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ORIGINAL RESEARCH

Carbapenem-Resistant *Klebsiella pneumoniae* in Southwest China: Molecular Characteristics and Risk Factors Caused by KPC and NDM Producers

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Background: Carbapenem-resistant *Klebsiella pneumoniae* (CRKP) infection has attracted worldwide concern and became a serious challenge for clinical treatment. The aims of this study were to evaluate the molecular characteristics and risk factors for CRKP infection.

Methods: All the CRKP strains were screened for antimicrobial resistance genes, virulence genes, and integron by polymerase chain reaction (PCR). Plasmid typing was performed by plasmid conjugation assay and PCR-based replicon typing (PBRT). The genetic environments of bla_{KPC-2} and bla_{NDM-1} were analyzed by using overlapping PCR and molecular typing was performed by multi-locus sequence typing (MLST). Risk factors for CRKP infection were analyzed by logistic regression model.

Results: All the 66 CRKP isolates were multidrug-resistant, but all of them were susceptible to tigecycline and polymyxin B. Among the CRKP isolates, 42 bla_{KPC-2}-positive strains were identified carrying IncFII plasmids. Meanwhile, 24 bla_{NDM}-positive strains were found on lncX3 plasmids, including 20 bla_{NDM-1} isolates and 4 bla_{NDM-5} isolates. Most of CRKP isolates contained several virulence genes and the class I integron (*intl1*). The genetic environments of bla_{KPC-2} and bla_{NDM-1} revealed that the conserved regions (*tnpA*-*tnpR*-IS *kpn8*-bla_{KPC-2}) and (bla_{NDM-1}-ble_{MBL}-*trpF*-*tat*) were associated with the dissemination of KPC-2 and NDM-1. ST11 was the most common type in this work. Hematological disease, tracheal cannula, and use of β-lactams and β-lactamase inhibitor combination were identified as independent risk factors for CRKP infection.

Conclusion: This study established the resistance pattern, molecular characteristics, clonal relatedness, and risk factors of CRKP infection. The findings of the novel strain that co-harboring bla_{NDM-5} and $bla_{IMP-4,}$ and the novel ST4495 indicated that the brand-new types have spread in Southwest China, emphasizing the prevent and control the further dissemination of CRKP isolates are highly needed.

Keywords: carbapenem-resistant *Klebsiella pneumoniae*, molecular characteristics, genetic environments, plasmid, risk factors

Introduction

Klebsiella pneumoniae, belonging to the family *Enterobacteriaceae*, is an important pathogen that causes opportunistic infections in hospitalized patients, and plays a primary role in pneumonia and neonatal sepsis.[1] With the increase of prevalence of multidrug-resistant (MDR) strains that producing extended-spectrum β-lactamase (ESBL) and/or carbapenemase, *K. pneumoniae* has emerged as a major threat in clinical and public health. Carbapenem-resistant *K. pneumoniae* (CRKP) has become

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one of the particularly important problems worldwide due to the gradually higher morbidity and subsequently worrying mortality.[2,3] According to the 2020 CHINET Resistance Monitoring Network, *K. pneumoniae* resistance to imipenem has risen rapidly from 16.1% in 2016 to 23.3% in 2020.

The resistance mechanism of *Enterobacteriaceae* to carbapenem antibiotics is usually caused by two major mechanisms: (i) Nonenzymatic resistance mechanisms and (ii) Hydrolysis of carbapenems by carbapenemases production.[4] Nonenzymatic resistance mechanisms mainly include overexpression of efflux pump-encoding genes and the mutation of porins.[5,6] As for later mechanism, carbapenemases are classified into three groups (ie, A, B, D) based on their molecular structure. Class A and D carbapenemases require an acyl enzyme that forms from an active serine site to hydrolyze their substrates, while class B metalloenzymes utilize the active zinc ion site to induce hydrolysis of carbapenem.[7] *Klebsiella pneumoniae* carbapenemase (KPC) is the most common class A enzyme in *Enterobacteriaceae* and have widely disseminated due to the encoding genes located on plasmid, especially for KPC-2.[8] In addition, New Delhi metallo-β-lactamase (NDM) is one of the important member in class B carbapenemases, and the NDM-1 is the most prevalent type globally. NDM-1-producing clinical strains have caused several outbreaks in China since it was first reported in 2012.[9–11] In other regions, such as Colombia, Italy, and Brazil, these strains also lead to health crisis.[12–14] Furthermore, a more pathogenic and virulent phenotype, also known as hypervirulent *K. pneumoniae* (HVKP), has been well-separated quickly. Importantly, the emergence of multidrug-resistant HVKP (MDR-HVKP) has been gradually reported due to the horizontal transfer of mobile genetic elements, bringing a remarkable challenge to healthcare system.[15–17]

The objectives of this study were to systematically analyze the clinical characteristics, molecular epidemiology, and risk factors for CRKP infection in a tertiary hospital. These findings will provide valuable information for further monitoring and controlling the dissemination of KPC and NDM *K. pneumoniae* strains in Southwest China.

Materials and Methods

Data Collection

The isolates were defined as CRKP if they were resistant to at least one of the carbapenem agents, including imipenem, ertapenem, and meropenem (www.cdc.gov/HAI/organisms/cre). A total of 66 non-repetitive clinical CRKP isolates were collected from September 2016 to August 2019 in the Affiliated Hospital of Southwest Medical University (Luzhou, China), and all the CRKP isolates were confirmed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS, Bruker Daltonics, Bremen, Germany).

In order to investigate the risk factors for CRKP infection, a stepwise matching method at a ratio of 1:1 has been used to identify appropriate control cases from patients who are infected with carbapenem-susceptible *K. pneumoniae* (CSKP). The same site of infection, the same gender, age ± 2 years, and the same year of hospital admission were considered as the matching criteria. The relative clinical data were retrospectively collected from medical records of each patient, including basic demographic information, underlying diseases and comorbidities, invasive procedures, antibiotic treatment, and clinical outcomes.

Antimicrobial Susceptibility Testing (AST)

The antimicrobial susceptibilities of all the 66 clinical CRKP isolates to 16 antimicrobials were tested by VITEK 2 Compact system (bioMérieux, Marcy l'Etoile, Lyon, France), including cefepime, cefotaxime, cefazolin, cefuroxime, ceftazidime, cefoxitin, piperacillin/tazobactam, ampicillin/sulbactam, amikacin, tobramycin, gentamicin, levofloxacin, ciprofloxacin, sulperazone, compound sulfamethoxazole, and aztreonam. The minimum inhibitory concentrations (MICs) of meropenem, imipenem, ertapenem, tigecycline, and polymyxin B were determined by broth microdilution method, and the results were interpreted according to the standards of the Clinical and Laboratory Standards Institute (CLSI) 2020-M100.[18] *Pseudomonas aeruginosa* ATCC27853 and *Escherichia coli* ATCC25922 were used as quality control strains (purchased from China National Health Inspection Center).

Screening of CRKP and Phenotypic Detection of Carbapenemase

The metallo-β-lactamase-producing isolates were differentiated from all the 66 CRKP by using the imipenem-EDTA double-disk synergy method and the carbapenem inactivation method (CIM). Briefly, the bacterial suspension (0.5 McFarland standard) was diluted from the overnight culture, and then smeared on Mueller-Hinton (MH) agar plate (Haibo, Qingdao, China). A disk containing 10 μg imipenem (OXOID, ThermoFisher Scientific, Massachusetts, USA) and a blank disk with 1.5mg/mL EDTA were placed 10 mm between each disk on the plate. After 18 h incubation, an enlarged zone of inhibition was considered as EDTA-synergy test positive.[18,19]

The CIM was performed as described previously.[18,20] A single colony of CRKP isolates was inoculated into a tube containing 2 mL trypticase soy broth (TSB) (Haibo, Qingdao, China) and a tube containing 2 mL TSB with 5mM EDTA, respectively. A 10 μg meropenem disk was placed in each tube. After 4 h incubation, these disks were took out and placed on a MH agar plate that was inoculated with a lawn of the meropenem-susceptible *Escherichia coli* ATCC25922 (0.5 McFarland standard). The results were interpreted according to CLSI.[18]

The Detection of Resistance Genes, Virulence Genes, and Integrase-Associated Genes

All of template DNAs were extracted by bacterial DNA Kit (Tiangen, Beijing,China) and used to detect carbapenemase genes, ESBL genes, AmpC β-lactamase genes, virulence genes, and integrase-associated genes by polymerase chain reaction (PCR). The primers were shown in Tables S1 and S2. The PCR conditions were described previously.[21–23] Positive products were sequenced by Shanghai Jieli Biotechnology, and the variable region of the class I integron (*intl1*) and the insertion sequence common region I (ISCR I) were analyzed by BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Plasmid Conjugation and Analysis

In order to evaluate whether the carbapenemase genes are located on the plasmid and whether these genes can be horizontally transferred, a plasmid conjugation transfer experiment was performed. Four hundred μL donor strain (CRKP strains) and 200 μL receptor strain (sodium azide-resistant *E. coli* strain J53) at logarithmic phase were added into a glass tube containing 800 μL Luria-Bertani (LB) broth and cultured at 37°C for 18 h. Transconjugants were selected by using LB plates that contains sodium azide (180 μg/mL) and imipenem (0.5 μg/mL).[21] The transconjugants were verified by MALDI-TOF MS, and the conjugative carbapenemase genes were confirmed by PCR. In addition, the successful conjugative plasmids were analyzed by PCR-based replicon typing according to previous study.[22]

Genetic Environments of KPC-2-Carrying Plasmids and NDM-1-Carrying Plasmids

The overlapping PCR was applied to investigate the genetic environments of the $bla_{\text{KPC-2}}$ and $bla_{\text{NDM-1}}$ (Tables S3 and S4).[23,24] The PCR products were sequenced by Shanghai Jieli Biotechnology, and then analyzed by NCBI GenBank database.[25]

Molecular Epidemiological Study

Multi-locus sequence typing (MLST) was performed to explore the genetic correlation of all the clinical CRKP isolates.[26] The positive products were sequenced by Shanghai Jieli Biotechnology, and the results were submitted to *K. pneumoniae* MLST database (https://bigsdb.pasteur.fr/cgi-bin/bigsdb/bigsdb.pl?db=pubmlst_klebsiella_seqdef) for comparison.

Statistical Analysis

All analyses and graphs were performed using SPSS v.24.0 software (SPSS Inc., Chicago, USA). The chi-square test or Fisher's exact test was used to analyze categorical variables. Continuous variables were presented as means ± standard deviation (SD), and were evaluated by Student's *t*-tests or Mann–Whitney *U*-test. Multivariable logistic regression analysis was operated to identify independent risk factors for CRKP infection. All biologically plausible variables with a value of $P < 0.1$ within univariate analysis were included in the following multiple logistic regression model. $P < 0.05$ was considered as statistically significant, and all probability values were two-tailed distribution.

Results

Distribution of Clinical CRKP Isolates

A total of 66 non-repetitive clinical CRKP isolates were collected from September 2016 to August 2019 in Southwest China. The clinical CRKP isolates were obtained from patients admitted to the 12 different departments, including the majority of neonatology department (27.3%, n = 18) and rehabilitation department (24.3%, n = 16) (Figure 1A). These isolates were mainly originated from sputum (34.8%, n = 23), followed by urine (25.8%, n = 17), blood (21.2%, n=14), secretion (6.1%, n= 4), pleuroperitoneal fluids (6.1%, n = 4), catheter tip (3.0%, n = 2), and pus (3.0%, n = 2) (Figure 1B).

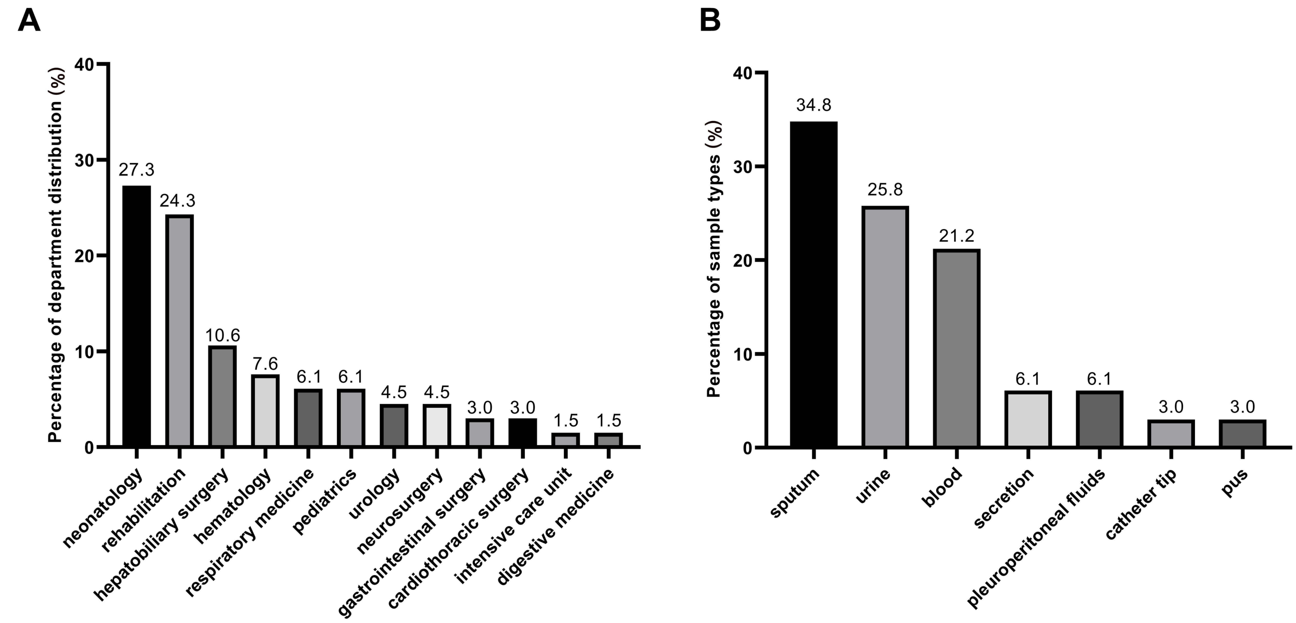

Figure 1 Distribution of 66 clinical CRKP strains. (**A**) Department distribution of 66 CRKP strains; (**B**) sample types of 66 CRKP strains.

Antimicrobial Susceptibility Profiles

All of 66 clinical CRKP isolates were multidrug-resistant and resistance rates to 12 antimicrobials reached 100%, which were cefepime, imipenem, piperacillin/tazobactam, ceftriaxone, ampicillin/sulbactam, cefotaxime, cefazolin, cefuroxime, ertapenem, ceftazidime, meropenem, and cefoxitin. In addition, the resistance rates to sulperazon, aztreonam, tobramycin, gentamicin, levofloxacin, ciprofloxacin, cotrimoxazole, and amikacin were 95.5%, 87.9%, 83.3%, 83.3%, 81.9%, 80.3%, 65.2%, and 54.5%, respectively. Remarkably, all the CRKP strains in this study were sensitive to tigecycline and polymyxin B (Table 1).

Carbapenem Resistance Phenotype and Molecular Characteristics

Both EDTA-synergy test and CIM test were shown that 24 (36.4%) clinical CRKP strains produced class B carbapenemases. The sequencing results confirmed that all of which isolates contained *bla*$_{NDM}$, in which 20 isolates carried *bla*$_{NDM-1}$ and 4 isolates carried *bla*$_{NDM-5}$, and 3 isolates possessed *bla*$_{NDM}$ and *bla*$_{IMP}$ simultaneously. The rest of 42 (63.6%) isolates contained *bla*$_{KPC-2}$. Notably, in addition to the production of carbapenemase, 100% and 95.5% of the clinical CRKP isolates were positive for ESBL and AmpC genes, respectively. Furthermore, virulence genes were detected in all isolates, 54 (81.8%) isolates detected three different virulence genes. Especially for strain Kpn497 and Kpn131, they carried 12 virulence genes and 11 virulence genes, respectively (Table 2).

Plasmid and Integron-Associated Analysis

KPC-encoding plasmids were positive in 35 transconjugants and all of these plasmids belonged to IncFII. Meanwhile, NDM-encoding plasmids that belong to IncX3 were detected in 24 transconjugants (Table 2).

Nearly 85% (n = 56) strains were *intI1*-positive, of which 46 strains contained variable region. A total of 6 cassette arrays were found among them. The cassette arrays were revealed in this study included: *addA2* (82.6%), *ddfrA12* (6.5%), *dfrA12-addA2* (4.3%), *dfrA27-arr3* (2.2%), *gcuF-dfrA12* (2.2%), and *bla*$_{OXA-10}$-*addA1-aacA4* (2.2%) (Table 2) In addition, ISCRI was detected in 25 strains.

Genetic Environments of *bla*$_{KPC-2}$ and *bla*$_{NDM-1}$

All of 42 KPC-2-producing strains could be divided into three different types (A, B, and C) based on the genetic structures that compared with pKP048 (GenBank Accession No.FJ628167). Type C was the most prevalent (n = 31), followed by type A (n = 8) and type B (n = 3). Type A exhibited the same structure as pKP048. The downstream of IS*Kpn6-like* was deletion in type B, and three genetic elements downstream of *bla*$_{KPC-2}$ were

Table 1 Antimicrobial Susceptibility Profiles of 66 Clinical CKRP Strains

Antimicrobial Agent	No.	%R	No.	%I	No.	%S
Amikacin	36	54.5	0	0	30	45.5
Cefepime	66	100	0	0	0	0
Imipenem	66	100	0	0	0	0
Piperacillin/tazobactam	66	100	0	0	0	0
Ceftriaxone	66	100	0	0	0	0
Ampicillin/sulbactam	66	100	0	0	0	0
Cefotaxime	66	100	0	0	0	0
Cefazolin	66	100	0	0	0	0
Cefuroxime	66	100	0	0	0	0
Ertapenem	66	100	0	0	0	0
Tobramycin	55	83.3	4	6.1	7	10.6
Sulperazon	63	95.5	2	3.0	1	1.5
Ceftazidime	66	100	0	0	0	0
Meropenem	66	100	0	0	0	0
Levofloxacin	54	81.9	0	0	12	18.1
Cefoxitin	66	100	0	0	0	0
Aztreonam	58	87.9	0	0	8	12.1
Ciprofloxacin	53	80.3	0	0	13	19.7
Gentamicin	55	83.3	0	0	11	16.7
Tigecycline	0	0	0	0	66	100
Compound sulfamethoxazole	43	65.2	0	0	23	34.8
Polymyxin B	0	0	0	0	66	100

Abbreviations: S, susceptible; I, intermediate; R, resistant.

deletion in type C, including IS*Kpn6-like, tnpR*, and *tnpA* (Figure 2A).

Furthermore, the genetic structure of pNDM-BJ01 (GenBank accession No. JQ001791) was used to compare with each NDM-1-producing strain, revealing A, B, and C types among these strains. As for type A (n = 12), the IS*Aba125* upstream of the bla_{NDM-1} was truncated, all downstream genes of *tat were* complete deletion. IS*Aba125* upstream of the bla_{NDM-1} was deletion in type B compared with type A. Type C presented as the IS*30* upstream of bla_{NDM-1} and followed by ble_{MBL}, *trpF, tat, cutA, groES, groEL* (Figure 2B).

MLST of Clinical CRKP Isolates

A total of 13 MLSTs were identified among all the clinical CRKP isolates, and ST11 (56.2%, n = 37) was the most frequent ST type, followed by ST4495 (19.7%, n = 13), ST2407 (4.6%, n = 3), ST147 (3.0%, n = 2), ST307 (3.0%, n = 2), ST37 (3.0%, n = 2), ST15 (1.5%, n = 1), ST17 (1.5%, n = 1), ST152 (1.5%, n = 1), ST23 (1.5%, n = 1), ST405 (1.5%, n = 1), ST318 (1.5%, n = 1), and ST467 (1.5%, n = 1).

Risk Factors and Multivariate Analysis of CRKP Infection

Statistically significant differences were observed for respiratory disease (P = 0.034), renal disease (P = 0.039), hematological disease (P = 0.02), tracheal cannula (P < 0.001), and use of β-lactams/β-lactamase inhibitor combination (P = 0.009) between CRKP and CSKP groups (Table 3). Multivariate analysis revealed that hematological disease (odds ratio [OR], 2.568; 95% confidence interval [95% CI], 1.106 to 5.964; P = 0.028), tracheal cannula (OR, 4.883; 95% CI, 1.797 to 13.265; P = 0.002), and use of β-lactams/β-lactamase inhibitor combination (OR, 4.271; 95% CI, 1.760 to 10.365; P = 0.001) were independent risk factors for CRKP infection.

Discussion

The clinical CRKP strains were first reported in 1997,[27,28] and this type of resistance did not become common within that decade. However, with the increasing use of carbapenems in recent years, CRKP strains have been distributed around the world at a boosting rate, and it was listed as one of the critical-priority bacteria by World Health

Table 2 Genotypes of 66 Clinical CRKP Strains

N	Carbapenemase	ESBLs	AmpC	Int	Virulence Gene	Gene Cassette	ISCR	MLST	Replicon Type
16	KPC-2	SHV+TEM+CTX-M	ACC	*IntI*	*entB+fimH+mrkD*	*addA2*	ISCR 1	ST11	IncFII
26	NDM-1	SHV+CTX-M	DHA		*entB+mrkD*		ISCR 1	ST4495	IncX3
27	NDM-1	SHV+CTX-M	DHA		*entB+fimH+mrkD*		ISCR 1	ST4495	IncX3
30	KPC-2	SHV+TEM+CTX-M	ACC	*IntI*	*entB+fimH+mrkD*	*addA2*	ISCR 1	ST11	IncFII
32	NDM-1	SHV+CTX-M	DHA		*entB+fimH+mrkD*		ISCR 1	ST4495	IncX3
34	NDM-1	SHV+CTX-M	DHA		*entB+fimH+mrkD*		ISCR 1	ST4495	IncX3
36	NDM-1	SHV+CTX-M	DHA		*entB+fimH+mrkD*		ISCR 1	ST4495	IncX3
37	NDM-1	SHV+CTX-M	DHA		*entB+fimH+mrkD*		ISCR 1	ST4495	IncX3
39	NDM-1	SHV	DHA		*entB+mrkD*		ISCR 1	ST4495	IncX3
40	NDM-1	SHV	DHA+ACC		*entB+fimH*		ISCR 1	ST37	IncX3
45	KPC-2	SHV+TEM+CTX-M	ACC	*IntI*	*entB+fimH+mrkD*	*addA2*		ST11	
46	NDM-1	SHV+CTX-M	DHA+ACC		*entB+fimH+mrkD*		ISCR 1	ST4495	IncX3
49	NDM-1	SHV+CTX-M	DHA+ACC		*entB+fimH+mrkD*		ISCR 1	ST4495	IncX3
51	KPC-2	SHV+CTX-M	ACC		*entB+fimH+mrkD*			ST11	
56	NDM-1	SHV+CTX-M	DHA+ACC		*entB+fimH+mrkD*		ISCR 1	ST4495	IncX3
57	NDM-1	SHV+CTX-M	DHA+ACC		*entB+fimH+mrkD*		ISCR 1	ST4495	IncX3
58	NDM-1+IMP-4	SHV+CTX-M	DHA+ACC		*entB+fimH*			ST4495	IncX3
101	NDM-1	SHV	ACC		*entB+fimH+mrkD*			ST37	IncX3
131	KPC-2	SHV+TEM	ACC	*IntI*	*entB+fimH+mrkD+rmpA/rmpA2+iucA +terB+aerobactin+HI1B+iroN+iutA*	*addA2*	ISCR 1	ST11	IncFII
210	KPC-2	SHV+TEM+CTX-M	ACC	*IntI*	*entB+fimH+mrkD*	*addA2*		ST11	IncFII
211	KPC-2	SHV+TEM+CTX-M	ACC	*IntI*	*entB+fimH+mrkD*	*addA2*		ST11	IncFII
214	KPC-2	SHV+TEM+CTX-M	ACC	*IntI*	*entB+fimH+mrkD*	*addA2*		ST11	IncFII
215	KPC-2	SHV+TEM+CTX-M	ACC	*IntI*	*entB+fimH+mrkD*	*addA2*		ST11	IncFII
221	KPC-2	SHV+TEM+CTX-M	ACC	*IntI*	*entB+fimH+mrkD*	*addA2*	ISCR 1	ST11	IncFII
223	KPC-2	SHV+TEM+CTX-M	ACC		*entB+fimH+mrkD*			ST147	
227	KPC-2	SHV+CTX-M	ACC	*IntI*	*entB+fimH+mrkD*	*addA2*		ST11	IncFII
228	KPC-2	SHV+CTX-M	ACC		*entB+fimH+mrkD*			ST11	IncFII
230	KPC-2	SHV+TEM+CTX-M	ACC	*IntI*	*entB+fimH+mrkD*	*addA2*		ST11	IncFII
232	KPC-2	SHV+TEM+CTX-M	ACC	*IntI*	*entB+fimH+mrkD*	*addA2*		ST11	IncFII
233	KPC-2	SHV	ACC	*IntI*	*entB+fimH+mrkD*	*dfrA12, addA2*		ST147	IncFII
234	KPC-2	SHV+TEM+CTX-M	ACC	*IntI*	*entB+fimH+mrkD*	*addA2*		ST11	InFIB
241	KPC-2	SHV+TEM+CTX-M	ACC	*IntI*	*entB+fimH+mrkD*	*addA2*		ST11	IncFII
244	KPC-2	SHV	ACC	*IntI*	*entB+fimH+mrkD*	*dfrA12*		ST23	IncFII

(*Continued*)

Table 2 (Continued).

N	Carbapenemase	ESBLs	AmpC	Int	Virulence Gene	Gene Cassette	ISCR	MLST	Replicon Type
245	KPC-2	SHV+TEM+CTX-M	ACC	*IntI*	*entB+fimH+mrkD*	*addA2*		ST11	IncFII
251	KPC-2	SHV+TEM+CTX-M	ACC	*IntI*	*entB+fimH+mrkD*	*addA2*		ST11	IncFII
252	KPC-2	SHV+TEM+CTX-M		*IntI*	*entB+fimH+mrkD*	*addA2*		ST11	IncFII
257	KPC-2	SHV+TEM+CTX-M	ACC	*IntI*	*entB+fimH+mrkD*	*addA2*		ST11	
258	KPC-2	SHV+TEM+CTX-M	ACC	*IntI*	*entB+fimH+mrkD*	*addA2*		ST11	IncFII
267	NDM-1	SHV	ACC	*IntI*	*entB+fimH+mrkD*	*addA2*	ISCR 1	ST11	IncX3
271	KPC-2	SHV+TEM+CTX-M	ACC		*entB+fimH+mrkD*			ST11	IncFII
285	KPC-2	SHV+TEM+CTX-M	ACC	*IntI*	*entB+fimH+mrkD*	*addA2*		ST11	IncFII
354	KPC-2	SHV+TEM+CTX-M	ACC	*IntI*	*entB+fimH+mrkD*	*addA2*		ST11	IncFII
406	KPC-2	SHV+TEM	DHA	*IntI*	*fimH+mrkD*	*OXA-10, addA1, aacA4*	ISCR 1	ST318	IncFII
436	KPC-2	SHV+TEM	DHA	*IntI*	*entB+fimH+mrkD*	*addA2*		ST17	IncFII
440	NDM-1	SHV+TEM	ACC	*IntI*	*mrkD*	*addA2*		ST307	IncX3
445	NDM-1	SHV+TEM	ACC		*mrkD*			ST307	IncX3
473	NDM-1+IMP-4	SHV+TEM	ACC	*IntI*	*entB+fimH+mrkD*	*addA2*	ISCR 1	ST152	IncX3
475	NDM-1	SHV+TEM	ACC	*IntI*	*fimH+mrkD*	*addA2*	ISCR 1	ST15	IncX3
478	KPC-2	SHV+TEM+CTX-M	ACC	*IntI*	*entB+fimH+mrkD*	*addA2*	ISCR 1	ST11	IncFII
479	KPC-2	SHV+TEM+CTX-M	ACC	*IntI*	*entB+fimH*	*addA2*		ST11	IncFII
480	KPC-2	SHV+TEM+CTX-M	ACC	*IntI*	*entB+fimH+mrkD*	*addA2*		ST11	IncFII
483	KPC-2	SHV+TEM+CTX-M	ACC	*IntI*	*entB+fimH+mrkD*	*addA2*		ST11	IncFII
489	KPC-2	SHV+TEM+CTX-M	ACC	*IntI*	*entB+fimH+mrkD*	*dfrA12, addA2*		ST11	
490	KPC-2	SHV	ACC	*IntI*	*entB+fimH+mrkD*	*dfrA27, arr3*		ST467	IncFII
494	NDM-5	SHV+CTX-M	DHA	*IntI*	*entB+fimH+mrkD*	*addA2*	ISCR 1	ST2407	IncX3
495	KPC-2	SHV+TEM+CTX-M		*IntI*	*entB+fimH+mrkD*	*dfrA12*	ISCR 1	ST11	IncFII
496	KPC-2	SHV+TEM	ACC	*IntI*	*entB+fimH+mrkD*	*gcuF, dfrA12*		ST405	
497	KPC-2	SHV+TEM		*IntI*	*entB+fimH+mrkD+rmpA/rmpA2+iucA + terB+wcaG+ aerobactin+HI1B+iroN +iutA*	*addA2*		ST11	IncFII
498	KPC-2	SHV+CTX-M	ACC	*IntI*	*entB+mrkD*	*dfrA12*		ST11	IncFII
499	NDM-5	SHV+CTX-M	DHA	*IntI*	*entB+fimH+mrkD*	*addA2*	ISCR 1	ST2407	IncX3
500	KPC-2	SHV+TEM+CTX-M	ACC		*entB+fimH+mrkD*			ST11	
502	NDM-5	SHV+CTX-M	DHA	*IntI*	*entB+fimH+mrkD*	*addA2*		ST2407	IncX3
504	NDM-5+IMP-4	SHV+TEM+CTX-M	DHA	*IntI*	*entB+fimH+mrkD*	*addA2*		ST11	IncX3

(*Continued*)

Table 2 (Continued).

N	Carbapenemase	ESBLs	AmpC	Int	Virulence Gene	Gene Cassette	ISCR	MLST	Replicon Type
505	KPC-2	SHV+TEM+CTX-M	ACC	*IntI*	*entB+fimH+mrkD*	*addA2*		ST11	IncFII
506	NDM-1	SHV+CTX-M	DHA	*IntI*	*entB+fimH+mrkD*	*addA2*		ST4495	IncX3
507	KPC-2	SHV+CTX-M	ACC	*IntI*	*entB+fimH+mrkD*	*addA2*		ST11	IncFII

Abbreviations: ESBL, extended-spectrum β-lactamase; Int, integron; ISCR, insertion sequence common region.

Organization (WHO).[29] In this work, the antimicrobial susceptibility profiles, molecular characteristics, plasmid and integron-associated analysis, genetic environments of bla_{KPC-2} and bla_{NDM-1}, and MLST were evaluated among 66 clinical CRKP isolates. Meanwhile, the risk factors for CRKP infection were also investigated. All the CRKP strains were MDR or even extensively drug-resistant (XDR), in this case, the tigecycline and polymyxin B were the last resort options for CRKP infection. However, the treatment was hardly successful because of the low blood concentrations when tigecycline was used in monotherapy.[30] Adverse events, such as hypofibrinogenemia, also resulted in treatment termination associated with tigecycline therapy.[31] In addition, nephrotoxicity and unclear dosing also limited the widespread clinical usage of polymyxin B.[32,33] Importantly, tigecycline- and polymyxin B-resistant CRKP strains have been reported in many regions,[34,35] therefore, the optimal therapeutic strategy for CRKP infection still needs to be explored.

Focusing on the phenotype and molecular analysis of CRKP isolates, the results revealed that the primary resistance mechanism of CRKP was carbapenemase production, which was consistent with other researches.[36,37] The bla_{KPC-2} and bla_{NDM-1} were two major carbapenemase genes in our study, accounting for 63.6% and 30.3%, respectively. KPC-2 is the most commonly identified enzyme among over 20 reported KPC variants so far,[38] and the majority of NDM-1-producers also harbored various other resistance genes but mostly were still susceptible to polymyxin B and tigecycline,[39] which were highly correlated with our results. Moreover, IMP is another important metallo-β-lactamase, and bla_{IMP-1} emerged in Japan in the late 1980s.[40] The bla_{IMP-4} was one of the most prevalent bla_{IMP} variants since it was first identified in *Acinetobacter spp*. in the mid-1990s,[41] particularly in China and Australia.[42,43] It is worth noting that bla_{IMP} often co-exists with other resistance genes,[44] which is consistent with our results. In our work, 3 strains were identified co-carrying bla_{NDM} and bla_{IMP} genes, including two isolates with bla_{NDM-1} and bla_{IMP-4} and one with bla_{NDM-5} and bla_{IMP-4}. Coexistence of bla_{NDM-1} and bla_{IMP-4} was still rarely distributed in China since first reported in 2012, but the strain co-harboring bla_{NDM-5} and bla_{IMP-4} has never been published before.[45]

In this work, bla_{KPC-2} was only found on IncFII plasmids, and bla_{NDM} was only detected on IncX3 plasmids. The IncFII plasmid family is commonly low copy number, not only carries multiple antimicrobial resistance and virulence genes, but also can replicate and disseminate in many different species of Enterobacteriaceae.[46] Furthermore, several studies have revealed that IncX3 plasmids were the predominant plasmid type that harboring a variety of bla_{NDM} genes.[47–49] Of note, IncX3 plasmids can frequently disseminate in humans, animals, and environment, and more commonly spread in Asia, including China,[49,50] Myanmar,[51] South Korea,[52] and India.[53] In addition, 46 CRKP strains carried variable regions of *intI1*, and 7 resistance gene cassettes were detected among them. The *addA2* (40/46) and *addA1* (1/46) were the genes responsible for aminoglycoside resistance. The *dfrA12* (6/46), *dfrA27* (1/46) contributed to trimethoprim resistance. Furthermore, a β-lactams-resistant gene bla_{OXA-10}, a gentamicin-resistant gene *arr3*, and a rifampicin-resistant gene *aacA4* were also detected.

A previous study has proved that the genetic environment of bla_{KPC-2} in pKP048 from China was considered as a combination of the Tn*3*-based transposon and the partial Tn*4401* structure.[54] In our study, KPC-2 producers presented a similar structure (*tnpA-tnpR-ISkpn8*-bla_{KPC-2}), but the environment surrounding bla_{KPC-2} was partly different from other reports in China due to the specific insertions and deletions.[55,56] Additionally, all the NDM-1 producers carried the highly conserved region (bla_{NDM-1}-ble_{MBL}-*trpF-tat*) surrounding the bla_{NDM-1} gene, which was

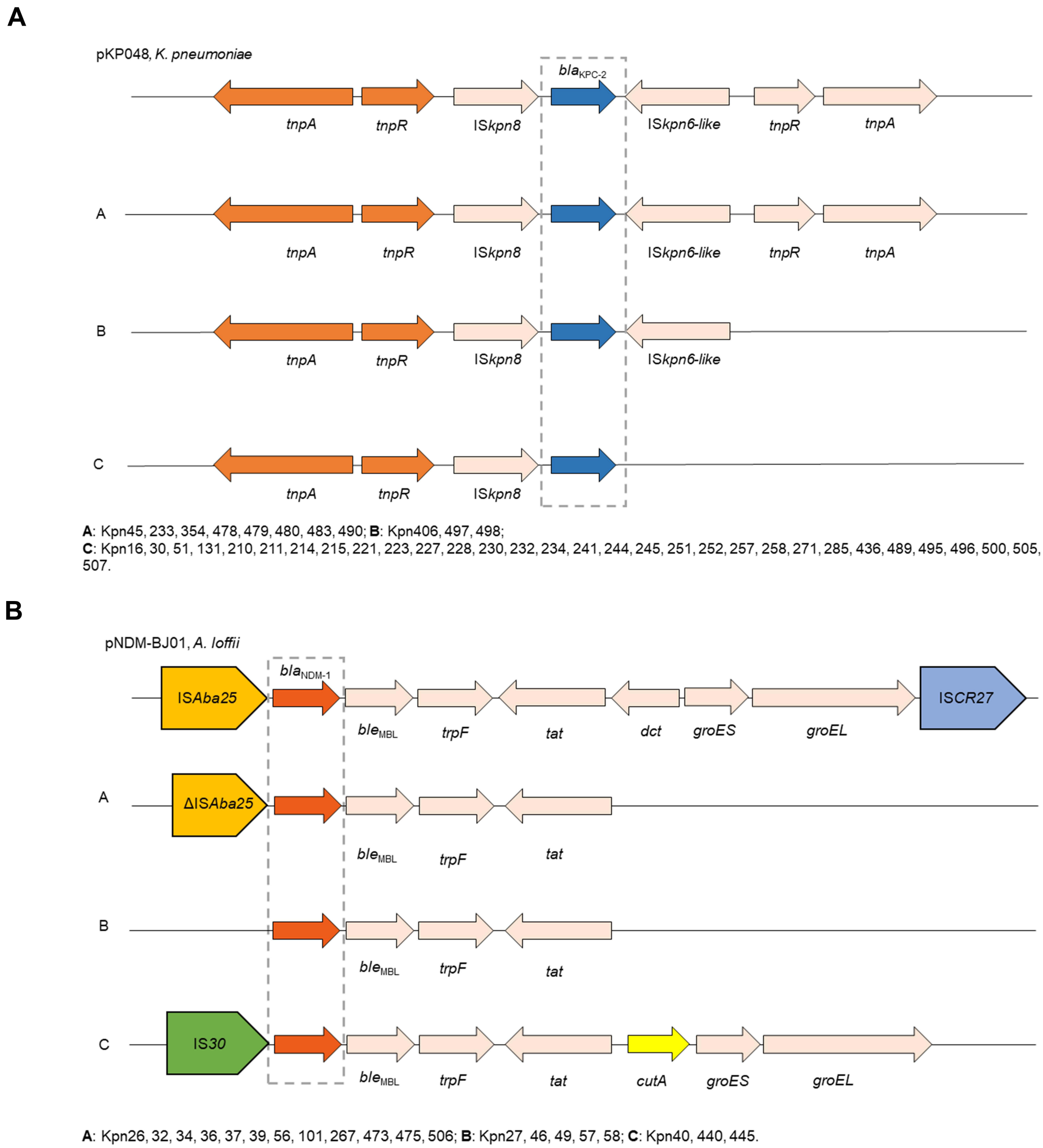

Figure 2 Comparison of the genetic elements surrounding the bla_{KPC-2} and bla_{NDM-1} genes identified in this study. (**A**) Comparison of the genetic environments of bla_{KPC-2}, reference sequences: *K. pneumoniae* (pKP048, GenBank Accession No.FJ628167); (**B**) comparison of the genetic environments of bla_{NDM-1}, reference sequences: *A. lwoffii* (pNDM-BJ01, GenBank accession NO. JQ001791).

identified not only in *K. pneumoniae*, but also in *Acinetobacter lwoffii* and *Enterobacter Cloacae*.[25,57,58]

Furthermore, the results showed that more than 80% clinical CRKP strains simultaneously carried virulence genes *entB, fimH*, and *mrkD*. The enterobactin that encoded by *entB* is one of the ubiquitous siderophores in *K. pneumoniae*, which is an important factor involving in iron acquisition.[59] The *fimH* and *mrkD* mediate bacterial adhesion through encoding type I and type III fimbriae of *K. pneumoniae* implied that these two genes may contribute to biofilm formation and

Table 3 Clinical Characteristics of CRKP and CSKP Strains

Variable	CRKP (n=66)	CSKP (n=66)	*P*-value
Demographic, n (%) or IQR			
Age (years)	44.5 (0,60)	46.5 (0.61)	0.754
Sex (male)	47 (71.2%)	46 (69.7%)	0.894
Length of hospital stays	15 (7, 27)	25 (10, 38)	0.1
Admission to ICU	14 (21.1%)	7 (10.6%)	0.096
Co-morbidity, n (%)			
Malignant disease	7 (10.6%)	15 (22.7%)	0.062
Diabetes mellitus	11 (16.7%)	12 (18.2%)	0.819
Hypertension	16 (24.2%)	16 (24.2%)	1
Heart disease	9 (13.6%)	11 (16.7%)	0.627
Hepatobiliary disease	12 (18.2%)	15 (22.7%)	0.517
Respiratory disease	45 (68.2%)	33 (50%)	**0.034**
Renal disease	7 (10.6%)	16 (24.2%)	**0.039**
Urinary tract infection	10 (15.2%)	9 (13.6%)	0.804
Craniocerebral disease	15 (22.7%)	9 (13.6%)	0.176
Hematological disease	32 (48.5%)	19 (28.8%)	**0.02**
Gastrointestinal disease	8 (12.1%)	5 (7.6%)	0.381
Invasive procedures and devices			
Tracheal cannula	24 (36.4%)	7 (10.6%)	**<0.001**
Central venous catheter	5 (7.6%)	12 (18.2%)	0.069
Foreign material in the body	14 (21.2%)	7 (10.6%)	0.096
Surgical operations after admission	9 (13.6%)	16 (24.2%)	0.12
Colostomy	3 (4.5%)	1 (1.5%)	0.612
Gastrostomy	2 (3%)	0	0.476
Antibiotic treatment, n (%)			
Penicillins	2 (3%)	5 (7.6%)	0.437
First, second-generation cephalosporins	7 (10.6%)	15 (22.7%)	0.21
Third, fourth-generation cephalosporins	17 (25.8%)	19 (28.8%)	0.696
Aminoglycosides	4 (6.1%)	1 (1.5%)	0.362
Quinolones	20 (30.3%)	21 (31.8%)	0.851
Metronidazole	0	1 (1.5%)	1
Carbapenems	30 (45.5%)	32 (48.5%)	0.727
β-lactams and β-lactamase inhibitor combination	40 (60.6%)	25 (37.9%)	**0.009**
Clinical outcomes, n (%)			
Patient outcome: mortality	5 (7.6%)	0	0.068

Note: Bold indicates $P < 0.05$.
Abbreviations: IQR, interquartile range; CR, carbapenem-resistant; CS, carbapenem-susceptible; ICU, intensive care unit.

development. Zhang et al have also observed that 95% tested *K. pneumoniae* isolates co-carrying *fimH* and *mrkD* genes in their study.[60] Additionally, the genes *peg-344, iroB, iucA, rmpA*, and *rmpA2* locate on the HVKP virulence plasmid and are regarded as regulators of hypervirulent phenotype.[61] Notably, two carbapenem-resistant HVKP (CR-HVKP) strains were found in this work, which contained $bla_{KPC\text{-}2}$ gene. There are two hypotheses to explain the evolution of this novel phenotype. First, CRKP strains acquire HVKP-specific virulence plasmid; second, HVKP strains obtain the carbapenem-resistant genes by acquisition of resistance plasmid or by the insertion of resistance determinants into HVKP-specific virulence plasmid.[59,62] Compared with the second hypothesis, the first one was more likely to occur.[63–65]

According to the results of MLST, the 42 KPC-2-producing CRKP strains were grouped into 7 ST types, of which 35 strains belonged to ST11. It has been reported that most of KPC-2-producing strains belong to clone group (CG) 258; meanwhile, ST11 and ST258 are the dominant ST types.[66] ST258 has spread around the world since it emerged in the early 21st century, particularly in North America, Latin America, and several European countries.[66,67] However, ST11 is more prevalent in Asia, and usually accounts for more than half of all KPC-2-producing CRKP strains in China.[47,68,69] In addition, the 24 NDM-producing CRKP strains were classified into 8 ST types, and ST4495 was the most common ST type. Remarkably, ST4495 (4-1-99-1-9-5-5) has never been detected before, indicating a novel clone carrying $bla_{\text{NDM-1}}$ has spread in Southwest China.

Hematological disease, tracheal cannula, and exposure to β-lactams and β-lactamase inhibitor combination were independent risk factors for CRKP infection. Many studies have demonstrated that the history of tracheal cannula was independent risk factor for CRKP infection.[70–73] Meanwhile, exposure to antibiotic was also associated with CRKP infection, such as exposure to quinolones,[74] β-lactams and β-lactamase inhibitor combination, and carbapenems,[75] which was consistent with our study. Notably, hematological disease was rarely found to be a risk factor for CRKP infection in other studies, but it is reasonable due to patients with hematological disease were accompanied with severe immune function deficiency and were more prone to be infected. There are some limitations in our study, first, the number of patients included in this study is relatively small, although this is a common problem in studies assessing risk factors for multidrug-resistant microbial infections.[74] Secondly, the study was performed in a single-center setting, so that some important risk factors may be missed.

Conclusion

The current study revealed the high prevalence of CRKP infection caused by NDM-1 and KPC-2 producers, and ST11 was the most common lineage. To the best of our knowledge, the novel ST4495 and co-exist of $bla_{\text{NDM-5}}$ and $bla_{\text{IMP-4}}$ were the first reported in this work. Moreover, plasmid, integron, ISCR, and other mobile genetic elements endowed bacteria a rapid adaptation ability in changing environments; meanwhile, the genetic environments of $bla_{\text{KPC-2}}$ and $bla_{\text{NDM-1}}$ showed polymorphism. Logistic regression model indicated that hematological disease, tracheal cannula, and use of β-lactams and β-lactamase inhibitor combination were independent risk factors for CRKP infection, suggesting that appropriate clinical management and antimicrobials treatment were necessary for retarding the selection of CRKP strains. Effective measures to prevent and control the further dissemination of CRKP strains are highly needed.

Data Sharing Statement

The data used and/or analyzed in this study are available from the corresponding author on reasonable request.

Ethics Approval and Consent to Participate

This study was approved by the Institutional Review Board of affiliated hospital of southwest medical university (KY2020043). As for neonate, a parent or legal guardian provided informed consent for these patients, and that this study was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all participants.

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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.

Disclosure

The authors declare that they have no conflict of interest.

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