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ORIGINAL RESEARCH

Epidemiological and Molecular Characteristics of bla_{NDM-1} and bla_{KPC-2} Co-Occurrence Carbapenem-Resistant *Klebsiella pneumoniae*

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Objective: Carbapenem-resistant *Klebsiella pneumoniae* (CRKP) has emerged and spread worldwide. It can usually cause a serious threat complicating treatment options in clinical settings. However, treatment options are limited. The present study investigates the prevalence and genetic characteristics of bla_{NDM-1} and bla_{KPC-2} co-harboring clinical isolates of *Klebsiella pneumoniae*.

Methods: In this study, Multiplex polymerase chain reaction (PCR) was performed to detect the carbapenem-resistant genes, and the broth microdilution method was used to determine the minimum inhibitory concentrations (MICs) of antibacterial drugs. The transferability of carbapenem-resistant phenotypes was examined using filter mating assays. Overall, we used Illumina sequencing to evaluate the epidemiological and molecular characteristics of bla_{NDM-1} and bla_{KPC-2} (genes encoding carbapenemase) co-occurrence in CRKP strains.

Results: All strains exhibited resistance to carbapenems and other antibiotics. However, they were still susceptible to polymyxin E. Among them, 18 isolates were positive for bla_{KPC-2}, bla_{NDM-1}, and multiple virulence determinants, such as genes encoding the virulence factor aerobactin, yersiniabactin, and the regulator of the mucoid phenotype (*rmpA* and *rmpA2*). Whole genome sequencing revealed that the 18 CRKP strains belonged to ST11 and capsular serotype KL64, and could be grouped into two evolutionary branches. Furthermore, these strains displayed hypervirulence potential since all of them carried pLVPK-like plasmid.

Conclusion: These findings suggested that ST11-KL64 CRKP strains are major threats in terms of nosocomial infections in this hospital. Hence, new strategies should be urgently developed to monitor, diagnose, and treat this high-risk CRKP clone.

Keywords: *Klebsiella pneumoniae*, carbapenem resistance, illumina sequencing, bla_{KPC-2}, bla_{NDM-1}

Introduction

Klebsiella pneumoniae is an important clinical pathogen. With the widespread use of antibiotics, its multidrug resistance has gradually increased, particularly to carbapenems.[1] The first carbapenemase in *K. pneumoniae* was discovered in 1996, which was encoded by bla_{KPC} gene.[2] Subsequently, other carbapenemase genes were discovered, including bla_{NDM}, bla_{OXA-48}, bla_{VIM}, and bla_{IMP}.[3–6] Among them, KPC-2, one of class A carbapenemase, is relatively common in China. With the widespread popularity of carbapenem and wide distribution of carbapenemase genes, carbapenem-resistant *K. pneumoniae* (CRKP) isolation rates are gradually increasing globally. At present, *K. pneumoniae* carbapenemase (KPC) is one of the most important carbapenemases in clinical setting, and its rapid spread has posed a huge challenge to global public health.[7] In China, CRKP isolates mainly carry bla_{KPC-2} or bla_{NDM-1} genes for carbapenem resistance, and multilocus sequence typing is mainly ST11.[8,9]

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K. pneumoniae exhibits two pathogenic types: hypervirulent *K. pneumoniae* (hvKP) and classical *K. pneumoniae* (cKP).[10] These two types cause a great challenge globally in terms of hospital infections.[11] cKP forms multidrug-resistant (MDR) and extensively drug-resistant strains by acquiring various antimicrobial resistance genes.[1] A typical representative is CRKP, which is related with high morbidity and mortality rates.[12] hvHP easily causes liver abscess, pneumonia, meningitis, and endophthalmitis in healthy individuals and has been reported to be closely related to iconic virulence factors including regulators of myxoid phenotypes (*rmpA* and *rmpA2*) and virulence genes (yersiniabactin and aerobactin).[10] For a long time, *K. pneumoniae* did not encode both multidrug resistance and high virulence phenotypes simultaneously.[13] However, in recent years, reports are emerging continuously regarding the simultaneous emergence of carbapenem resistance and virulence in a single epidemic clone, which has become a serious public health threat.[14–16] ST11-CR-hvKp is the most representative clade detected in China.[15,17,18] Therefore, the genomic characterization of CRKP should be urgently studied to prevent, diagnose, and treat infections caused by *K. pneumoniae*.

In this study, we obtained 60 CRKP isolates from inpatients in a tertiary hospital in China, among which the NDM and KPC co-producing ST11-KL64 CRKP clone is a major threat in terms of nosocomial infections at this hospital, and these

strains displayed hypervirulence potential since all of them carried pLVPK-like plasmid. In addition, the transmission routes and genetic characterizations of these strains were investigated as well.

Materials and Methods

Patients and Isolates

From January 2019 to June 2021, 60 CRKP isolates were isolated from sputum, Tracheal aspiration, BAL, blood, urine, pus, and ascites fluid samples from the general ward and intensive care unit (ICU) of a tertiary hospital in Yangzhou, Jiangsu Province, China. There are 35 beds in the ICU of the hospital. The clinical data of patients were reviewed from the patient management system, including gender, age, in-patient department, underlying disease, clinical diagnosis, prognosis, duration of hospital stay, invasive procedures performed before sample collection, and antibiotic exposure. The underlying diseases included diseases of immune, respiratory, urinary, digestive, and blood systems, as well as hypertension, diabetes, and tumors. Invasive operations included invasive ventilator, tracheotomy and intubation, and drainage. The study was approved by the Research Ethics Committee of the Affiliated Hospital of Yangzhou University and was followed the Declaration of Helsinki. No identifiable patient information was collected in this study. All isolates were isolated for routine clinical experiment.

Bacterial Identification and Antimicrobial Susceptibility

The samples were plated onto 5% sheep blood agar and were cultured at 37°C for bacterial isolation. All isolates were identified using the VITEK-2 system. A multiplex PCR method was used to assess whether these isolates contained carbapenemase genes including bla_{NDM}, bla_{KPC}, bla_{SIM}, bla_{GIM}, bla_{SPM}, bla_{AIM}, bla_{OXA-48}, bla_{BIC}, bla_{DIM}, bla_{IMP}, bla_{VIM} (Table S1), referring to the primer sequence.[19] The isolates carrying the corresponding carbapenemase-encoding gene were used as positive controls. Antimicrobial susceptibility was investigated using VITEK-2 system and the broth microdilution methods. The susceptibility breakpoint was interpreted according to 2018 Clinical and Laboratory Standards Institute guideline except for tigecycline and polymyxin E, which followed the criteria of European Committee on Antimicrobial Susceptibility Testing (version 12.0). *Escherichia coli* ATCC25922 was used as the quality control strain for antimicrobial susceptibility testing.

Filter Mating Assay

The transferability of carbapenem-resistant phenotypes was measured using filter-mating assay. *K. pneumoniae* YZ6 Hyg^r was used as the recipient strain, and the 60 CRKP isolates were used as the donor strains. Transconjugants were selected on LB agar plates supplemented with hygromycin (200 mg/L) and meropenem (2 mg/L).[20] The transconjugants carrying the gene encoding carbapenemase were confirmed using multiplex PCR and antimicrobial susceptibility testing.

Genome Extraction, Bioinformatics Analysis, and Phylogenetic Tree Construction

The epidemiological and molecular features of bla_{KPC} and bla_{NDM} co-occurrence were further studied using 18 CRKP isolates with bla_{KPC-2} and bla_{NDM-1} co-occurrence. FastPure Bacteria DNA Isolation Mini Kit (Vazyme, Nanjing, China) was used to extract the genomic DNA from all CRKP isolates with bla_{KPC-2} and bla_{NDM-1} co-occurrence. The extracted genomic DNA was subjected to 1% agarose gel electrophoresis, quantified using the Qubit fluorometer, and further subjected to short-read sequencing (2 × 150 bp) on the Illumina HiSeq 2500 platform. The short-read Illumina raw sequences of 18 CRKP isolates with bla_{KPC-2} and bla_{NDM-1} co-occurrence were screened for quality and assembled through SPAdes. The contigs smaller than 500 bp were purposefully removed. The sequence types (STs), capsule types, insertion sequences (IS), and multiple antimicrobial-resistance and virulence genes of these isolates were identified using CGE server (https://cge.cbs.dtu.dk) and Kleborate. The phylogenetic trees for the 18 CRKP isolates with bla_{KPC-2} and bla_{NDM-1} co-occurrence were constructed using Roary and FastTree. Further, visualization and modification were performed using iTOL5.

Results

Clinical Characteristics of CRKP Isolates

From January 2019 to June 2021, 60 strains of CRKP were isolated from 54 inpatients in a tertiary hospital in Yangzhou (Table 1). Among the 54 inpatients, 39 were males (72.22%) and 15 were females (27.78%), with an average age of 65.0 years. Clinical isolates were obtained from sputum (35/60, 58.33%), Tracheal aspiration (8/60,13,33%), BAL (5/60,8.34%), blood (6/60, 10.00%), urine (4/60, 6.67%), pus (1/60, 1.67%), and ascites (1/60, 1.67%) samples. The inpatients were from ICU (38/54, 70.37%), respiratory medicine (3/54, 5.56%), neurosurgery (3/54, 5.56%), general surgery (3/54, 5.56%), emergency (2/54, 3.70%), thoracic surgery (1/54, 1.85%), neurology (1/54, 1.85%), gastroenterology (1/54, 1.85%), cardiology (1/54, 1.85%), and nephrology (1/54, 1.85%) departments. Most patients were exposed to antibiotics, mainly cephalosporins, carbapenems, and enzyme inhibitors, and 45 patients had undergone invasive operation before sample collection.

Antimicrobial Susceptibility and Transferability

According to the drug sensitivity test data (Table 2), all CRKP isolates exhibited multiple-drug resistance. Moreover, most strains displayed resistance to doxycycline (58/60, 96.7%), chloramphenicol (59/60, 98.3%), and aztreonam (59/60, 98.3%) but remained susceptible to polymyxin (59/60, 98.3%). Multiple PCR revealed that a total of 18 isolates simultaneously carried bla_{NDM-1} and bla_{KPC-2}. The results of conjugation assay (Figure 1) revealed that most of the bla_{KPC-2}-carrying CRKP isolates could not successfully transfer their carbapenemase genes into the recipient strain. By contrast, among the 18 strains with bla_{KPC-2} and bla_{NDM-1} co-occurrence, 16 strains could successfully transfer the carbapenem resistance phenotype to the recipient strain YZ6 Hyg^r, indicating that their carbapenem encoding genes were located on the conjugate plasmids.

STs, Capsular Types, Virulence Genes, and Phylogenetic Analysis of CRKP Strains with bla_{NDM-1} and bla_{KPC-2} Co-Occurrence

MLST is a well-known gene typing method that can be used to monitor and control the spread of pathogens in hospitals.[21] According to the *K. pneumoniae* MLST database, 18 CRKP strains with bla_{NDM-1} and bla_{KPC-2} co-occurrence were all identified as ST11, and the capsular type were KL64, suggesting that there was nosocomial infection of ST11 CRKP at this hospital. In addition, phylogenetic analysis revealed that 18 CRKP isolates with bla_{NDM-1} and bla_{KPC-2} co-occurrence were divided into two clades (Figure 2). Clade 1 included 9 strains (KP72, KP74, KP37, KP60, KP52, KP63, KP53, KP64 and KP62), whereas clade 2 contained 9 strains (KP58, KP44, KP43, KP80, KP78, KP20, KP81, KP73, and KP57).

Table 1 Characteristics of the 60 CRKP Isolates from the 54 Inpatients

Patient	Isolate Number	Gender	Age (Years)	Isolate Type	Isolate Date	Ward	Outcome	Invasive Operation Before Isolation of Strains	Antibiotics Used Before Isolation of Strains	PCR Result
Pa1	KP1	Male	71	Sputum	11/01/2019	ICU	Discharged	No	Penicillin, enzyme inhibitors, cephalosporins, quinolones	bla_{KPC}
Pa3	KP3	Male	49	Sputum	11/14/2019	ICU	Discharged	Yes	Cephalosporins, penicillin, enzyme inhibitors, carbapenem, polypeptide	bla_{KPC}
Pa5	KP5	Male	88	Sputum	11/22/2019	Respiratory medicine	Discharged	No	Cephalosporins, quinolones, carbapenem	bla_{KPC}
Pa8	KP8	Male	54	Ascites fluid	12/30/2019	ICU	Discharged	Yes	Carbapenem	bla_{KPC}
Pa11	KP11	Male	93	Tracheal aspiration	01/22/2019	ICU	Died	No	Penicillin, enzyme inhibitors	bla_{KPC}
Pa12	KP12	Male	85	Tracheal aspiration	02/07/2020	ICU	Discharged	Yes	Penicillin, enzyme inhibitors, quinolones	
Pa13	KP13	Male	71	Blood	02/18/2020	ICU	Died	Yes	Enzyme inhibitors, cephalosporins, carbapenem	bla_{KPC}
Pa17	KP17	Male	72	BAL	03/28/2021	ICU	Automatic discharge*	Yes	Penicillin, enzyme inhibitors, polypeptide	bla_{KPC}
Pa18	KP18	Male	58	Tracheal aspiration	04/11/2021	ICU	Discharged	Yes	Penicillin, enzyme inhibitors, cephalosporins, glycylcycline	bla_{KPC}
Pa19	KP19	Male	5	Sputum	04/16/2021	ICU	Automatic discharge*	Yes	Enzyme inhibitors, cephalosporins, polypeptide	bla_{KPC}
Pa20	KP20	Male	54	Sputum	04/16/2021	ICU	Automatic discharge*	Yes	Penicillin, enzyme inhibitors	$bla_{KPC,}$ bla_{NDM}
	KP27	Male	54	Blood	04/19/2021	ICU	Automatic discharge*	Yes	Penicillin, enzyme inhibitors,	bla_{KPC}
Pa21	KP21	Male	67	Tracheal aspiration	04/15/2021	ICU	Automatic discharge*	Yes	Penicillin, enzyme inhibitors,	bla_{KPC}
Pa22	KP22	Male	68	Sputum	04/18/2021	Neurosurgery	Discharged	Yes	Penicillin, enzyme inhibitors, polypeptide, carbapenem, glycylcycline	bla_{KPC}
Pa24	KP24	Male	79	Sputum	04/17/2021	Cardiology	Discharged	Yes	Penicillin, enzyme inhibitors	bla_{KPC}
Pa25	KP25	Male	65	Sputum	04/20/2021	Emergency	Discharged	Yes	Penicillin, enzyme inhibitors	bla_{KPC}
Pa26	KP26	Female	78	BAL	04/20/2021	ICU	Automatic discharge*	Yes	Penicillin, enzyme inhibitors, cephalosporins, quinolones	bla_{KPC}
	KP45	Female	78	Blood	05/08/2021	ICU	Automatic discharge*	Yes	Glycylcyclin, carbapenem, polypeptide	bla_{KPC}
Pa28	KP28	Male	65	Sputum	04/21/2021	ICU	Automatic discharge*	Yes	Penicillin, enzyme inhibitors	bla_{KPC}
Pa29	KP29	Male	55	Sputum	04/23/2021	Emergency	Discharged	Yes	Penicillin, enzyme inhibitors, cephalosporins, quinolones, carbapenem, polypeptide	bla_{KPC}
Pa30	KP30	Male	40	Sputum	04/26/2021	ICU	Automatic discharge*	Yes	Penicillin, enzyme inhibitors, cephalosporins, macrolide	bla_{KPC}
Pa31	KP31	Female	89	Tracheal aspiration	04/26/2021	ICU	Automatic discharge*	Yes	Carbapenem	bla_{KPC}
Pa32	KP32	Female	79	Sputum	04/26/2021	ICU	Died	Yes	Carbapenem, enzyme inhibitors, cephalosporins	bla_{KPC}
Pa36	KP36	Female	15	Pus	05/01/2021	General surgery	Discharged	No	–	
Pa37	KP37	Male	70	Sputum	05/01/2021	Gastroenterology	Discharged	Yes	Carbapenem, quinolones	bla_{KPC} bla_{NDM}
Pa38	KP38	Male	69	Sputum	04/28/2021	ICU	Discharged	Yes	Penicillin, enzyme inhibitors, carbapenem	bla_{KPC}
Pa39	KP39	Male	69	BAL	05/10/2021	ICU	Died	Yes	Penicillin, enzyme inhibitors, carbapenem, polypeptide, glycylcyclin	bla_{KPC}
Pa40	KP40	Female	47	Sputum	05/10/2021	ICU	Discharged	Yes	Cephalosporins	bla_{KPC}
	KP52	Female	47	Urine	05/19/2021	ICU	Discharged	Yes	Cephalosporins, aminoglycoside, polypeptide	bla_{KPC} bla_{NDM}
Pa41	KP41	Male	59	Sputum	05/11/2021	ICU	Automatic discharge*	Yes	Penicillin, enzyme inhibitors, glycylcyclin, polypeptide, cephalosporins	bla_{KPC}
Pa42	KP42	Female	53	Sputum	05/11/2021	ICU	Automatic discharge*	Yes	Penicillin, enzyme inhibitors, carbapenem, polypeptide, glycylcyclin, cephalosporins	bla_{KPC}

Pa43	KP43	Male	73	BAL	05/11/2021	ICU	Discharged	Yes	Cephalosporins	bla_{KPC}, bla_{NDM}
Pa44	KP44	Female	49	Urine	05/12/2021	Nephrology	Discharged	Yes	Enzyme inhibitors, carbapenem, polypeptide, glycylcyclin, cephalosporins, aminoglycoside	bla_{KPC}, bla_{NDM}
Pa46	KP46	Female	72	Sputum	05/11/2021	ICU	Automatic discharge*	Yes	Penicillin, enzyme inhibitors, glycylcyclin, polypeptide, cephalosporins, quinolones	bla_{KPC}
Pa48	KP48	Male	74	Sputum	05/15/2021	ICU	Discharged	Yes	Penicillin, enzyme inhibitors	bla_{KPC}
Pa49	KP49	Female	88	Tracheal aspiration	05/17/2021	ICU	Discharged	Yes	Cephalosporins, enzyme inhibitors	bla_{KPC}
Pa50	KP50	Male	76	Sputum	05/15/2021	ICU	Automatic discharge*	No	Carbapenem	bla_{KPC}
Pa51	KP51	Male	89	Sputum	05/15/2021	ICU	Discharged	No	Carbapenem	bla_{KPC}
Pa53	KP53	Male	43	Sputum	05/19/2021	ICU	Automatic discharge*	Yes	Enzyme inhibitors, cephalosporins, polypeptide, glycylcyclin	bla_{KPC}, bla_{NDM}
Pa57	KP57	Male	71	Sputum	05/25/2021	ICU	Automatic discharge*	Yes	Cephalosporins, enzyme inhibitors, polypeptide, carbapenem	bla_{KPC}
Pa58	KP58	Male	68	Sputum	05/25/2021	Respiratory medicine	Discharged	No	Cephalosporins, enzyme inhibitors	bla_{KPC}, bla_{NDM}
Pa59	KP59	Female	87	Tracheal aspiration	05/23/2021	ICU	Automatic discharge*	Yes	Cephalosporins, carbapenem	bla_{KPC}
Pa60	KP60	Male	57	Sputum	05/31/2021	ICU	Discharged	Yes	Cephalosporins, carbapenem, enzyme inhibitors, glycylcyclin	bla_{KPC}, bla_{NDM}
Pa61	KP61	Male	54	Sputum	03/24/2020	Neurosurgery	Discharged	Yes	Cephalosporins, carbapenem, enzyme inhibitors, polypeptide, quinolones	bla_{KPC}
Pa62	KP62	Female	69	Sputum	05/31/2021	ICU	Discharged	Yes	Cephalosporins, enzyme inhibitors, glycylcyclin	bla_{KPC}, bla_{NDM}
Pa63	KP63	Male	65	Blood	05/30/2021	Thoracic surgery	Discharged	Yes	Cephalosporins, carbapenem, enzyme inhibitors, quinolones	bla_{KPC}, bla_{NDM}
	KP66	Male	65	Sputum	05/26/2021	Thoracic surgery	Discharged	Yes	Cephalosporins, carbapenem, enzyme inhibitors, quinolones	bla_{KPC}, bla_{NDM}
Pa64	KP64	Male	76	Blood	05/24/2021	ICU	Died	Yes	Cephalosporins, carbapenem, enzyme inhibitors, glycylcyclin	bla_{KPC}, bla_{NDM}
	KP81	Male	76	Urine	06/24/2021	ICU	Died	Yes	Cephalosporins, carbapenem, enzyme inhibitors, polypeptide	bla_{KPC}, bla_{NDM}
Pa65	KP65	Male	7 months	Sputum	04/09/2020	ICU	Discharged	No	–	bla_{KPC}
Pa67	KP67	Male	75	Sputum	06/03/2021	General surgery	Discharged	No	–	
Pa70	KP70	Male	71	Sputum	06/03/2021	Neurology	Automatic discharge*	Yes	Cephalosporins, carbapenem, enzyme inhibitors, polypeptide, fosfomycin, penicillin	
Pa71	KP71	Female	16	Sputum	06/14/2021	ICU	Discharged	Yes	Cephalosporins, enzyme inhibitors, glycylcyclin	bla_{KPC}
Pa72	KP72	Female	75	Sputum	06/17/2021	ICU	Discharged	Yes	Cephalosporins, carbapenem, enzyme inhibitors, quinolones	bla_{KPC}, bla_{NDM}
Pa73	KP73	Male	83	Tracheal aspiration	06/18/2021	General surgery	Discharged	Yes	Cephalosporins, carbapenem, enzyme inhibitors, quinolones	bla_{KPC}, bla_{NDM}
Pa74	KP74	Male	85	Blood	06/19/2021	ICU	Discharged	Yes	Cephalosporins, carbapenem, enzyme inhibitors	bla_{KPC}, bla_{NDM}
	KP80	Male	85	Urine	06/23/2021	ICU	Discharged	Yes	Cephalosporins, carbapenem, enzyme inhibitors	bla_{KPC}, bla_{NDM}
Pa76	KP76	Male	89	Sputum	06/22/2021	Respiratory medicine	Discharged	Yes	Cephalosporins, carbapenem, enzyme inhibitors, quinolones, polypeptide, glycylcyclin	
Pa78	KP78	Female	72	Sputum	06/11/2021	Neurosurgery	Discharged	Yes	Penicillin, enzyme inhibitors	bla_{KPC}, bla_{NDM}
Pa79	KP79	Female	71	BAL	06/22/2021	ICU	Discharged	Yes	Cephalosporins, carbapenem, enzyme inhibitors, glycylcyclin, fosfomycin	bla_{KPC}

Note: * indicates that the patients did not get better, but they chose to give up further treatment and leave the hospital.

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Table 2 MICs (Mg/L) of All CRKP Strains in This Study

	DOX	CST	CIP	MEM	CHL	GEN	ATM	TGC
KP1	64	≤0.25	>128	>128	64	>128	>128	4
KP3	>128	≤0.25	>128	>128	>128	>128	>128	8
KP5	>128	≤0.25	>128	>128	>128	>128	>128	8
KP8	64	0.25	>128	>128	>128	>128	>128	8
KP11	>128	≤0.25	>128	128	>128	>128	>128	2
KP12	64	≤0.25	>128	128	>128	>128	>128	2
KP13	64	≤0.25	>128	>128	>128	64	>128	4
KP17	>128	0.5	>128	>128	>128	>128	>128	16
KP18	64	≤0.25	>128	>128	64	>128	>128	4
KP19	64	≤0.25	>128	>128	64	32	>128	8
KP20	64	≤0.25	>128	>128	>128	>128	>128	2
KP21	>128	≤0.25	>128	>128	>128	32	>128	16
KP22	32	≤0.25	>128	>128	>128	>128	>128	1
KP24	64	≤0.25	>128	>128	>128	>128	>128	2
KP25	32	≤0.25	>128	>128	>128	>128	>128	1
KP26	>128	≤0.25	>128	>128	>128	>128	>128	2
KP27	>128	≤0.25	>128	>128	>128	>128	>128	4
KP28	64	≤0.25	>128	>128	>128	>128	>128	2
KP29	64	≤0.25	>128	>128	>128	>128	>128	4
KP30	64	≤0.25	>128	>128	>128	>128	>128	4
KP31	64	≤0.25	>128	>128	>128	>128	>128	2
KP32	64	≤0.25	>128	>128	16	128	>128	8
KP36	>128	0.5	>128	>128	>128	>128	>128	4
KP37	64	≤0.25	>128	>128	>128	>128	>128	4
KP38	64	≤0.25	>128	>128	>128	128	>128	8
KP39	64	≤0.25	>128	>128	>128	>128	>128	2
KP40	64	≤0.25	>128	>128	64	>128	>128	4
KP41	128	≤0.25	>128	>128	128	64	>128	4
KP42	>128	≤0.25	>128	>128	>128	>128	2	4
KP43	64	2	>128	>128	32	>128	>128	4
KP44	128	≤0.25	>128	>128	>128	>128	>128	4
KP45	64	2	>128	>128	32	>128	>128	4
KP46	32	≤0.25	>128	>128	128	>128	>128	1
KP48	8	≤0.25	>128	8	>128	128	>128	1
KP49	>128	≤0.25	>128	>128	>128	>128	>128	2
KP50	64	≤0.25	>128	>128	>128	>128	>128	2
KP51	>128	≤0.25	>128	>128	>128	>128	>128	2
KP52	>128	1	>128	>128	>128	>128	>128	4
KP53	128	≤0.25	>128	>128	64	>128	>128	8
KP57	64	≤0.25	>128	>128	128	>128	>128	8
KP58	>128	≤0.25	>128	>128	64	>128	>128	8
KP59	64	≤0.25	>128	>128	>128	>128	>128	4
KP60	64	≤0.25	>128	>128	128	>128	>128	8
KP61	>128	≤0.25	>128	>128	>128	>128	>128	4
KP62	0.5	≤0.25	>128	>128	128	>128	>128	4
KP63	64	≤0.25	>128	>128	>128	>128	>128	4
KP64	64	≤0.25	>128	>128	128	>128	>128	4
KP65	64	2	>128	>128	>128	>128	>128	2
KP66	64	≤0.25	>128	>128	128	>128	>128	4
KP67	64	1	>128	>128	>128	>128	>128	4
KP70	64	≤0.25	>128	>128	>128	>128	>128	8

(*Continued*)

Table 2 (Continued).

	DOX	CST	CIP	MEM	CHL	GEN	ATM	TGC
KP71	>128	16	>128	>128	>128	>128	>128	8
KP72	64	≤0.25	>128	>128	64	>128	>128	8
KP73	128	≤0.25	>128	>128	>128	>128	>128	8
KP74	64	≤0.25	>128	>128	64	>128	>128	8
KP76	>128	≤0.25	>128	>128	64	>128	>128	8
KP78	>128	≤0.25	>128	>128	64	>128	>128	4
KP79	64	≤0.25	>128	>128	64	>128	>128	8
KP80	64	≤0.25	>128	>128	64	>128	>128	8
KP81	64	≤0.25	>128	>128	64	>128	>128	8

Abbreviations: ATM, Aztreonam; MEM, Meropenem; GEN, Gentamicin; DOX, Doxycycline; CIP, Ciprofloxacin; CHL, Chloramphenicol; CST, Colistin; TGC, Tigecycline.

Identification of Antimicrobial Resistance Genes and Virulence Genes

According to the WGS results, the antibiotic-resistance genes and virulence genes of 18 CRKP strains with $bla_{\text{NDM-1}}$ and $bla_{\text{KPC-2}}$ co-occurrence were identified (Figure 2). All isolates contained resistance genes against β-lactams ($bla_{\text{CTX-M-65}}$, $bla_{\text{KPC-2}}$), tetracyclines [*tet*(A)], aminoglycosides (*rmtB*), sulfonamides (*sul2*), trimethoprim (*dfrA14*), fosfomycin (*fosA*), and quinolones (*qnrS1*). The β-lactam resistance gene $bla_{\text{TEM-1B}}$ was present in 17 CRKP isolates; however, it was not present in KP74 isolates. The resistance genes against β-lactams ($bla_{\text{ADC-25}}$, $bla_{\text{OXA-23}}$, and $bla_{\text{OXA-66}}$), aminoglycosides [*aph(3')-Ic, strB*, and *strA*], tetracyclines [*tet*(B)], macrolides [*mph*(E), *msr*(E)] were observed in KP62 isolates. However, these resistance genes were not observed in the remaining 17 isolates. Analysis combined with clinical data (Table 1), KP62 strains were isolated from ICU, and the patients had undergone invasive operations before sample collection. In addition, all CRKP strains with $bla_{\text{NDM-1}}$ and $bla_{\text{KPC-2}}$ co-occurrence contained yersiniabactin and aerobactin, which represented the potential of CR-hvKP phenotype. Except KP62, the regulatory factors of mucus phenotype gene (*rmpA2*) were detected in all CRKP isolates with $bla_{\text{NDM-1}}$ and $bla_{\text{KPC-2}}$ co-occurrence.

To determine the type of virulence plasmid harbored by these strains, plasmid pLVPK (GenBank accession AY378100), a classical virulence plasmid carrying a set of virulence genes, including *iroBCDN, iucABCD, rmpA*, and *rmpA2*, was used as reference plasmid. Surprisingly, we found that the virulence plasmids carried by all strains were

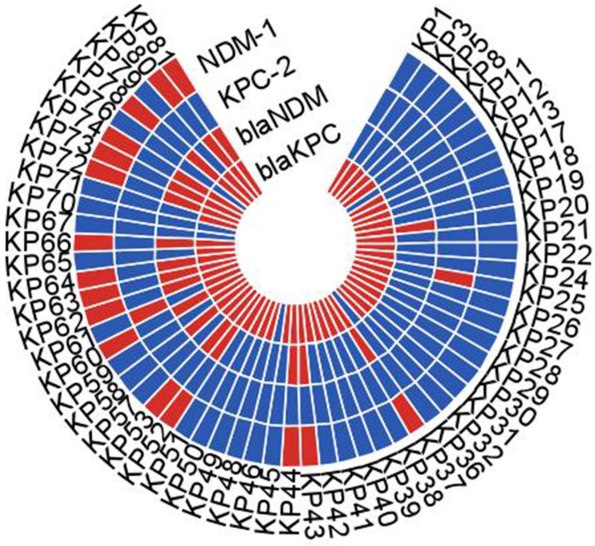

Figure 1 The results of conjugation assay. Red and blue colors, respectively, indicate positive and negative PCR results for bla_{NDM} and bla_{KPC}. Red color of NDM-1 and KPC-2 indicates successful conjugation transfer test results, blue color indicates failure results.

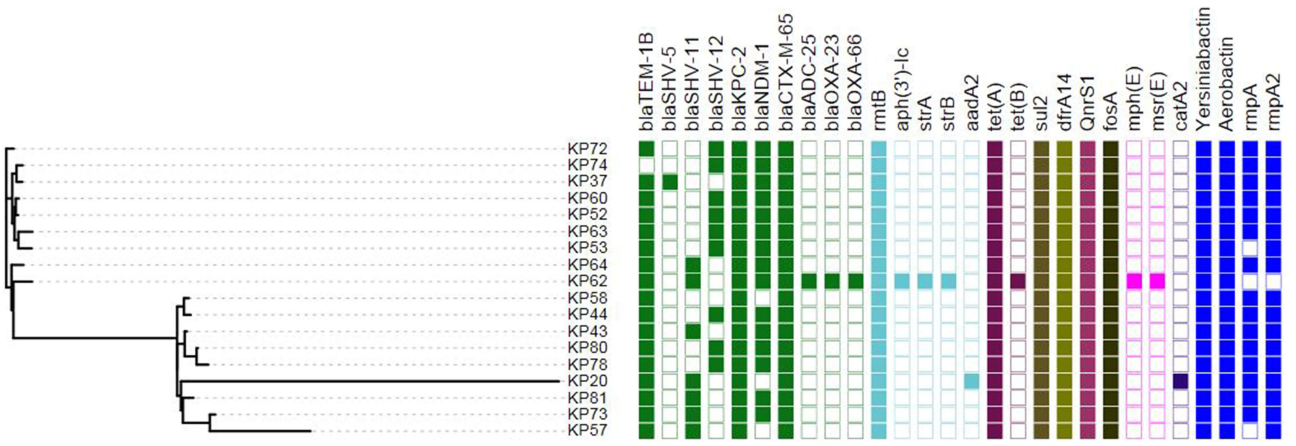

Figure 2 Phylogenetic analysis of 18 CRKP strains with *bla*$_{NDM-1}$ and *bla*$_{KPC-2}$ co-occurrence. Distribution of antibiotic resistance genes and virulence genes in CRKP isolates. The cells of different colors indicate the presence of different genes. Blue represents virulence gene; remaining each color represents a type of drug resistance gene, and the blank cells represent the deletion of genes.

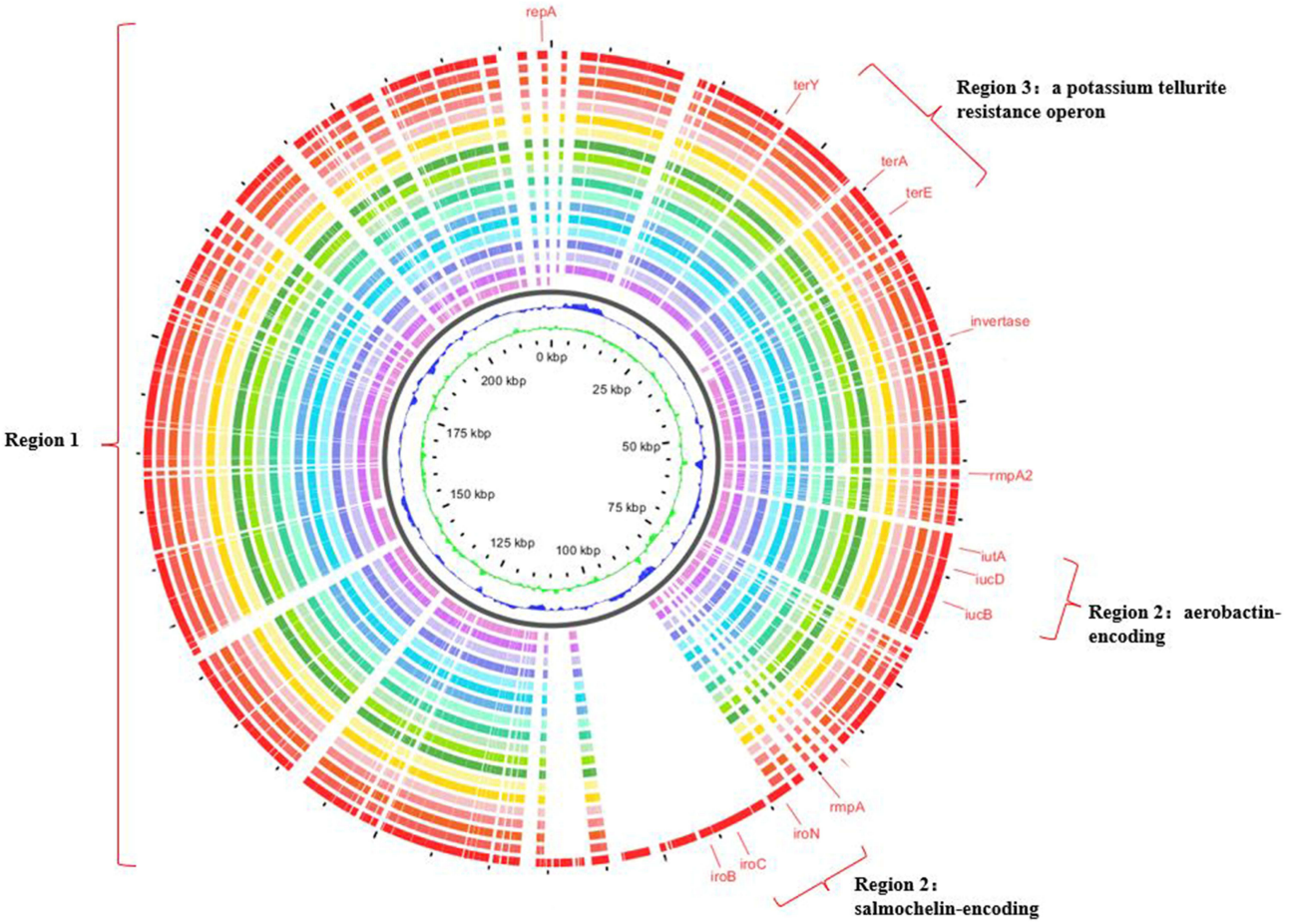

Figure 3 Sequence comparison of 18 CRKP strains plasmids with pLVPK virulence plasmids. Sequence comparison revealed that region 1 of pLVPK plasmids was very similar to 18 *bla*$_{NDM-1}$ and *bla*$_{KPC-2}$ co-occurrence strains. Virulence factors, such as *iroBCDN, iucABCD, iutA*, and *rmpA*2, and a potassium tellurite resistance operon, such as *terA, terE, terY,* located on regions 2 and 3, respectively. The outermost circle annotates the genetic information, and different plasmids are assigned different colors.

aligned well with pLVPK based on Illumina-based contigs analysis (Figure 3), suggesting the identified virulence genes in these strains might be closely related to pLVPK-like plasmids.

Discussion

ICU is the main site of nosocomial infections and has been generally considered as a suitable place to study the epidemic characteristics of MDR strains,[22,23] particularly *K. pneumoniae*.[24] The phenomenon of drug resistance in bacterial isolates from ICU is becoming more and more serious in recent years. It is closely related to factors such as inpatients suffering from various basic diseases, the use of invasive surgical treatment, prolonged hospitalization, and extensive use of broad-spectrum antibiotics, causing a great challenge in terms of clinical antibacterial treatment.[25,26] In the study, among the 60 CRKP strains, 42 (70.00%) were isolated from the ICU. This exhibited a high prevalence rate in inpatients, which was consistent with previous studies.[27,28]

According to MLST typing, all 18 CRKP strains with $bla_{\text{NDM-1}}$ and $bla_{\text{KPC-2}}$ co-occurrence belonged to ST11, indicating that *K. pneumoniae* positive for ST11 $bla_{\text{KPC-2}}$ was the dominant strain in this hospital. This was consistent with previous studies in China.[27,29] ST11 is the single locus variant of ST258, and they belong to the same clonal members of CG258,[7] which has greatly contributed to the global spread of KPC-producing CRKP during the past 20 years.[9,30] In contrast, the ST258 is prevalent in North America and Europe, whereas ST11 is the main type in Asia.[31,32] Additionally, ST11 is the main sequence type of KPC producing CRKP in China and has been reported globally, including the United States, Europe, and Asia.[9,12,31–37]

Previously, *K. pneumoniae* with the ST11 phenotype was a widely and commonly occurring MDR clone, exhibiting resistance to carbapenems; however, it was not highly virulent. However, ST11 has recently attracted considerable attention because of its feature of co-occurrence of resistance and hypervirulence genes in a single strain.[12] In our study, the analysis by Kleborate revealed that all 18 CRKP strains with $bla_{\text{NDM-1}}$ and $bla_{\text{KPC-2}}$ co-occurrence exhibited synthesis of aerobactin, which has been considered as the major siderophore system in the hvKP. The *rmpA* and *rmpA2* virulence genes have been thought to control the capsular polysaccharide biosynthesis and symbolize a hypermucoviscous phenotype, which also existed in most of the 18 strains. Therefore, 18 CRKP strains with $bla_{\text{NDM-1}}$ and $bla_{\text{KPC-2}}$ co-occurrence isolated in this study exhibited hypervirulence phenotype and deserved our attention.

In addition, CRKP strains with $bla_{\text{NDM-1}}$ and $bla_{\text{KPC-2}}$ co-occurrence belonged to serotype KL64 in this study, which was different from previously reported KL1, KL2, and KL62 serotypes.[9] Clinically, the ST11-KL64 CRKP isolates carrying the *rmpA* and *rmpA2* virulence genes and producing KPC-2 were more survivable in the environment and could cause more severe infection.[34] It is reported that the inpatients infected with ST11-KL64 CRKP had a higher mortality. The results of this study revealed that one patient died among 16 patients with the infection of ST11-KL64 CRKP carrying *rmpA* and *rmpA2* genes, indicating a high mortality rate of ST11-KL64 CRKP. This revealed the highly virulent nature of these ST11-KL64 CRKP isolates, and targeted surveillance was urgently needed in this regard. It is necessary to further conduct genomic epidemiological and evolutionary analyses throughout the country to elucidate the genetic basis and evolutionary characteristics of the widely spread carbapenem-resistant and highly virulent ST11-KL64 *K. pneumoniae* in China.

The results of phylogenetic tree revealed that 18 ST11-KL64 CRKP strains with $bla_{\text{NDM-1}}$ and $bla_{\text{KPC-2}}$ co-occurrence could be divided into two evolutionary clades, indicating two independent transmission events. Some patients of the two evolutionary clades overlapped in terms of strain isolation time or inpatient departments, which may be the main reason for the spread of *K. pneumoniae* in this hospital. For example, KP43, KP53, KP57, KP60, KP62, and KP64 were isolated from 8 hospitalized ICU patients with similar sampling time, indicating that transmission events occurred in a short period of time. The spread of ST11-CRKP in different departments or different wards of the same department in the hospital has been frequently reported.[6,9,11,32,38] The results revealed two independent outbreaks of ST11-KL64 CRKP strains with $bla_{\text{NDM-1}}$ and $bla_{\text{KPC-2}}$ co-occurrence in the ICU and respiratory ward from 2019 to 2021. These results confirmed that ST11-CRKP strains were prone to transfer. Further, we should further analyze the drug-resistant gene transfer of coexisting strains, and analyze the genetic environment of $bla_{\text{NDM-1}}$ and $bla_{\text{KPC-2}}$ in combination with the long

read sequencing results. Therefore, practical approaches must be implemented to control transmission and reduce the occurrence of nosocomial infections.

Multiplex PCR revealed that among 60 CRKP isolates, 54 strains produced carbapenemase and contained bla_{KPC} gene. This was the most common mechanism of carbapenem resistance in *K. pneumoniae*. In China, it is reported that KPC-producing *K. pneumoniae* was the main strain causing outbreak.[39,40] Among the 54 CRKP strains with KPC-producing gene, 18 strains carried bla_{NDM} gene at the same time. In addition, the Illumina sequencing analysis revealed that apart from the many types of β-lactam-resistance genes, other resistance genes such as *tet*(A), *rmtB, sul2, dfrA14, fosA*, and *qnrS1* were present in all 18 ST11-KL64 CRKP isolates with bla_{NDM-1} and bla_{KPC-2} co-occurrence, conferring resistance to tetracyclines, aminoglycosides, sulfonamides, trimethoprim, fosfomycin, and quinolones, respectively. The co-existence of carbapenemase, β-lactamases, and many types of drug resistance genes led to the multidrug resistance. Undoubtedly, the existence of resistance genes enables *K. pneumoniae* isolates to survive the attack of antibacterial drugs. Thus, treatment of infections caused by these multi-resistant CRKP strains is a great challenge because of limited availability of antimicrobials. Fortunately, polymyxin was effective in vitro, suggesting that it might be a valuable therapeutic option for ST11-KL64 CRKP infections.

Conclusion

Our study confirmed that the CRKP strains isolated from the hospital mainly had ST11-KL64 phenotype and mostly carried bla_{KPC-2} resistance genes. The ICU was the main site of nosocomial infection and rapid transmission of CRKP. The strains exhibited high pathogenicity by acquiring various drug-resistance genes and virulence genes, causing a major challenge to public health. Therefore, it is urgent to develop effective strategies to control and prevent further nosocomial infection.

Data Sharing Statement

The datasets presented in this study can be found in online (https://doi.org/10.6084/m9.figshare.21360120).

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Author Contributions

All authors contributed to data analysis, drafting or revising the article, have agreed on the journal to which the article will be submitted, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare no conflicts of interest in this work.

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