

## Whole-Genome Analysis of KPC-Producing *Klebsiella Pneumoniae* Isolates from Hospital Acquired Post-Neurosurgical Meningitis

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**Received:** December 14, 2021

**Published:** January 31, 2022

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### Abstract

**Background:** Nosocomial bacterial infections from carbapenem-resistant *Klebsiella pneumoniae* (CRKP) are associated with high mortality in neurosurgical patients. This study examined the post-neurosurgical meningitis outbreak caused by CRKP of patients with nervous system tumours, and analysed the molecular characteristics of the causative strain.

**Methods:** Neurosurgical cancer patients with meningitis caused by CRKP between 2017-2019 were retrospectively analysed. Identification of strains and antimicrobial susceptibilities was conducted using BD Phoenix-100, 16S rRNA gene sequencing and broth microdilution. Multilocus sequence typing (MLST) and pulsed-field gel electrophoresis (PFGE) were used to identify the subtypes of *K. pneumoniae*. The genotype correlation, resistance genes and plasmid of isolates were assessed by whole-genome sequencing (WGS).

**Results:** Isolates were resistant to almost all of the tested antimicrobial agents except polymyxin and tigecycline. The PFGE and MLST revealed all isolates were the same strain - ST11-while WGS phylogenetic analysis indicated they were closely related. The isolates harboured *blaKPC-2* and an IncFII-type plasmid; the *blaKPC-2* gene had a similar genetic environment across isolates.

**Conclusions:** The results of molecular analysis showed that ST11 and IncFII-type plasmid in CRKP have close correlations and indicate a long-term retrospective genomic study throughout the hospital for KPC-producing *K. pneumoniae* is necessary.

**Keywords:** *K. pneumoniae*; Meningitis; Whole-Genome Sequencing; Incfii-Type Plasmid

### Introduction

Patients (especially in nervous system cancer patients who receive cancer care) due to exposure to various risk factors (such as catheters and tubes, neurological deterioration, multiple injuries and damage to the immune system) and more susceptible to hospital infections [1-3]. During neurosurgical procedures, especially in the ICU, invading microorganisms overcome complex host defence mechanisms and cause infections with high morbidity and mortality [4-8]. Meningitis is several infections, defined as inflammation

of the meninges; it may be caused by a variety of infectious agents, which include bacteria, viruses, parasites and fungi, as well as non-communicable process. Although the most common viral meningitis, bacterial meningitis but may be more severe, potentially life-threatening [9].

Last several years, carbapenem-resistant *Klebsiella pneumoniae* (CRKP) has become one of the biggest global public health concerns [10,11]. Despite this, there are few reports in the literature on meningitis caused by CRKP [12,13]. This study reports ten cases

of post-neurosurgical nosocomial meningitis due to CRKP over three years; six isolate strains were further studied. From 2017 to 2019, every July to September, there were nosocomial meningitis outbreaks in cancer patients of central nervous system in our hospital's neurosurgery department; the culprit was identified as CRKP. 30 pathogens were isolated from different sites on the patients, and six isolate strains were further studied and whole genome sequenced to identify the relationship between the strains. To our knowledge, this report is the first CRKP meningitis outbreak in cancer patients described in central China [12,13].

## Materials and Methods

### Bacterial isolation, species identification and antimicrobial susceptibility profiles

Identification of strains was performed by using BD Phoenix-100 (BD, American) and 16S rRNA gene sequencing. Antimicrobial susceptibilities were initially tested using BD Phoenix-100 (BD, American), following which MICs were measured using broth microdilutions according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [14]. *Escherichia coli* (*E. coli*) strain ATCC 25922 was used as the quality control. The CLSI breakpoints (M100-S27) and the 2018 EUCAST breakpoints (only for polymyxin and tigecycline) were used to interpret the MIC results.

### Pulsed-field gel electrophoresis (PFGE) and Multilocus sequence typing (MLST)

All isolates for PFGE. Briefly, genomic DNA was digested with XbaI (Bio-Rad Laboratories, Hercules, CA, USA), restriction fragments were separated in CHEF Mapper XA System (Bio-Rad Laboratories). Cluster analysis using Bionumerics software (Applied Maths NV, Sint-Martens-Latem, East Flanders, Belgium) was performed with the Dice similarity coefficients and unweighted-pair group matching algorithm. The same PFGE pattern were defined in strains which shared with > 75% similarity.

The sequence types of the six carbapenemase producers were determined by multilocus sequence typing (MLST) analysis with seven housekeeping genes (*gapA*, *infB*, *mdh*, *pgi*, *phoE*, *rpoB* and *tonB*) amplified and sequenced according to Diancourt, *et al.* Alleles and sequence types were assigned using the MLST database (<http://www.pasteur.fr/mlst/Kpneumoniae.html>) [15].

### Conjugation and plasmid analysis

In plasmid conjugation experiments, *E. coli* J53 as a recipient strain were performed. Next, the transconjugants were cultured on

agar (OXOID, UK) medium supplemented with 200 mg/L sodium azide and 2 mg/L meropenem. Finally, BD Bruker MALDI Biotyper system was used to identify transconjugants and amplify *blaKPC* by PCR to ensure that the plasmid was successfully transferred to the recipient strain. Plasmid comparisons were performed by using BRIG (<http://brig.sourceforge.net/>) and Easyfig (<http://mj-sull.github.io/Easyfig/>) tools.

## WGS

The preferred embodiment, genomic extracted with Wizard Genomic DNA Purification Kit (Promega, Madison, WI). Paired-end sequencing (2 × 150 bp) was used to obtain the whole genome sequence (WGS) of six strains in the Illumina Hiseq 2500 and Nanopore MinION (long-read) sequencing with Rapid Barcoding Kit (RBK004) in the R9.4.1 flow cell. As previously reported, use the hybrid de novo assembly strategy with Unicycler tools to assemble the genome [16]. Use different databases, including Plasmid Finder, Res Finder and ISfinder, to study the distribution of plasmid replicons, resistance genes and inserted sequences in the genome. The genetic environments surrounding the carbapenemase genes were annotated using Easyfig 2.2.3.

### Phylogenetic Reconstruction and analysis

Use kSNP program to identify core genome single nucleotide polymorphism SNPs on WGS data of six isolates [17]. kSNP is a programme in view of k-mer analysis. Before running kSNP, use Kchooser to evaluate the best value of k-mer. After running the kSNP program, use the output file for further analysis [18]. Use iTOL to generate the maximum likelihood tree output from the core SNP matrix of kSNP (<https://itol.embl.de/>).

### Nucleotide sequence accession numbers

The sequences of the plasmid of *blaKPC-2* were submitted to NCBI GenBank with accession numbers: pKP6106\_KPC (MW465706) and pKP7849\_KPC (MW478298). All relevant data is available from the corresponding author upon reasonable request.

## Results

### Clinical characteristics of CRKP meningitis

The medical records of patients from whom CRKP were isolated are listed in Supplement table 1. A total of 10 cases bacterial meningitis and 30 pathogens were identified between July-August of 2017-2019. Although there were few confirmed cases, there are suspected outbreaks in neurosurgery department of our hospital

from July to August every year. Multidrug-resistant bacteria with the same drug resistance spectrum were isolated from different sites from each patient, and the antimicrobial susceptibility patterns were tested (Supplement table 2). The strain isolated from patients 1-4 was resistant to all of the tested antimicrobial agents used for treatment of infections caused by *Enterobacteriaceae*, except chloramphenicol, minocycline, polymyxin and tigecycline. The strains isolated from patients 5-6 and patients 8-9 were susceptible to trimethoprim-sulfamethoxazole, minocycline, polymyxin and tigecycline, and to chloramphenicol, polymyxin and tigecycline, respectively. The strains isolated from patient 7 and patient 10 were susceptible to minocycline, polymyxin and tigecycline, and amikacin, polymyxin, and tigecycline, respectively.

Among the 26 *K. pneumoniae* causing meningitis, 20 strains of bacteria were not preserved and 6 isolates were available for further experiments, which were isolated from 6 different patients. The six strains include three from 2017, one from 2018 and two from 2019.

PFGE and multilocus sequence typing (MLST)

MLST analysis found that the six clinically derived carbapenemase-producing *K. pneumoniae* were all ST11; this is the dominant clone of KPC-Kp and has been widely reported in different regions in China. The PFGE results confirmed the homology of the six isolates (Figure 1). Using the interpretation criteria of PFGE patterns proposed by Tenover, et al. [19], the six strains of CRKP from different years were identified as the same strain.

Genomic characteristics of strains studied

The complete genome sequences of isolates 8297, 7213, 6106, 9168, 9070, and 7849 were successfully obtained by combining Illumina short-read and Nanopore long-read data. The results of the analysis indicated that all isolates carried *blaKPC-2*. Strain 8297 had one chromosome of 5,962,663 bp and seven plasmids. Strain 7213, 6106, 7849 and 9070 had six plasmids and chromosomes of 5,957,769 bp;

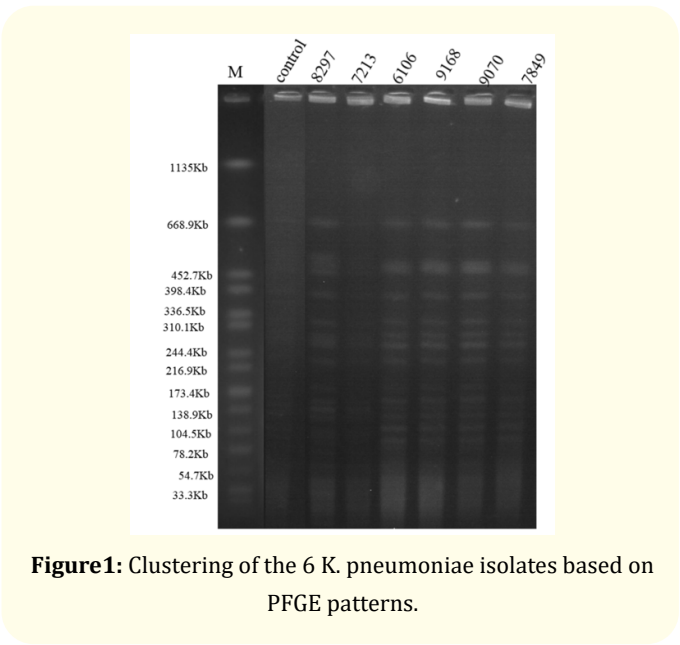


Figure1: Clustering of the 6 *K. pneumoniae* isolates based on PFGE patterns.

5,961,238 bp; 5,987,968 bp and 5,986,653 bp, respectively. Strain 9168 had one chromosome of 5,624,028 bp and four plasmids. The plasmids which include *blaKPC-2* are the same size in strain 6106, 7213 and 8297; all were isolated in 2017. The size of pKP9070\_KPC and pKP7849\_KPC are also the same, which were isolated in 2019.

Associated with the XDR phenotype, resistance determinants were identified in each strain (Table 1). The chromosomal and plasmid-carried determinants were relevant to the resistance of the tested antimicrobials, including  $\beta$ -lactams (*blaKPC-2*, *blaCTX-M-65*, *blaSHV-12* and *blaSHV-11*), aminoglycosides (*aadA2*, *aadA1* and *rmtB*), quinolones (*qnrS1*, *oqxA* and *oqxB*), chloramphenicol (*catA2*), doxycycline [*tet(A)*], trimethoprim-sulfamethoxazole (*sul1*, *sul2*, *dfrA12* and *dfrA14*), fosfomycin (*fosA*) and macrolides [*mph(A)*]. These genotypes were consistent with phenotypic test results (Supplement table 2).

Year	Patient no.	Sex	Age (year)	Admission time	Cancer	Neurosurgical procedures	Source	The studied strain ID and Source	The date of CRKPN was first detected	Treatment	Outcome
2017	1	M	48	12-Jul-17	Right frontal glioma	Yes	Ca, dn, sf	8297, Ca	21-Jul-17	CL, MI	The infection is controlled
	2	F	70	11-Jul-17	Left temporo-occipital triangle glioma	Yes	sf, bl	Not studied	21-Jul-17	CL, MI, TGC	The infection is controlled
	3	F	53	10-Jul-17	Giant meningioma of left sphenoid crest	Yes	sf, bl, ps	7213, sf	23-Jul-17	CL, MI, TGC	The infection is controlled
	4	F	70	20-Jun-17	Left cerebellar tumor	Yes	bl, sf	6106, bl	6-Jul-17	TGC	Died

2018	5	M	57	19-Jul-18	Left cerebello-pontine angle tumor	Yes	bl, sf	9168, bl	3-Aug-18	TGC, MEM, POY	Died
	6	M	70	10-Jul-18	Left frontotemporal glioma	Yes	sp, bl, sf	Not studied	20-Jul-18	C/S, LEV, SXT	The infection is controlled
	7	F	57	1-Aug-18	Meningioma	Yes	dn, bl, sf	Not studied	10-Aug-18	MI, TGC	The infection is controlled
2019	8	M	63	23-Jul-19	Glioma	Yes	Ca, bl, sf	9070, bl	31-Jul-19	MEM, TGC, CL	Died
	9	F	49	24-Jul-19	Epidermoid galactoma	Yes	sf, bl	7849, sf	16-Aug-19	TGC, CL, POY	Died
	10	M	62	2-Aug-19	Right thalamic tumor	Yes	sp, bl, sf	Not studied	20-Aug-19	TGC, POY	The infection is controlled

Supplement Table 1: Characteristics of neurosurgical patients with nosocomial meningitis during the period 2017-2019.

**Abbreviations:** Ca: Catheter; dn: Drain; sf: cerebrospinal fluid; bl: blood; ps: pus; sp, sputum; cl: chloramphenicol; MI: minocycline; TGC: Tigecycline; MEM: Meropenem; POY, Polymyxin; C/S: cefoperazone/sulbactam; LEV: Levofloxacin; SXT: Trimethoprim/sulfamethoxazole.

Year	Patient	MIC															
		GEN	AMP	FEP	FOX	CRO	TZP	SXT	LVX	CIP	MEM	IPM	AMK	CL	MI	POY	TGC
2017	1	16	32	32	32	64	128	8	8	4	16	16	64	8	4	0.25	1
	2	16	32	32	32	64	128	8	8	4	16	16	64	8	4	0.25	0.5
	3	16	32	32	32	64	128	8	8	4	16	16	64	4	4	0.25	0.25
	4	16	32	32	32	64	128	4	8	4	16	16	64	8	4	0.25	0.25
2018	5	16	32	32	32	64	128	0.5	8	4	16	4	64	32	4	0.125	0.25
	6	16	32	32	32	64	128	0.5	8	4	16	16	64	32	4	0.5	1
	7	16	32	32	32	64	128	8	8	4	16	16	64	32	4	0.5	1
2019	8	16	32	32	32	64	128	4	16	4	16	16	64	8	16	0.25	0.5
	9	16	32	32	32	64	128	4	16	4	16	16	64	8	16	0.125	0.5
	10	16	32	32	32	64	128	4	16	4	16	16	8	32	16	0.125	0.25

Supplement Table 2: Characteristics of antimicrobial susceptibilities in the ten patients.

**Abbreviations:** GEN: Gentamicin; AMP: ampicillin; FEP: Cefepime; FOX: Cefoxitin; CRO: Ceftriaxone; TZP: Piperacillin-Tazobactam; SXT: Trimethoprim-Sulfamethoxazole; LVX: Levofloxacin; CIP: Ciprofloxacin; MEM: Meropenem; IPM: Imipenem; AMK: Amikacin; CL: Chloramphenicol; MI: Minocycline; POY: Polymyxin; TGC: Tigecycline

Isolates	Source	Resistance determinants	Chromosome size	Plasmid size
8297	ca	aadA2 blaCTX-M-65 blaKPC-2 blaSHV-12 dfrA12 fosA mph(A) rmtB sul1	5 962 663bp	5 485 939bp、 229 339bp 129 531bp、 98 788bp(pKP8297_KPC) 10 060bp、 5 596bp、 3 410bp
7213	sf	aadA2 blaCTX-M-65 blaKPC-2 blaSHV-12 dfrA12 fosA mph(A) rmtB sul1	5 957 769bp	5 485 163bp、 228 622bp、 129 540bp、 98 788bp(pKP7213_KPC) 、 10 060bp、 5 596bp
6106	bl	aadA2 blaCTX-M-65 blaKPC-2 blaSHV-12 dfrA12 fosA mph(A) rmtB sul1	5 961 238bp	5 487 558bp 、 229 696bp 、 129 540bp、 98 788bp(pKP6106_KPC)、 10 060bp 、 5 596bp
9168	bl	aadA1 blaCTX-M-65 blaKPC-2 blaSHV-11 blaSHV-12 catA2 fosA oqxA oqxB rmtB	5 624 028bp	5 480 246bp、 128 126bp(pKP9168_KPC)、 10 060bp、 5 596bp
9070	bl	QnrS1 aadA1 blaCTX-M-65 blaKPC-2 blaSHV-11 blaSHV-12 dfrA14 fosA rmtB sul2 tet(A)	5 986 653bp	5 519 453bp、 217 877bp、 148 968bp(pKP9070_KPC)、 82 789bp、 11 970bp、 5 596bp
7849	sf	QnrS1 aadA1 blaCTX-M-65 blaKPC-2 blaSHV-11 blaSHV-12 dfrA14 fosA rmtB sul2 tet(A)	5 987 968bp	5 520 759bp、 217 877bp、 148 977bp(pKP7849_KPC) 、 82 789bp、 11 970bp、 5 596bp

**Table 1:** Molecular characteristics of the strains and their plasmids.  
**Abbreviations:** Ca: Catheter; sf: Cerebrospinal Fluid; bl: Blood.

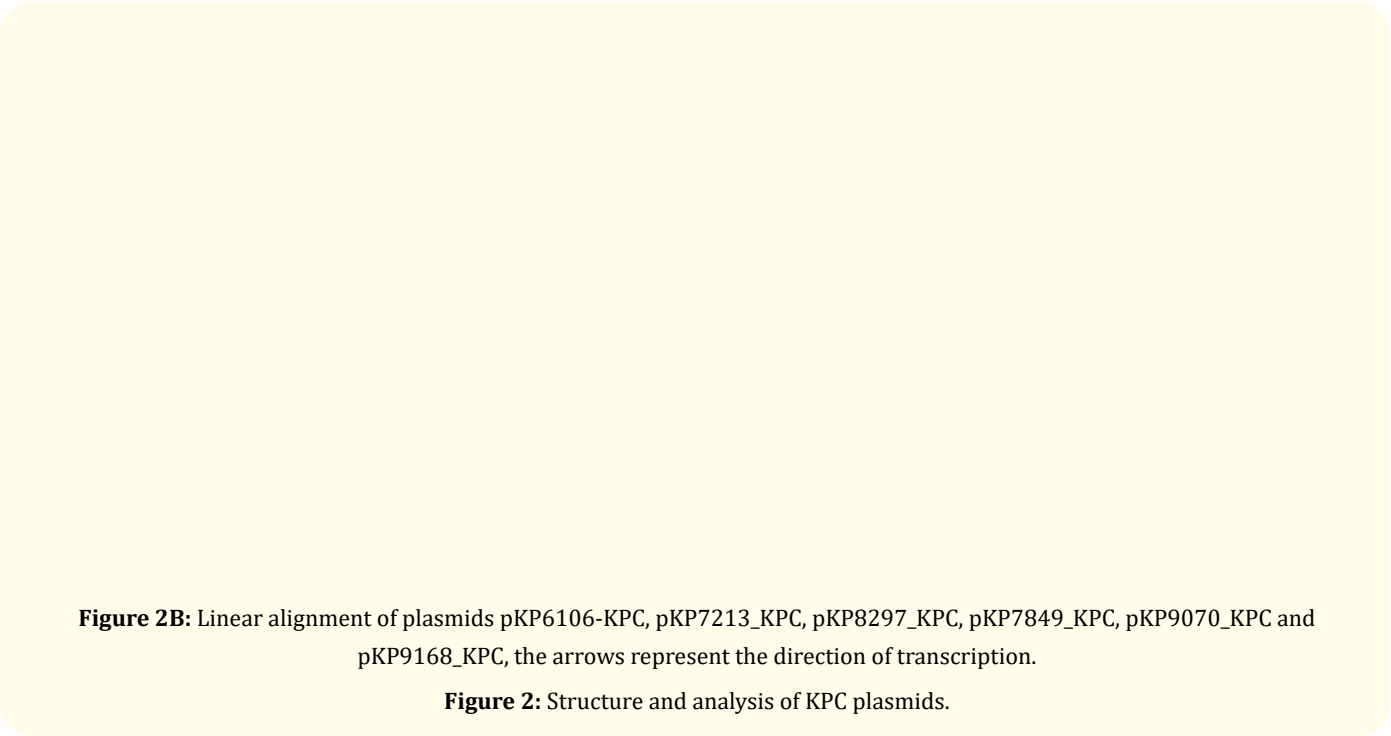
Plasmid and genome Phylogenetic analysis based WGS

According to the analysis, the plasmids harbouring *blaKPC-2* (pKP6106\_KPC, pKP7213\_KPC and pKP8297\_KPC) had the same sequence; pKP7849\_KPC and pKP9070\_KPC also had the same sequence. The detailed comparison of the strains is shown in Figure 2A. Comparing the plasmid sequences, the six isolates all had IncFII-type plasmids, which is proposed to promote the spread of the *blaKPC* gene in *K. pneumoniae* ST11 in China. Moreover, their *blaKPC-2* genes had the same genetic environment in pKP6106\_KPC, pKP7213\_KPC, pKP7849\_KPC, pKP8297\_KPC and pKP9070\_KPC; the exception was pKP9168\_KPC, which differed (Figure 2B). pKP9168\_KPC has the same identity as CP025463.1 as identified by nucleotide Blast; CP025463.1 is the accession number of strain p44-2, which was recovered from Huashan Hospital (Shanghai, China) in 2016. These analyses revealed the plasmids harbouring *blaKPC-2* were the same between 2017 and 2019.

Phylogenetic analysis based on the k-mer algorithm shows that the strains isolated from 2017 (7213, 6106 and 8297) and 2019 (7849 and 9070) share certain phylogenetic relationships (Figure 3). 9168 was found to be closely related to the genomes of other strains.

Discussion

Bacterial meningitis in hospitals, especially intracranial infections caused by bacteria which resistant to carbapenems, is a life-threatening complication in neurosurgery patients [20, 21]. The most common pathogen of hospital-associated meningitis is *Staphylococcus*, and Gram-negative bacteria with multi-drug resistance and extensive drug resistance, including *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Klebsiella pneumoniae*; CRKP is rarely isolated from CSF samples [12,13,22,23]. Nevertheless, in many countries, including Turkey, the United States and





**Figure 3:** SNP analysis of strain 7213, 6106, 8297, 7849, 9070 and 9168, performed using kSNP. The maximum likelihood analysis of the core SNP matrix output for kSNP is performed in iTOL.

China, meningitis caused by CRKP neurosurgery has been reported [13,23-25]. To our knowledge, this is the first CRKP meningitis outbreak in cancer patients described in central China.

From July to September from 2017 to 2019, there were 3-4 cases of meningitis in a short period caused by CRKP in the neurosurgical ward of our hospital. Strictly implement infection control measures every year, including that all CRKP carriers should be isolated in a room under strict contact prevention measures and marked in the hospital information system; workers strengthen the disinfection of chlorhexidine in patient wards, And implement a culture of monitoring of hands and the environment; the hospital health nurse manager emphasizes the cleaning of high contact surfaces such as door handles, bedside lockers, chairs and bed rails to reduce cross transmission. Similarly, all exposed patients undergo rectal and pharynx screening once a week. However, despite these measures of surveillance, the samples from environmental and colonisation screening were negative for CRKP and the source of the infection could not be found.

It was very difficult to clarify transmission events between patients based on epidemiological data alone [26]. The combination of genetic information and clinical epidemiology can better explain the spread of outbreaks. Phylogenetic analysis based on the difference and SNP, in this study identified isolates exhibit closely relat-

ed, and the difference in binding between the patient/department and SNP difference, the pathogen may be determined from a patient to another Patient transmission. The spread over three years suggests that CRKP may still be present in the hospital; the results of this study enforce the importance of continuing active surveillance cultures, and provide support for including healthcare workers, common environmental areas and jointly used medical devices in surveillance procedures. Increasing these protocols could help in limiting spread of CRKP and improving outbreak management.

Previous studies have shown, ST11 is a typical advantage of KPC-Kp clone, and has been widely reported in different parts of China [12,15,27-31]; all the strains identified in this study were ST11, and the plasmids carrying *blaKPC* were IncFII-type. Previous studies have demonstrated the diversity of plasmids carried by *blaKPC*, including IncFII, IncN, IncL/M, IncR and ColE1 groups, ranging in size from 10 to 300 kb [32-36]. IncF replicons may be further divided into FIA, FIB, FIC and FII groups. IncFII family plasmid present in a variety *Enterobacter* and plays an important role in the spread of antimicrobial resistance gene or the like as *blaKPC* [28,37]. There is a close correlation between ST11 KPC-Kp and IncFII-like plasmids, which indicates that ST11 KPC-Kp seems to be a good settler because it can capture IncFII-like plasmids that are widely spread in clinical settings [27]. It is known that horizontal gene transfer mediated by IncFII-like plasmids plays an important role in the pandemic spread of *blaKPC-2* in *Klebsiella pneumoniae* ST11 in China. In addition, the genetic environment surrounding *blaKPC-2* was investigated in the isolates collected in this study; the results suggest the genetic background of *blaKPC-2* is relatively similar in all plasmids, as the *blaKPC-2* genes were located within the same genetic context. Although the six isolates all carried IncFII-type plasmids, the size of the plasmid carrying the *blaKPC-2* differed between isolates, indicating that the *blaKPC-2* in the hospital strains may have been from various origins, spread throughout the hospital for many years. It is necessary to conduct a long-term retrospective genomic study of KPC-producing *K. pneumoniae* throughout the hospital to elucidate the evolution of KPC-producing *K. pneumoniae*.

Despite its significant results, this study did have limitations. Firstly, too few strains were studied; although the study contained strains isolated from different years, it is a retrospective analysis of medical records and therefore not all species and isolates from

those periods were collected. The collected samples, whether from the environment, the patient, or the medical staff themselves did not have detected CRKP at the time; the source of CRKP has not been traced back to until now.

## Conclusions

In summary, this study reports an outbreak in patients with nervous system tumours caused by closely related CRKP that lasted three years. When an outbreak occurs, the patient and the environment should be disinfected immediately, and the patient should be given appropriate treatment and be isolated; these measures can effectively contain the outbreak. In addition, the results of molecular analysis showed that ST11 and IncFII-type plasmids in CRKP have a close correlation and indicate that a long-term retrospective genomic study throughout the hospital for KPC-producing *K. pneumoniae* is necessary.

## Declarations

## Funding

This study was supported through Hospital Nursery Fund: Grant No. 2017011.

## Conflicts of Interest

Competing interests (Not applicable).

## Ethics Approval

This study obtained permissions from the Bioethics Committee of Affiliated Cancer Hospital of Zhengzhou University and Henan Cancer Hospital and participants (consent to participate was obtained from participants) to review patient records and use the data.

## Consent to Participate

Yes.

## Consent to Publication

Yes.

## Availability of Data and Material

Yes.

## Code Availability

Not applicable.

## Authors' Contributions

MYS performed experimental work and drafted manuscript; WQX and YMC analyzed the study data; XKW and YYQ provided interpretation of data; QXX conceived the study and provided data interpretation. All authors read and approved the final manuscript.

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