

Rapid Emergence of a Pandrug-Resistant *Klebsiella pneumoniae* ST11 Isolate in an Inpatient in a Teaching Hospital in China After Treatment with Multiple Broad-Spectrum Antibiotics

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Purpose: Pandrug-resistant *K. pneumoniae* is still rare in China, and the lack of studies limits our understanding of the emergence mechanism of pandrug-resistant *K. pneumoniae*. Here, we report the rapid emergence of a pandrug-resistant *K. pneumoniae* ST11 strain in an inpatient after treatment with multiple broad-spectrum antibiotics in China.

Patients and Methods: *K. pneumoniae* strain KP65 was isolated from a 55-year-old male patient hospitalized in the Department of Intensive Care Unit (ICU) of a teaching hospital in China. Antimicrobial susceptibility testing was conducted according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI). The complete genome sequence of the strain was determined using the Illumina NovaSeq 6000 platform and long-read MinION sequencer. Genomic features and resistance mechanisms of the strain were comprehensively analysed using various bioinformatics approaches.

Results: *K. pneumoniae* strain KP65 was found to be resistant to all antibiotics tested, including beta-lactams, aminoglycosides, quinolones, tigecycline and colistin. Seven resistance genes were identified in the genome. The carbapenem-resistant gene *bla*_{KPC-2} and extended-spectrum β -lactamase (ESBL)-producing gene *bla*_{CTX-M-65} are located on the IncFII-type plasmid pKPC-2-KP65. No *mcr* genes were detected in the genome, but an IS5 insertion element was found at position 117 of the *mgrB* gene. Regarding the *rpsJ* gene, single-base substitution, G169C, leading to the amino acid substitution V57L was also identified. According to in silico MLST analysis, *K. pneumoniae* KP65 belongs to sequence type ST11. The closest relative of *K. pneumoniae* KP65 is another ST11 *K. pneumoniae* strain, which was isolated from a bloodstream infection in Hangzhou, differing by only 53 cgMLST loci.

Conclusion: Under the selective pressure of antibiotics, the KPC-2-producing *K. pneumoniae* ST11 strain can easily evolve pandrug resistance through chromosomal mutations. More attention is required to monitor the prevalence of the KPC-2-producing *K. pneumoniae* ST11 strain in China.

Keywords: *Klebsiella pneumoniae*, *mgrB*, *rpsJ*, ST11, *bla*_{KPC-2}

Introduction

Klebsiella pneumoniae is an important pathogen that often causes nosocomial infections in hospitalized patients. In the past decade, the clinical isolation rate of *K. pneumoniae* has been rising, and in China, *K. pneumoniae* has become the second largest clinically isolated gram-negative bacilli after *Escherichia coli*.¹

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The emergence of carbapenem-resistant *K. pneumoniae* poses a serious threat to public health due to the limited therapeutic options to treat such infections.² *K. pneumoniae* carbapenemase (KPC)-type carbapenemase, an Ambler class A β -lactamase, is one of the most important carbapenemases commonly found in *K. pneumoniae*. Acquisition of KPC-type carbapenemase has contributed to resistance to all β -lactams. Additionally, nationwide surveillance of clinical carbapenem-resistant *K. pneumoniae* strains has revealed that sequence type (ST) 11 is the dominant clone of KPC-producing *K. pneumoniae* in China.³

Most *bla*_{KPC}-carrying *K. pneumoniae* strains are multi-drug-resistant (MDR) bacteria that are often co-resistant to β -lactams, quinolones, aminoglycosides and fosfomycin.⁴ Tigecycline and colistin are two effective antibiotics for KPC-producing *K. pneumoniae* infections.⁵ Tigecycline was effective against most KPC-producing *K. pneumoniae* at the beginning of its clinical use,^{6,7} and colistin is currently recognized as the last resort for treating infections due to carbapenem-resistant *K. pneumoniae* strains.⁸ Carbapenem-resistant *K. pneumoniae* that are co-resistant to tigecycline and colistin often represent the emergence of pandrug-resistant bacteria, which are called super bacteria and make clinical treatment difficult.

Pandrug-resistant *K. pneumoniae* has been reported sporadically in many countries.^{9–13} However, pandrug-resistant *K. pneumoniae* in China is still rare and has only been reported by a few studies.¹⁴ In the present study, one pandrug-resistant *K. pneumoniae* strain, KP65, was collected from a urine specimen of an inpatient in a teaching hospital in China. The entire genome sequence of the strain was determined, and genomic features, plasmids and resistance mechanisms were comprehensively analysed.

Materials and Methods

Patient and Isolate

A 55-year-old male patient diagnosed with hypoxic-ischaemic encephalopathy and aspiration pneumonia was hospitalized in the Department of Intensive Care Unit (ICU) of a teaching hospital in Zhejiang Province, China, in March 2018. Two days after admission, he experienced fever and showed elevated inflammatory markers (including CRP, PCT, among others) suggestive of infection. He was administered piperacillin/tazobactam as empiric treatment. One week later, a carbapenem-resistant *Acinetobacter baumannii* strain was isolated from his sputum sample. The

antibiotic treatment was changed to cefoperazone/sulbactam combined with tigecycline. This treatment lasted for twenty days until a carbapenem-resistant *K. pneumoniae* strain was isolated from both his sputum and urine samples. Antibiotic treatment was then changed to imipenem combined with tigecycline. Three days later, a carbapenem-resistant *K. pneumoniae* strain was isolated from his blood sample, and antibiotic treatment was changed to polymyxin B for one week. A pandrug-resistant *K. pneumoniae* strain, KP65, was collected from the urine sample of the patient six days after the end of polymyxin B treatment. The isolate was preliminarily identified using the VITEK MS system (bioMérieux, France) and was further confirmed by 16S rRNA gene sequencing.

Antimicrobial Susceptibility Test

Antimicrobial susceptibility testing was conducted using the VITEK 2 system (bioMérieux, France) with a gram-negative antimicrobial susceptibility testing card (AST-GN16) and standard broth microdilution tests following the guidelines of the Clinical and Laboratory Standards Institute (CLSI). For tigecycline MIC detection, the medium must be prepared fresh on the day of use. As there are no CLSI breakpoints for tigecycline and colistin, the EUCAST standard was adopted.¹⁵

Whole-Genome Sequencing

The whole-genome sequence of the strain was determined using the Illumina NovaSeq 6000 platform (Illumina Inc., San Diego, CA, USA) and a long-read MinION sequencer (Nanopore, Oxford, UK). Both short Illumina reads and long MinION reads were hybrid assembled using Unicycler (v0.4.7) under the conservative mode. Complete circular contigs were generated and corrected using Pilon with Illumina reads for several rounds until no change was detected. The complete genome sequence was automatically annotated by the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) server.

Genomic Features and Plasmid Characterization

Multilocus sequence typing (MLST), antimicrobial resistance genes and plasmid replicons were analysed using the BacWGSTdb server.^{16,17} Circular comparisons based on concentric rings between the *bla*_{KPC-2}-carrying plasmid and similar plasmids were conducted with a BLAST Ring Image Generator (BRIG).¹⁸

Phylogenetic Relationship Analysis

The phylogenetic relationship between KP65 and other *K. pneumoniae* strains was analysed using the BacWGSTdb server with core genome multilocus sequence typing (cgMLST) approaches with a 100 threshold for phylogenetic analysis.¹⁶ The database currently contains 7480 *K. pneumoniae* strains.

Nucleotide Sequence Accession Numbers

The complete sequences of the isolate *K. pneumoniae* KP65 and plasmids have been deposited in GenBank under accession numbers CP044258-CP044260. The version described in this paper is the first version.

Table 1 MICs of the Antibiotics Tested in *K. pneumoniae* KP65

Antibiotics	MIC (mg/L)	
	BKPI 19	Interpretive Categories ^c
Cefazolin ^a	≥64	R
Ceftriaxone ^a	≥64	R
Cefepime ^a	≥64	R
Cefoxitin ^a	≥64	R
Aztreonam ^a	≥64	R
Piperacillin/tazobactam ^a	≥128	R
Imipenem ^b	>128	R
Meropenem ^b	>128	R
Amikacin ^a	≥64	R
Ciprofloxacin ^a	≥4	R
Levofloxacin ^a	≥8	R
Tigecycline ^b	32	R
Colistin ^b	>64	R

Notes: ^aTested by AST-GNI6. ^bTested by standard broth microdilution tests. ^cMIC, minimum inhibitory concentration.

Abbreviation: R, resistant.

Ethical Approval

This study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Zhejiang Provincial People's Hospital. Written informed consent from the patient was exempted by the Ethics Committee of Zhejiang Provincial People's Hospital because the present study focused only on bacteria. Isolation of clinical *K. pneumoniae* strain KP65 was part of routine hospital laboratory procedure.

Results and Discussion

K. pneumoniae strain KP65 is a pandrug-resistant strain resistant to all antibiotics tested, including beta-lactams, aminoglycosides, quinolones, carbapenems, tigecycline and colistin. The MICs of the antibiotics tested are presented in Table 1.

The genome of *K. pneumoniae* strain KP65 consisted of 3 contigs comprising 5,592,496 bp. Among them, one contig (contig 1: 5,438,014 bp) belongs to the chromosome and the others to two plasmids (contig pKPC-2-KP65: 144,422 bp and contig 3: 10,060 bp). Three plasmid replicons were identified in the genome: one on contig3 (ColRNAI) and another two on plasmid pKPC-2-KP65 (IncFII and IncR). The PGAP server predicted a total of 5384 protein-coding sequences, 85 tRNA genes and 25 rRNA operons.

The resistance genes and plasmid replicons present in the genome of the isolate are presented in Table 2. We identified the beta-lactam resistance genes *bla*_{SHV-11}, *bla*_{TEM-1B}, *bla*_{CTX-M-65} and *bla*_{KPC-2}; the aminoglycoside resistance gene *rmtB*; and the fosfomycin resistance genes *fosA* and *fosA3*. Among them, *bla*_{SHV-11} and *fosA* are located on the chromosome. The other resistance genes, including the carbapenem-resistance gene *bla*_{KPC-2} and extended-spectrum β-lactamase

Table 2 Antimicrobial Resistance Genes and Plasmid Replicons in Isolates *K. pneumoniae* Strain KP65

Antimicrobial Resistance Gene	Contig	Identity (%)	Position	Antimicrobial Resistance Category
<i>bla</i> _{CTX-M-65}	pKPC-2-KP65	100.00	5660..6535	Beta-lactam
<i>fosA3</i>	pKPC-2-KP65	100.00	12397..12813	Fosfomycin
<i>bla</i> _{TEM-1B}	pKPC-2-KP65	100.00	15580..16440	Beta-lactam
<i>rmtB</i>	pKPC-2-KP65	100.00	16610..17365	Aminoglycoside
<i>bla</i> _{KPC-2}	pKPC-2-KP65	100.00	92566..93447	Beta-lactam
<i>bla</i> _{SHV-11}	I	100.00	2783701..2784561	Beta-lactam
<i>fosA</i>	I	99.27	4752290..4752701	Fosfomycin
Plasmid replicon	Contig	Identity (%)	Position	Annotation
IncR	pKPC-2-KP65	99.20	27677..27927	Gram-negative
IncFII	pKPC-2-KP65	100.00	144166..144422	Gram-negative
ColRNAI	3	98.46	5781..5910	Gram-negative

(ESBL)-producing gene *bla*_{CTX-M-65}, are located on plasmid pKPC-2-KP65. The pKPC-2-KP65 plasmid contributes the most to the KP65 resistance phenotype.

Local alignment of plasmid pKPC-2-KP65 with similar plasmids deposited in the NCBI GenBank database indicated that it is a novel plasmid that has not been reported (Figure 1). Several similar *bla*_{KPC-2}-carrying plasmids were found in the database, with the most closely related plasmid showing 90% coverage and 99.98% identity to pKPC-2-KP65 (*K. pneumoniae* strain 283747 plasmid p283747-KPC, accession no. MF168406).¹⁹

No *mcr* genes were detected in the genome, but an IS5 insertion element was found at position 117 of the *mgrB* gene, a negative regulator of the PhoP/PhoQ two-component system contributing to colistin resistance. The insertion interrupts the coding sequence of *mgrB* and may contribute to the colistin resistance of KP65.²⁰ Moreover, a single-base substitution, G169C, leading to the amino acid substitution V57L, was detected in the *rpsJ* gene. This mutation may contribute to tigecycline resistance in this isolate.^{21,22} Both

mutations occur on the chromosome. As the strain was isolated from urine samples of the patient after a period of tigecycline and colistin use, these mutations may have occurred due to antibiotic stress from tigecycline and colistin. Our findings support the hypothesis that there is a risk of mutation-mediated resistance under antibiotic pressure in KPC-producing *K. pneumoniae* during tigecycline and colistin treatment, leading to treatment failure. This is also an important mechanism for the emergence of pandrug-resistant *K. pneumoniae* strains in hospitalized patients.

The phylogenetic relationship between *K. pneumoniae* KP65 and other *K. pneumoniae* strains currently deposited in the BacWGSTdb server was analysed by cgMLST approaches (Figure 2). A total of 122 phylogenetically related strains were identified in the database; among them, 117 strains were from China. The data suggest that the closest relative of *K. pneumoniae* KP65 is another ST11 *K. pneumoniae* strain, skl10042 (Accession No. NXDY00000000), which was isolated from a bloodstream infection in Hangzhou in 2013; the sequences differ by only 53 cgMLST loci. Compared with

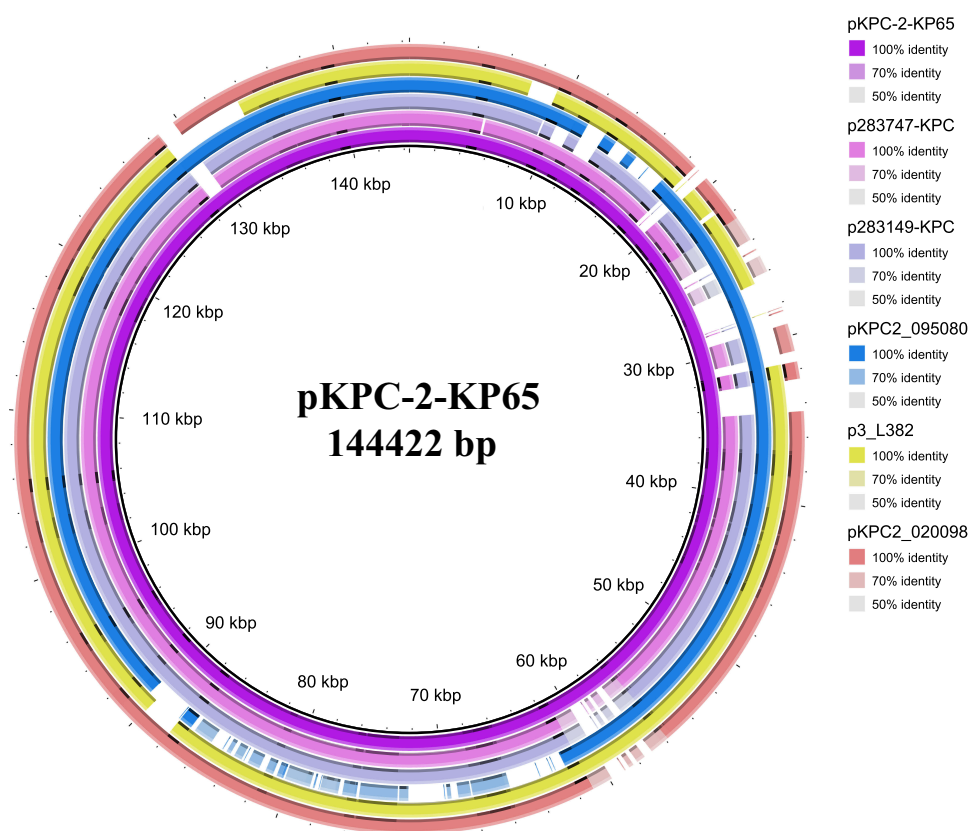


Figure 1 Circular comparison between the *bla*_{KPC-2}-carrying plasmid pKPC-2-KP65 and similar plasmids. p283747-KPC: *K. pneumoniae* strain 283747 plasmid p283747-KPC, accession no. MF168406, size 148,021 bp. p283149-KPC: *K. pneumoniae* strain 283149 plasmid p283149-KPC, accession no. MK036886, size 136,186 bp. pKPC2_095080: *K. pneumoniae* strain WCHKP2080 plasmid pKPC2_095080, accession no. CP036362, size 120,195 bp. p3_L382: *K. pneumoniae* strain L482 plasmid p3_L382, accession no. CP033962, size 136,159 bp. pKPC2_020098: *K. pneumoniae* strain WCHKP020098 plasmid pKPC2_020098, accession no. CP036306, size 149,519 bp.

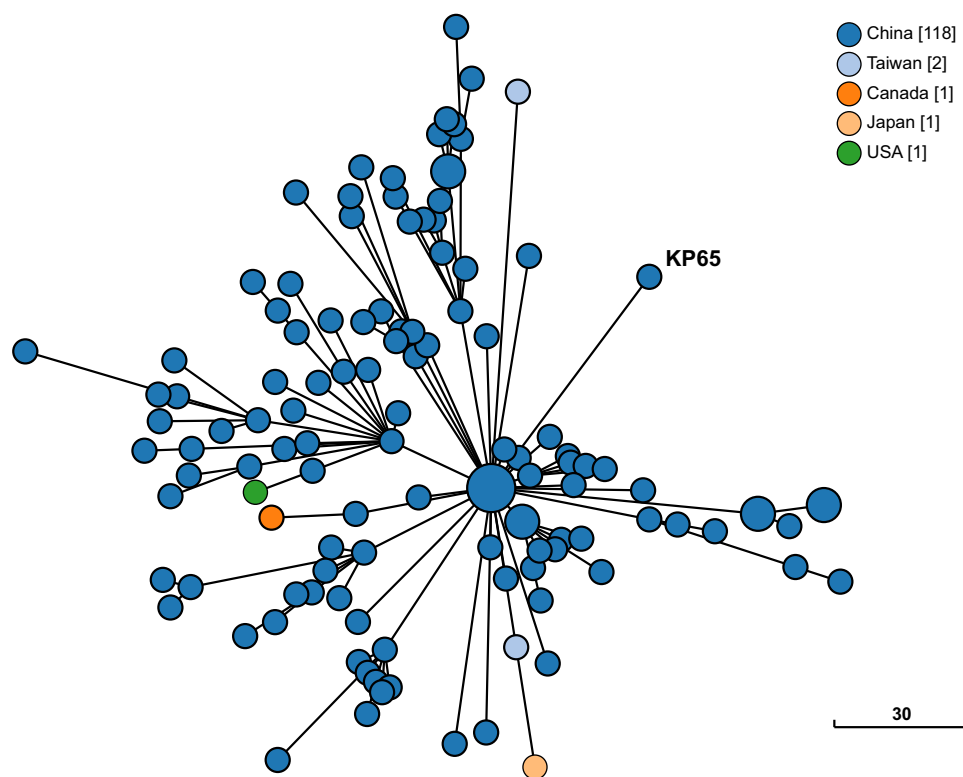


Figure 2 Phylogenetic relationship between *K. pneumoniae* strain KP65 and closely related *K. pneumoniae* strains currently available in BacVGSTdb. Clonal relationships between different isolates are depicted by the line length connecting each circle. The numbers given in square brackets indicate the number of isolates from each country.

K. pneumoniae KP65, no insertion, deletion or point mutation events were found in the *mgrB* and *rpsJ* genes in these classic ST11 KPC-2-producing strains, constituting a different antimicrobial resistance profile in these strains.

The emergence of pandrug-resistant *K. pneumoniae* strains makes clinical treatment difficult. Moreover, clonal dissemination of such pandrug-resistant *K. pneumoniae* clinical strains has led to public health concerns. Therefore, the prevalence of the KPC-2-producing *K. pneumoniae* ST11 strain, especially the emergence of colistin and tigecycline co-resistant strains, needs our attention. Classification management systems of antibiotics, strict medication guidelines for tigecycline and colistin, and combined use of two or three antibiotics may be an effective way to prevent the emergence of pandrug-resistant *K. pneumoniae*.

Conclusion

Our study reports the rapid emergence of a pandrug-resistant *K. pneumoniae* ST11 strain in an inpatient after treatment with multiple broad-spectrum antibiotics in China. More attention is required to monitor the prevalence of the KPC-2-producing *K. pneumoniae* ST11 strain in China. These

strains can easily evolve pandrug resistance through chromosomal mutations under the selective pressure of antibiotics.

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Disclosure

The authors report no conflicts of interest in this work.

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