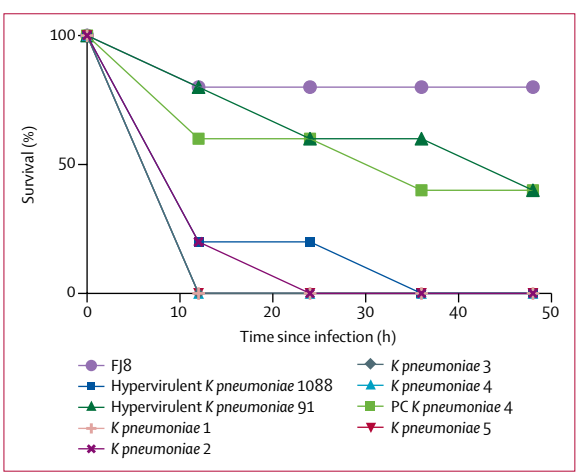

Figure 3: **Virulence potential of *Klebsiella pneumoniae* strains in a *Galleria mellonella* infection model**
The effect of 1×10^6 colony-forming units of each *K pneumoniae* strain on survival was assessed in *G mellonella*. The results for other doses of each *K pneumoniae* strain are shown in the appendix (pp 22, 23). FJ8 is a classic ST11 *K pneumoniae* strain that did not a harbour virulence plasmid. Hypervirulent *K pneumoniae* 1088 and 91 are two ST23 K1 hypervirulent *K pneumoniae* strains reported in a previous study.[29] *K pneumoniae* 1–5 are two ST11 hypervirulent *K pneumoniae* strains tested in this study. Strain PC *K pneumoniae* 4 is a mutant of strain *K pneumoniae* 4, in which the virulence plasmid has been removed in plasmid curing experiments.

infection; survival was 0% with the K1 hypervirulent *K pneumoniae* strain 1088 at 48 h and 40% with the K1 hypervirulent *K pneumoniae* strain 91 at 36 h post-infection. The ST11 carbapenem-resistant *K pneumoniae* outbreak strains *K pneumoniae* 1, 3, 4, and 5 resulted in 0% survival by 12 h; 0% survival was reached with *K pneumoniae* 2 after 24 h, suggesting that strains *K pneumoniae* 1–5 were more virulent than the K1 hypervirulent *K pneumoniae* strains 1088 and 99, as well as the classic ST11 *K pneumoniae* strains, FJ8 and FJ9 (figure 3). Data on the effects of the other inoculums of these strains are available in the appendix (pp 22, 23). The consistency between the clinical data and the results of the phenotypic assays supported the notion that the ST11 carbapenem-resistant *K pneumoniae* strains, *K pneumoniae* 1–5, were ST11 carbapenem-resistant hypervirulent *K pneumoniae*.

Pangenome analysis of nine ST11 strains (SH-1, *K pneumoniae* 1–5, FJ8, HS11268, and JM45) identified a unique set of genes in the five ST11 carbapenem-resistant hypervirulent *K pneumoniae* strains, which aligned well with the virulence plasmid pLVPK (AY378100), a 219 385 bp IncHI1B/IncFIB plasmid recovered from *K pneumoniae* strain CG43 (appendix pp 24, 25). In the virulence gene analysis, we found that all ST11 strains tested, except for HS11286, had type 3 fimbriae (*mrkABCDF*) and type 1 fimbrial adhesion genes (*fimA–H*), as well as the *iroE*, *iutA*, *kpn*, and *ycfM* genes, which encode various virulence factors, suggesting that these virulence genes might be typical of ST11 *K pneumoniae* (figure 4; appendix p 11, 12). The K1 hypervirulent *K pneumoniae* strains NTUH-K2044 and 1088 lacked these virulence genes typical of ST11 strains, but harboured a different set of virulence genes including *iroBCDN*, *iucABCD*, *rmpA* and *rmpA2*, and *irp1* and *irp2*, which were located in the virulence plasmid pLVPK (figure 4; appendix pp 11, 12).[5] In addition to harbouring the typical virulence genes for ST11 strains, the five outbreak strains also contained the *iucABCD*, *rmpA2*, and *iutA* genes. This finding was consistent with the results of the pangenome analysis in that virulence plasmid-related genes were unique to ST11 carbapenem-resistant hypervirulent *K pneumoniae*, but not the classic ST11 carbapenem-resistant *K pneumoniae* strains (figure 4; appendix pp 24, 25).

Alignment of contigs showed that all five ST11 carbapenem-resistant hypervirulent *K pneumoniae* outbreak strains carried a plasmid that aligned well to most parts of the pLVPK plasmid, including the region in which the *rmpA2*, *iucABCD*, and *iutA* genes were located, but not to a roughly 50 kbp region carrying the *rmpA* and *iroBCDN* (salmochelin) genes. Southern hybridisation confirmed the presence of a roughly 170 kbp virulence plasmid in all five strains (figure 5). The complete sequence of the virulence plasmid from strain *K pneumoniae* 4, pVir-CR-HvKP4 (178 154 kbp), aligned well to pLVPK (figure 5). pVir-CR-HvKP4 is an IncHI1B/IncFIB-type plasmid with a length of 178 154 bp and an average GC content of 50·5%, which encodes 216 predicted open reading frames. It shares 99% identity with pRJF999 (GenBank CP014011), pK2044 (GenBank AP006726), pRJA166b (GenBank CP019049), pRJF293 (GenBank CP014009), and pLVPK (GenBank AY378100), with query coverages ranging from 90% to 97%. We also obtained the complete sequence of a bla_{KPC-2}-carrying plasmid from *K pneumoniae* 4, pKPC-CR-HvKP4

(MF437312), and found it to be a similar size (177 585 bp) to pVir-CR-HvKP4 (MF437313; appendix p 26). To test whether acquisition of the roughly 170 kbp virulence plasmid contributed to the hypervirulence phenotype of the ST11 carbapenem-resistant hypervirulent *K pneumoniae* strains, we used the plasmid curing approach to remove the virulence plasmid pVir-CR-HvKP4 from *K pneumoniae* 4, to produce the strain PC-*K pneumoniae* 4 (appendix pp 27, 28). Subsequently, we found that strain PC-*K pneumoniae* 4 had a negative string test phenotype and substantially reduced survival in human neutrophils (figure 2) and virulence in *G mellonella* (figure 3), supporting the notion that pVir-CR-HvKP4 contributed to the hypervirulence phenotype of *K pneumoniae* 4 (appendix p 22, 23) and, by extension, the other newly emerged ST11 carbapenem-resistant hypervirulent *K pneumoniae* strains.

We retrospectively screened 387 clinical ST11 carbapenem-resistant *K pneumoniae* strains collected from 25 provinces and municipalities in China in 2015 for the presence of the virulence plasmid. 11 (3%) of the 387 ST11 carbapenem-resistant *K pneumoniae* strains carried the virulence plasmid. Of these 11 isolates, two carried the *rmpA2* and *iucA* genes. The other nine isolates had two additional virulence genes, *rmpA* and *iroN*, suggesting that these isolates could harbour the full length of virulence plasmid pLVPK. Whole genome sequencing of SH-1, one of the 11 strains, confirmed that it had virulence plasmid highly similar to pLVPK (figure 5; appendix p 13). These 11 carbapenem-resistant *K pneumoniae* strains, all of which carried the bla_{KPC-2} gene, were collected from three different provinces. Strains from within the same region were more closely genetically related than those recovered from different regions (appendix pp 16–18). Retrospective analysis of the clinical records showed that these 11 ST11 *K pneumoniae* were recovered from blood and sputum culture of the patients, and that five of the 11 patients died and the other six patients were discharged with critical illnesses, suggesting that these 11 isolates could cause substantial morbidity and mortality.

Discussion

Our results show the emergence of new ST11 carbapenem-resistant hypervirulent *K pneumoniae* strains that caused fatal hospital infections. Due to acquisition of a virulence plasmid by classic ST11 carbapenem-resistant *K pneumoniae* strains, these new strains are simultaneously hypervirulent, multidrug resistant, and transmissible, and should therefore be regarded as a real superbug that could pose a serious threat to public health.

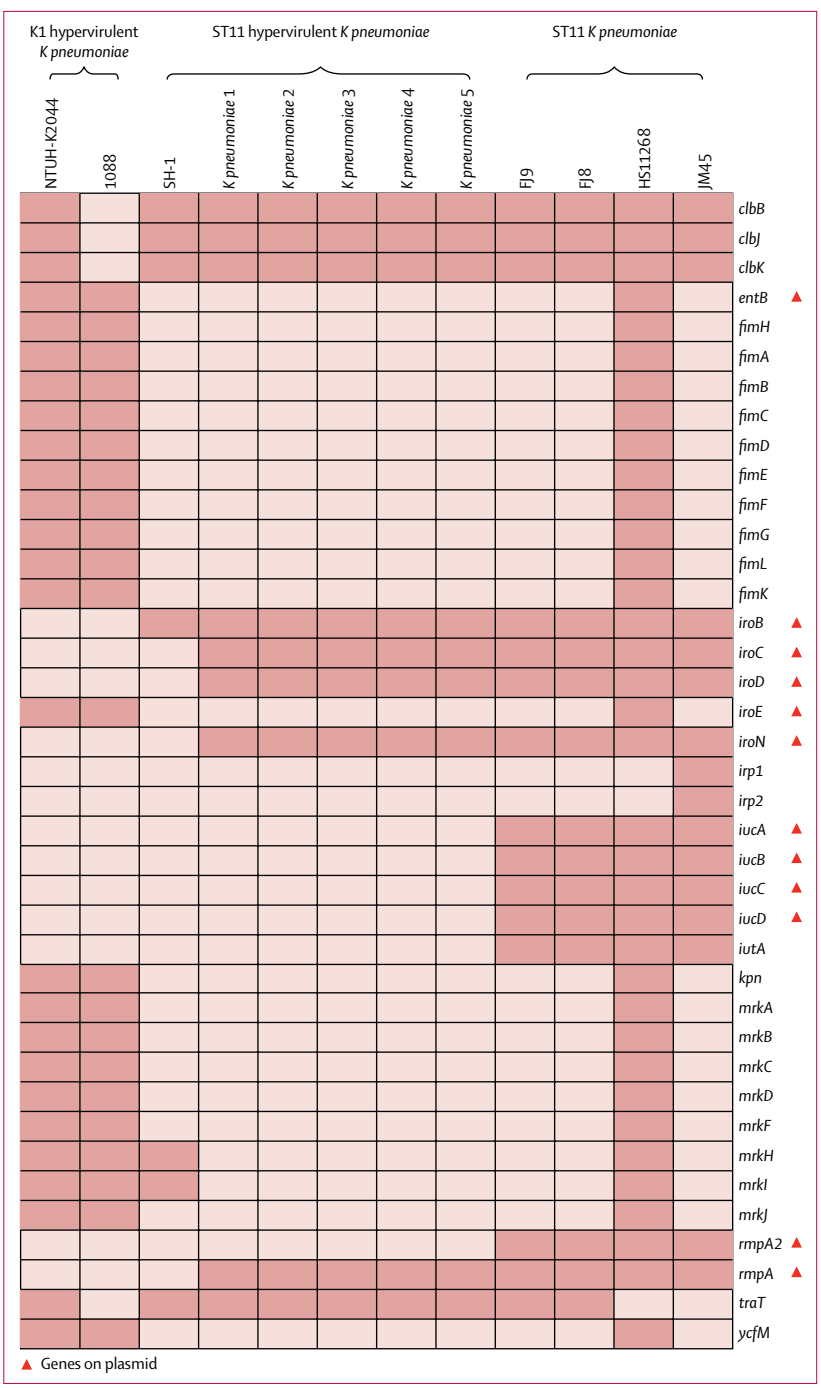

Figure 4: **Virulence gene analysis of *Klebsiella pneumoniae* strains**
Heatmaps were generated by aligning the draft genome sequence of each strain to the virulence gene database. The presence of virulence genes in a specific genome is represented by the light red box and the absence of virulence genes is represented by a dark red box. Red triangles show virulence genes located in a plasmid. FJ8 and FJ9 are classic ST11 *K pneumoniae* strains that did not a harbour virulence plasmid. Hypervirulent *K pneumoniae* 1088 is an ST23 K1 hypervirulent *K pneumoniae* strain reported in a previous study.[29] *K pneumoniae* 1–5 are ST11 hypervirulent *K pneumoniae* strains tested in this study. NTUH-K2044 is a publicly available K1 hypervirulent *K pneumoniae* strain; HS11268 and JM45 are two publicly available classic ST11 carbapenem-resistant *K pneumoniae* strains; and SH-1 is a clinical carbapenem-resistant *K pneumoniae* strain isolated from a hospital in Shanghai in 2015 that carried the virulence plasmid. *clbBJK*=colibactin synthesis loci. *entB*=enterobactin-related genes. *fimA–K*=type 1 fimbriae genes. *iroBCDEN*=iron acquisition system. *irp1*/2=yersiniabactin-related genes. *iucABCD*=aerobactin-related genes. *iutA*=ferric aerobactin receptor. *mrkA–J*=type 3 fimbriae genes. *rmpA*/*rmpA2*=cps transcriptional activator. *kpn*=FimH-like adhesion gene. *ycfM*=outer membrane lipoproteins. *traT*=outer membrane lipoprotein.

Known virulence factors of *K pneumoniae* that are responsible for disease progression include, but are not limited to surface antigens, especially CPS (K antigen), siderophores that are responsible for binding ferric iron secreted by the iron-binding proteins of the host, and adherence factors that are responsible for attachment to host cell surfaces, such as type 1 and type 3 fimbriae and non-fimbrial adhesion proteins.[30] Consistently, K1 hypervirulent *K pneumoniae* strains such as NTUH-K2044 and 1088 have been shown to produce virulence factors associated with surface antigen and siderophores, which are encoded by genes located in a virulence plasmid. ST11 *K pneumoniae* strains, however, have been shown to express various adherence factors. The putative functional roles of fimbriae and outer membrane lipoproteins in biofilm formation and the development of antibiotic resistance might explain why the ST11 *K pneumoniae* strains were more resistant to antibiotics than hypervirulent *K pneumoniae*.[31] It has previously been common for ST11-type *K pneumoniae* to be resistant to carbapenems, but not hypervirulent. The acquisition of a roughly 170 kbp virulence plasmid represents an important evolution event underlying the emergence of ST11 carbapenem-resistant hypervirulent *K pneumoniae* strains that have virulence factors from both K1 and K2 hypervirulent *K pneumoniae* and ST11 carbapenem-resistant *K pneumoniae* strains. ST11 hypervirulent *K pneumoniae* strains are therefore not only highly resistant to antibiotics, but also hypervirulent. Clinically, the ST11 carbapenem-resistant hypervirulent *K pneumoniae* strains caused more severe disease than classic ST11 strains and high mortality. Despite being susceptible to tigecycline in in-vitro minimum inhibitory concentration tests, long-term treatment with this antibiotic (alone or in combination with several other antibiotics) or even polymyxin B, was not able to eradicate such organisms from the bloodstream, consistently resulting in a fatal outcome. Unfortunately, ceftazidime with avibactam is not available in China, so we could not test their efficacy in treating these ST11 carbapenem-resistant hypervirulent *K pneumoniae*.

Since none of the available antibiotics was effective in treating infections caused by ST11 carbapenem-resistant hypervirulent *K pneumoniae*, we have implemented a new infection prevention and control (IPC) policy to control the outbreak in the hospital. Current consensus IPC policy on multidrug-resistant bacterial pathogens in China includes hand hygiene management, isolation of patients, periodic environmental cleansing and equipment disinfection, investigation of outbreak agents, and cautious use of antibiotics. We tried to map route or routes of transmission of these ST11 carbapenem-resistant hypervirulent *K pneumoniae* strains, but we were unable to do so. We speculated that patients transferred from a small-scale local hospital, where the IPC standard was low, might be the sources of carbapenem-resistant *K pneumoniae* infections. In view of this risk, we modified the IPC policy to control the outbreak. First, we introduced pre-screening of carbapenem-resistant Enterobacteriaceae in faecal and sputum samples of patients before their admission to the

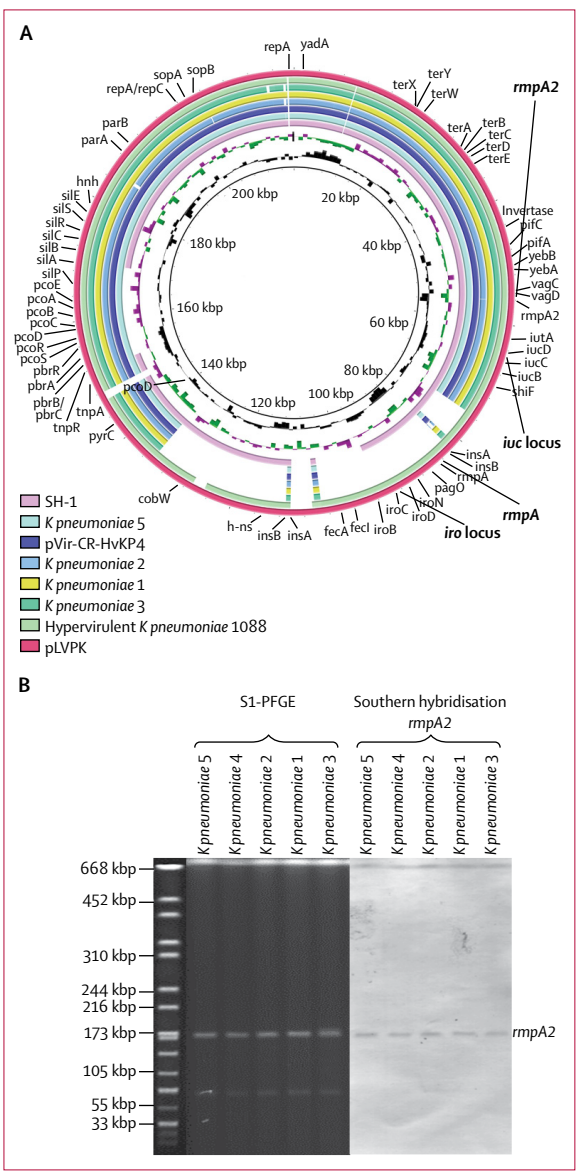

Figure 5: **Gene map of virulence plasmid harboured by *Klebsiella pneumoniae* outbreak strains**

(A) Alignment of the roughly 170 kbp plasmid recovered from the five ST11 carbapenem-resistant hypervirulent *K pneumoniae* strains, a plasmid recovered from strain K1 hypervirulent *K pneumoniae* 1088, and a plasmid harboured by strain ST11 *K pneumoniae* SH-1, against the known virulence plasmid pLVPK from the *K pneumoniae* strain CG43 (AY378100). The circular map was generated with the BLAST Ring Image Generator. Plasmids, pLVPK and pVir-CR-HvKP4 (accession number MF437313) were mapped with complete sequences, whereas others were generated by aligning the draft genome sequences to pLVPK. Plasmid pVir-CR-HvKP4 is the virulence plasmid from ST11 carbapenem-resistant hypervirulent *K pneumoniae* strain *K pneumoniae* 4. (B) S1-PFGE and Southern hybridisation of the marker gene of the virulence plasmid *rmpA2*, which was hybridised to the roughly 170 kbp plasmid to confirm the presence of the virulence plasmid in the five ST11 carbapenem-resistant hypervirulent *K pneumoniae* strains. PFGE=pulsed-field gel electrophoresis.

ICU. Second, we implemented stringent isolation procedures for carbapenem-resistant Enterobacteriaceae-bearing patients. Third, it was made necessary for medical personnel who came into contact with patients infected with carbapenem-resistant Enterobacteriaceae to go through a disinfection procedure. Finally, the ICU wards where the carbapenem-resistant Enterobacteriaceae-positive patients stayed were completely disinfected after the discharge of the patients and left unoccupied for more than 2 weeks after disinfection and before the admission of new patients. Since the implementation of these IPC procedures in this ICU, no fatal infections due to ST11 carbapenem-resistant hypervirulent *K pneumoniae* have occurred as of April, 2017. More evidence is needed to confirm that this IPC policy is effective in preventing carbapenem-resistant Enterobacteriaceae infections in hospitals.

The main limitation of this study is that only a small number of cases have been investigated. A large-scale study on the relationship between the clinical outcomes of infections caused by carbapenem-resistant *K pneumoniae* strains with or without the virulence plasmid is underway to provide more evidence on the clinical significance of these newly emerged strains.

Importantly, these ST11 carbapenem-resistant hypervirulent *K pneumoniae* were found to have disseminated in other parts of China, signifying that their threat to human health is imminent. Worldwide surveillance of these carbapenem-resistant hypervirulent *K pneumoniae* strains and implementation of stricter control measures are needed to prevent these novel strains from further disseminating in hospital settings and the community.

Contributors

DG collected the strains, did strain characterisation, and participated in manuscript writing. ND did the whole-genome sequencing and comparative genomics and participated in manuscript writing. ZZ did the *G mellonella* infection experiments. DL, LS, and JY participated in the strain characterisation and human neutrophil assays. MH and LW collected the clinical data and analysed the data. EW-CC participated in the research design, data interpretation, and manuscript writing. SC and RZ designed and supervised the study, interpreted the data and wrote the manuscript.

Declaration of interests

We declare no competing interests.

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