

Epidemiological and Antimicrobial Resistant Patterns, and Molecular Mechanisms of Carbapenem-Resistant *Klebsiella pneumoniae* Infections in ICU Patients

Fanbo Lu^{1,*}, Luwen Zhang^{1,*}, Juanjuan Ji², Yuanhong Xu¹, Bo Wang¹, Jinxing Xia¹

¹Department of Clinical Laboratory, First Affiliated Hospital of Anhui Medical University, Hefei, People's Republic of China; ²Department of Respiratory and Critical Care Medicine, First Affiliated Hospital of Anhui Medical University, Hefei, People's Republic of China

*These authors contributed equally to this work

Correspondence: Jinxing Xia; Bo Wang, Department of Clinical Laboratory, First Affiliated Hospital of Anhui Medical University, No. 218 Jixi Road, Hefei, Anhui, People's Republic of China, Email xiajinxing@ahmu.edu.cn; wangbo8711@126.com

Objective: To study the epidemiological and antimicrobial resistant patterns, clinical characteristics and risk factors of critically ill patients infected with carbapenem-resistant *Klebsiella pneumoniae* (CRKP) from intensive care units (ICUs). The potential molecular mechanisms of antimicrobial resistance and virulence of CRKP were investigated through evaluation of associated genes.

Methods: Totally, 201 ICU patients infected with *K. pneumoniae* were recruited from January 2020 through January 2021. *K. pneumoniae* strains were collected from diverse clinical specimens and identified by microbial cultures and matrix-assisted laser desorption ionization–time-of-flight mass spectrometry. Antimicrobial resistance was measured through broth micro-dilution or Kirby–Bauer assays. The carbapenemase-, virulence-, and capsular serotype-associated genes of CRKP were individually detected by PCR and sequencing. Demographic and clinical profiles were acquired from hospital databases to evaluate the correlation of CRKP infection incidence with clinical risk factors.

Results: Of the 201 *K. pneumoniae* strains, CRKP accounted for 41.29%. Seasonal bias existed in local prevalence of CRKP infections. CRKP strains mounted significantly strong resistance against major antimicrobial agents except ceftazidime–avibactam, tigecycline and minocycline. Recent exposure to certain antibiotics and prior treatment with invasive interventions were prone to increase CRKP infection risks with worsened infectious outcomes. The local top carbapenemase-encoding and virulence-associated genes of CRKP were *bla_{KPC}* and *irp2*, respectively. Nearly half of CRKP isolates harbored a capsular polysaccharide serotype of K14. K64 (*wzi-64*) which preferentially emerged in the cohort with worse outcomes of infection.

Conclusion: Featured epidemiology and typical clinical characteristics existed extensively in *K. pneumoniae* infections among ICU patients. The CRKP cohort exhibited substantially high antimicrobial resistance. Distinctive carbapenemase-, virulence-, and serotype-associated genes were intensively involved in the spread and pathogenesis of CRKP. These findings supported careful management of critically ill patients potentially infected with virulent CRKP in the ICUs.

Keywords: carbapenem-resistant *Klebsiella pneumoniae*, epidemiology, clinical features, antimicrobial resistance, virulence, ICU

Introduction

Klebsiella pneumoniae is classified as a common human- and animal-associated Gram-negative bacterium in the environment and has become a major cause of hospital-acquired infections worldwide.¹ *K. pneumoniae* belongs to the commensal bacterial family with the potential to engage in a wide range of infectious conditions including soft tissue, wound, and respiratory infections, especially in patients with compromised immune systems.² Numerous studies have reported *K. pneumoniae* infection frequencies can vary geographically and demographically, with a prevalence rate ranging from 1.30% to 16.00%.^{3–5} In China, *K. pneumoniae* was one of the three leading causative agents in patients

with bacterium-associated acute respiratory infections between 2009 and 2019.⁶ The critically ill patients in intensive care units (ICUs) are at a high risk for *K. pneumoniae* infections, mainly due to impaired immune functions, severe underlying diseases, hospitalization time, and/or multiple invasive therapeutic interventions.

Carbapenems remain one of the last-line therapeutic drugs against *K. pneumoniae* in gravely ill patients. Though a majority of the patients were routinely infected with carbapenem-sensitive *K. pneumoniae* (CSKP), the emergence of carbapenem-resistant *K. pneumoniae* (CRKP) strains has been increasingly reported.⁷ Plasmids encoding carbapenemase can be transferred among bacteria by horizontal gene transfer and readily disseminated in hospitals. Notably, recent research groups have confirmed carbapenem resistance was partly attributed to increased length of hospital stay and led to an elevated mortality in patients.⁸ Therefore, the carbapenemase-mediated resistance poses a serious public health threat.⁹

K. pneumoniae pathogenicity is also associated with certain virulence factors that help it to evade host immune surveillance, including capsules, lipopolysaccharides, exopolysaccharides, adhesins, and iron uptake systems.¹⁰ Interestingly, continuous evolution of hypervirulence plasmids gives rise to a pathotype of hypervirulent *K. pneumoniae*. This type of *K. pneumoniae* has been increasingly reported in association with plasmid-mediated pathogenicity loci of *iuc*, *iro*, *rmpA/rmpA2*, etc.^{11,12} Of them, the *iucABCD* and *iutA*, and *iroBCDN* are involved in the biosynthesis of aerobactin, and salmochelin, respectively.^{13–15} While, the *rmpA/rmpA2*-encoded proteins regulate capsule productions in *K. pneumoniae*.¹⁶ In China, *K. pneumoniae* carrying virulence plasmids is often subject to excess morbidity and mortality.^{17,18} The presence and spread of hypervirulent *K. pneumoniae* is of great concern due to its virulence and lack of therapeutic options. In addition, multidrug resistance has been increased all over the world that is also considered a public health threat. Several recent investigations reported the emergence of virulent multidrug-resistant (MDR) bacterial pathogens from different origins that increase the necessity of the proper use of antibiotics as well as the application of rapid accurate diagnostic tools for screening the emerging virulent MDR strains including *K. pneumoniae*.^{19–24}

In this study, we comprehensively investigated the epidemiology and drug sensitivities of CRKP and CSKP from ICU patients, and their clinical characteristics, multiple risk factors in combination with corresponding clinical data. In addition, typically important carbapenem-resistance genes, virulence-linked genes, as well as the *wzi* alleles that are conserved and used to predict capsular types of *K. pneumoniae*, were sequenced to explore the potential molecular mechanisms of CRKP.

Materials and Methods

Study Design, Bacterial Isolates and Patient Data Collection

The present study collected 201 cases infected with *K. pneumoniae* from the ICUs in the First Affiliated Hospital of Anhui Medical University, a Class A tertiary comprehensive hospital committed to delivering medical education and offering nearly 3000 beds for Anhui province in China, from January 2020 through January 2021. In cases with multiple detections of *K. pneumoniae*, the very first positive cultures we obtained were valid in this study. The relevant clinical data were accessed through the electronic medical database of the hospital. The information of all the cases generally included fundamental demographics, hospital stay length, concomitant diseases, invasive procedures or treatments, clinical medications and outcomes, as well as laboratory testing parameters.

Bacterial Isolation and Identification, and Antibiotic Susceptibility Testing

K. pneumoniae isolates were aseptically isolated and then identified to species level from blood, ascites, drainage fluid, cerebrospinal fluid, lower respiratory tract secretions and other materials of inpatients through an automatic microbial identification platform (BacT/AlerT 3D, BioMérieux, Marcy l'Etoile, France), blood agar cultures, and matrix-associated laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS, Vitek MS, BioMérieux, Marcy l'Etoile, France) within the hospital clinical laboratory according to the manufacturers' instructions.

Antimicrobial susceptibility testing of the *K. pneumoniae* isolates to common clinically used antibiotics was performed via the broth micro-dilution method using an automated Vitek 2 Compact system with Advanced Expert

System (BioMérieux, Marcy l'Etoile, France) or Phoenix 100 system (Becton Dickinson, Sparks, MD, USA), or by the Kirby–Bauer (Oxoid, Basingstoke, UK) approach according to the Clinical and Laboratory Standards Institute (CLSI) unified protocol.²⁵ Specifically, the tested antibiotics included penicillins (ampicillin), β -lactam combination agents (ampicillin–sulbactam, piperacillin–tazobactam, cefoperazone–sulbactam, and ceftazidime–avibactam), cephalosporins (cefazolin, ceftazidime, ceftriaxone, cefotaxime, cefuroxime, and cefepime), cephamycins (cefotetan, cefmetazole), monobactams (aztreonam), carbapenems (ertapenem, imipenem, and meropenem), aminoglycosides (amikacin, gentamicin, and tobramycin), fluoroquinolones (ciprofloxacin, levofloxacin), tetracyclines (tetracycline, minocycline), and folate pathway antagonists (trimethoprim–sulfamethoxazole). *Escherichia coli* ATCC25922 and *K. pneumoniae* ATCC700603 were used as quality controls as previously described.²⁶

Determination of Carbapenemase-Encoding Genes, Virulence-Associated Genes, and Capsular Polysaccharide Serotype-Associated Genes

Carbapenemase-encoding genes including *bla*_{KPC}, *bla*_{NDM}, *bla*_{OXA-23}, *bla*_{OXA-24}, *bla*_{OXA-48}, *bla*_{OXA-50}, *bla*_{OXA-55}, *bla*_{OXA-58}, *bla*_{OXA-60}, *bla*_{OXA-69}, *bla*_{VIM1}, *bla*_{VIM2}, *bla*_{IMP1}, *bla*_{IMP2}, *bla*_{SME}, *bla*_{GES}, *bla*_{GIM1}, *bla*_{IMI}, *bla*_{NMC}, *bla*_{SIM1} and *bla*_{SPM1}, and virulence-associated genes including *iucA*, *iroB*, *irp2*, *terB*, *peg344*, *peg589*, *peg1631*, *rmpA*, *rmpA2* and *crmpA* were determined by PCR and sequencing assays.^{27,28} The PCR primers were commercially synthesized by Shanghai Sangon Biotech Co., Ltd. (Shanghai, China), and the assays were performed in a DNA analyzer (Applied Biosystems 7500, Thermo Fisher Scientific, Waltham, MA, USA) with the PCR conditions as follows: (a) 5 minutes at 94 °C for initial denaturation, (b) 32 cycles of 30s at 94 °C for denaturation, 90s at a given temperature for annealing, and a given time period at 72 °C for extension), and (c) 10 minutes at 72 °C for last extension (Table 1). The resultant PCR products were confirmed by DNA agarose gel electrophoresis and quantified through a spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific, Waltham, MA, USA), and then the typical carbapenem-resistant gene fragments underwent the process of sequencing service commercially provided by Sangon Biotech Co., Ltd. (Shanghai, China).

The capsular polysaccharide serotypes of CRKP strains were identified through *wzi* gene by PCR with the specific primers commercially available (Shanghai Sangon Biotech Co., Ltd., Shanghai, China) (Table 1), and followed by sequencing of the PCR products for *wzi* alleles, as previously described.²⁹ The corresponding sequencing data then went through assignment analysis using an online database (<http://bigsd.b.pasteur.fr/klebsiella>).

Table 1 PCR Primer and Amplification Condition Information

Target Genes	Primers (5'-3')	Fragment Length (bp)	Annealing Temperature (°C)	Extension Time (sec)
Carbapenemase-encoding genes^a				
<i>bla</i> _{KPC}	F: ATGCTCACTGTATCGCCGCTCT R: TTTTCAGAGCCTTACTGCCC	893	58	60
<i>bla</i> _{OXA-23}	F: AAGCATGATGAGCGCAAAG R: AAAAGGCCCATTTATCTCAAA	1066	50	60
<i>bla</i> _{NDM}	F: GGTGTGGCGATCTGGTTTTTC R: CGGAATGGCTCATCACGATC	621	52	40
<i>bla</i> _{OXA-48}	F: TTGGTGGCATCGATTATCGG R: GAGCACTTCTTTTGATGGC	744	55	50
<i>bla</i> _{VIM1}	F: TTATGGAGCAGCAACCGATGT R: CAAAAGTCCCGCTCCAACGA	920	55	60
<i>bla</i> _{IMP1}	F: TGAGCAAGTTATCTGTATTC R: TTAGTTGCTTGGTTTTGATG	740	55	50
<i>bla</i> _{SME}	F: AGATAGTAAATTTTATAG R: CTCTAACGCTAATAG	1138	50	70
<i>bla</i> _{GES}	F: GTTTTGCAATGTGCTCAACG R: TGCCATAGCAATAGGCGTAG	371	55	30

(Continued)

Table 1 (Continued).

Target Genes	Primers (5'-3')	Fragment Length (bp)	Annealing Temperature (°C)	Extension Time (sec)
Virulence-associated genes				
<i>iucA</i>	F: AATCAATGGCTATTCGCGTG R: CGCTTCACTTCTTTCACTGACAGG	239	59	30
<i>iroB</i>	F: ATCTCATCATCTACCCTCCGCTC R: GGTTGCGCGTCGTTTCAA	235	59	30
<i>irp2</i>	F: GCTACAATGGGACAGCAACGAC R: GCAGAGCGATACGGAATGC	230	59	30
<i>terB</i>	F: TATCGCTGTTGCCAGTGAC R: CGGACAGCACTCTTCTCATC	288	59	30
<i>peg344</i>	F: CTTGAAACTATCCCTCCAGTC R: CCAGCGAAAGAATAACCCC	508	53	40
<i>peg589</i>	F: TGAACCCCTGAAGGTCTATC R: GTGATGAATAAACTACTGCGGC	236	55	30
<i>peg1631</i>	F: GGGATTATCAACCGCTTTG R: TCTCCAGCATCATCGTCA	503	59	40
<i>rmpA</i>	F: GAGTAGTTAATAAATCAATAGCAAT R: CAGTAGGCATTGCAGCA	332	50	40
<i>rmpA2</i>	F: GTGCAATAAGGATGTACATTA R: GGATGCCCTCCTCCTG	430	50	40
<i>crmpA</i>	F: GTAATAGAGATATAAATATCATATTGA R: CATCTTTCATCAACCATTTTC	588	50	40
The <i>wzi</i> allele gene				
<i>wzi</i>	F: CGAGCGCTTTCTATCTTGGT R: GAGAGCCACTGGTTCCAGAA	580	55	40

Notes: ^aThe information was listed on the PCR primers and conditions for the representative carbapenemase-encoding genes, the relevant information of the rest genes involved in this study can be found in Reference.²⁷

Abbreviations: PCR, polymerase chain reaction; bp, base pair; F, forward primer; R, reverse primer.

Statistical Analysis

The IBM Statistical Package for the Social Sciences (SPSS) (version 25.0, SPSS Inc., Chicago, IL, USA) and GraphPad Prism (version 5.0, GraphPad Software Inc., San Diego, CA, USA) were adopted to process statistically all the data collected. The χ^2 or Fisher's exact tests were applicable to the analysis regarding categorical variables when appropriate. The Student's *t*-test was available for data with normal distribution, otherwise, Mann-Whitney *U*-test was applied. A *P* value of < 0.05 was regarded as statistical significance.

Results

Bacterial Identification and Distribution of *K. pneumoniae*

Throughout the entire study period, 201 of *K. pneumoniae* isolates from ICU patients were collected and identified by microbial cultures for macroscopic/microscopic examinations and MALDI-TOF MS (Figure 1). To be specific, 83 (41.29%) of these isolates were CRKP, and the remaining ones (*n* = 118, 58.71%) were determined to be CSKP. All the specimens involved were from diverse source origins. Lower respiratory tract secretions (*n* = 141, 70.15%) accounted for the majority of source types, followed by blood (*n* = 40, 19.90%), ascites (*n* = 5, 2.49%), drainage fluid (*n* = 3, 1.49%) and others (*n* = 12, 5.97%). Upon comparison of the isolation rates during the whole year, the data showed that CRKP was relatively more prevalent during the seasons with cold/hot weather switching, eg, spring (esp., March) and autumn (esp., October); while CSKP dominated in the entire winter (*P* < 0.01, Figure 2).

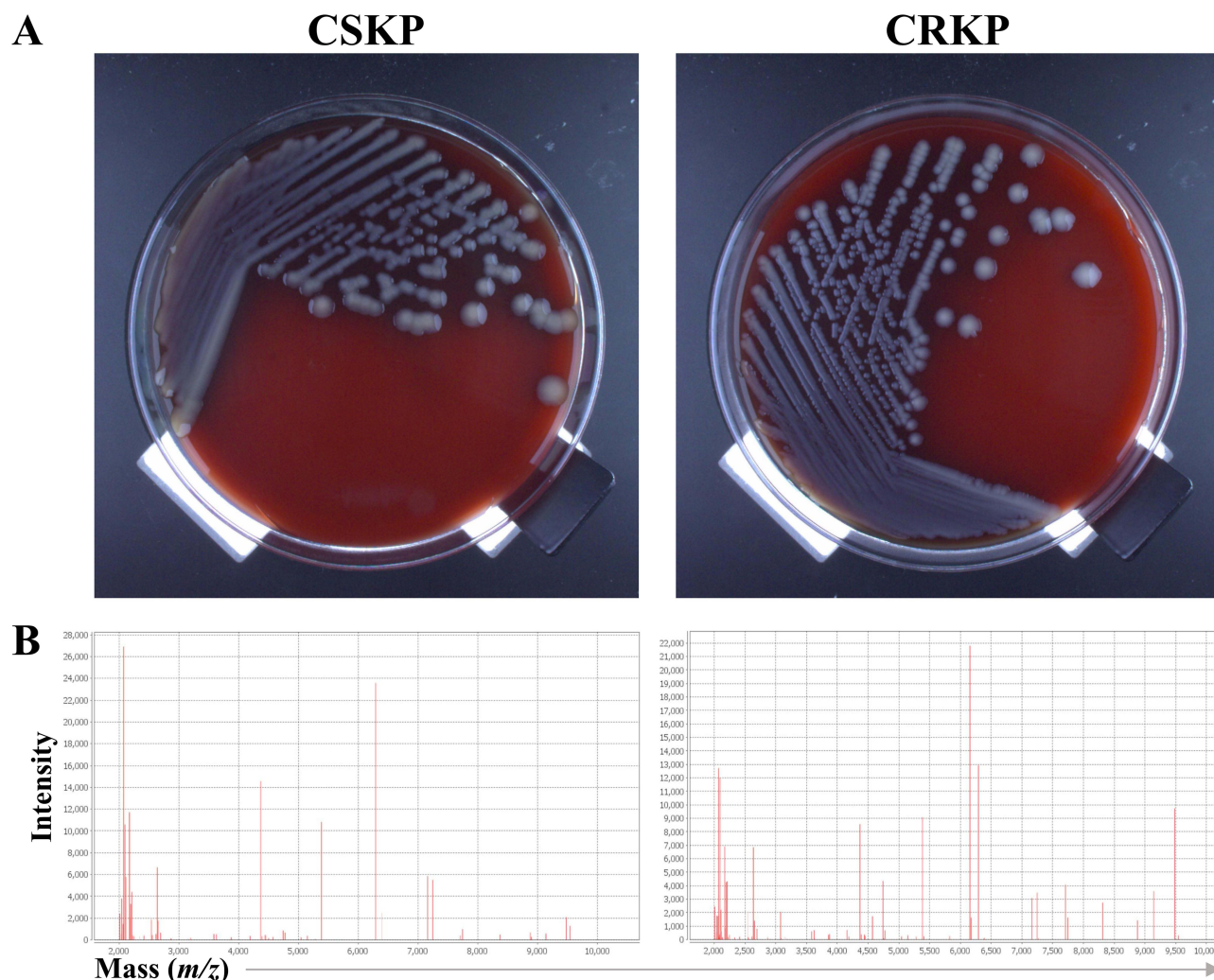


Figure 1 Identification of *K. pneumoniae* from the critically ill patients in the ICUs. **(A)** Representative microbial cultures of CSKP and CRKP for macroscopic/microscopic examinations. **(B)** Representative data from MALDI-TOF MS spectra for CSKP and CRKP identification.

Abbreviations: CSKP, carbapenem-sensitive *Klebsiella pneumoniae*; CRKP, carbapenem-resistant *Klebsiella pneumoniae*.

Clinical Features with *K. pneumoniae* Infections

The demographic and clinical profiles of patients infected with CSKP and CRKP are described in Table 2. Of these 201 patients, their average age was 58.25 ± 17.61 years, males outnumbered females by approximately 2.5-fold. The average time of hospital stay after CSKP and CRKP infections was 20.10 ± 23.35 , and 24.41 ± 26.27 days, respectively. The predominant concomitant diseases in all the infected patients were primarily involved with cerebral diseases (59.70%), renal diseases (34.83%), hypertension (32.34%), cardiac diseases (31.34%) and others, among which renal diseases in ICU patients infected with CRKP were likely to concomitantly occur more often in comparison to those with CSKP in the present study.

As to such various risk factors related to CRKP infections, prior treatments/processes of urinary catheter, gastric cannula, blood product transfusion, mechanical ventilation, peripheral arterial catheter, central venous catheter, thoracic drainage, continuous renal replacement therapy (CRRT), as well as recent prior exposure to certain antibiotics of glycopeptides, carbapenems, quinolones, aminoglycosides, were found statistically different between CRKP and CSKP infection groups ($P < 0.05$). Whereas, the remaining risk factor variables did not make significant differences within both groups, mainly including most of the comorbidities, surgery, fiberoptic bronchoscopy, extracorporeal membrane oxygenation (ECMO), hemodialysis and plasma exchange, as well as prior administration of β -lactam/ β -lactamase inhibitor,

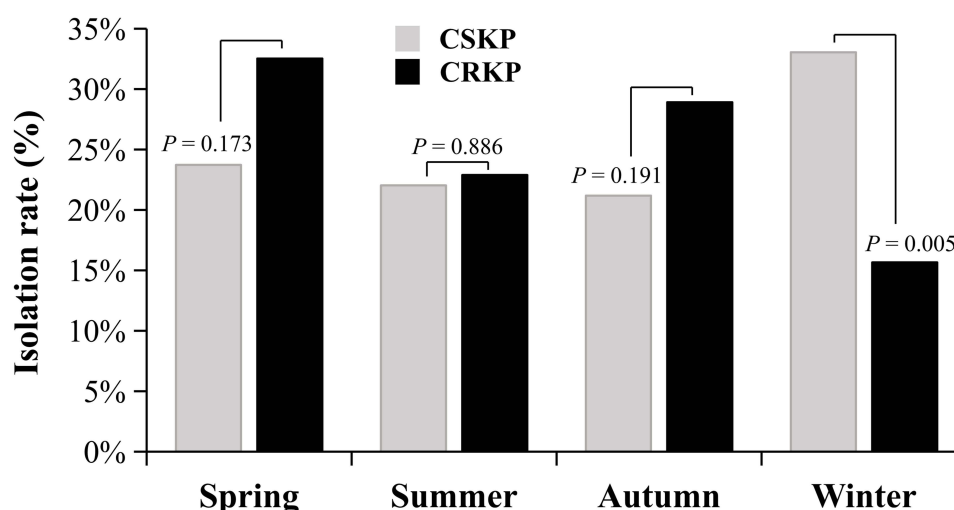


Figure 2 Comparison of the isolation rates of CSKP ($n = 118$) and CRKP ($n = 83$) from the ICU patients during the entire year with typical seasons.

Abbreviations: CSKP, carbapenem-sensitive *Klebsiella pneumoniae*; CRKP, carbapenem-resistant *Klebsiella pneumoniae*.

cephalosporins, linezolid, and antifungal agents. Furthermore, the clinical outcomes of CRKP-infected patients were worsened when compared to those infected with CSKP ($P = 0.000$), in terms of infection persistence (Table 2).

We next summarized and evaluated the laboratory testing parameters of the ICU patients infected with *K. pneumoniae*. As listed in Table 3, on average, white blood cell count (WBC, mainly neutrophils), prothrombin time (PT), activated partial thromboplastin time (APTT) and fibrinogen (FIB) levels in the entire infected population at admission were all beyond normal ranges, whereas, their mean concentrations of hemoglobin (Hb), hematocrit (Hct), total protein (TP, esp., albumin or ALB) were lower than normal levels. In comparison of CSKP and CRKP groups, we found significant differences in terms of red blood cell count (RBC), Hb and Hct ($P < 0.001$). We also observed

Table 2 Characteristics of the Critically Ill Patients Infected with *K. pneumoniae*

Variables	Total, $n = 201$ (%)	CSKP, $n = 118$ (%)	CRKP, $n = 83$ (%)	P^a
General information				
Gender: male	139 (69.15)	80 (67.80)	59 (71.08)	0.619
Age/years (mean \pm SD)	58.25 \pm 17.61	58.22 \pm 19.28	58.29 \pm 15.02	0.392
Length of hospital stay ^b /days (mean \pm SD)	21.88 \pm 24.63	20.10 \pm 23.35	24.41 \pm 26.27	0.147
Concomitant diseases				
Cerebral disease	120 (59.70)	70 (59.32)	50 (60.24)	0.896
Renal disease	70 (34.83)	35 (29.66)	35 (42.17)	0.067
Hypertension	65 (32.34)	35 (29.66)	30 (36.15)	0.333
Cardiac disease	63 (31.34)	39 (33.05)	24 (28.92)	0.534
Shock	55 (27.36)	30 (25.42)	25 (30.12)	0.462
Chronic pulmonary disease	50 (24.88)	33 (27.97)	17 (20.48)	0.227
Hepatic insufficiency	49 (23.38)	27 (22.88)	22 (26.51)	0.556
Diabetes mellitus	34 (16.92)	24 (20.34)	10 (12.05)	0.123
Malignancy	28 (13.93)	18 (15.25)	10 (12.05)	0.518
Therapeutic processes before infection				
Urinary catheter	174 (86.57)	95 (80.51)	79 (95.18)	0.003
Gastric cannula	168 (83.58)	89 (75.42)	79 (95.18)	0.000
Blood product transfusion	145 (72.14)	71 (60.17)	74 (89.16)	0.000
Mechanical ventilation	144 (71.64)	77 (65.25)	67 (80.72)	0.017

(Continued)

Table 2 (Continued).

Variables	Total, n = 201 (%)	CSKP, n = 118 (%)	CRKP, n = 83 (%)	P ^a
Peripheral arterial catheter	135 (67.16)	68 (57.63)	67 (80.72)	0.001
Central venous catheter	129 (64.18)	66 (55.93)	63 (75.90)	0.004
Surgery	95 (47.26)	53 (44.92)	42 (50.60)	0.427
Lumbar/abdominal drainage	56 (27.86)	28 (23.73)	28 (33.73)	0.119
Fiberoptic bronchoscopy	33 (16.42)	16 (13.56)	17 (20.48)	0.192
Thoracic drainage	28 (13.93)	10 (8.47)	18 (21.68)	0.008
Continuous renal replacement therapy	20 (9.95)	6 (5.08)	14 (16.87)	0.006
Extracorporeal membrane oxygenation	12 (5.97)	6 (5.08)	6 (7.23)	0.742
Hemodialysis and plasma exchange	11 (5.47)	4 (3.39)	7 (8.43)	0.218
Antibiotic exposure before infection				
β-Lactam/β-Lactamase inhibitor	142 (70.65)	80 (67.80)	62 (74.70)	0.290
Cephalosporins	65 (32.34)	34 (28.81)	31 (37.35)	0.203
Glycopeptides	65 (32.34)	25 (21.19)	40 (48.19)	0.000
Carbapenems	64 (31.84)	22 (18.64)	42 (50.60)	0.000
Quinolones	33 (16.42)	12 (10.17)	21 (25.30)	0.004
Aminoglycosides	24 (11.94)	5 (4.24)	19 (22.89)	0.000
Antifungal agents	20 (9.95)	9 (7.63)	11 (13.25)	0.190
Linezolid	17 (8.46)	7 (5.93)	10 (12.05)	0.125
Outcomes of infection				
Better	112 (55.72)	99 (83.90)	13 (15.66)	0.000
Worse	77 (38.31)	11 (9.32)	66 (79.52)	
Others	12 (5.97)	8 (6.78)	4 (4.82)	

Notes: ^aP values were from the comparisons between groups of CSKP and CRKP. ^bThe length of hospital stay meant the average day time after CSKP or CRKP infections.
Abbreviations: CSKP, carbapenem-sensitive *Klebsiella pneumoniae*; CRKP, carbapenem-resistant *Klebsiella pneumoniae*; SD, standard deviation.

Table 3 Summary of Blood Test Data of the ICU Patients Infected with *K. pneumoniae*

Laboratory Parameters from Blood ^a , mean ± SD	Total (n = 201)	CSKP (n = 118)	CRKP (n = 83)	P ^b
WBC # (10 ⁹ /L)	10.71 ± 5.81	10.95 ± 6.07	10.38 ± 5.44	0.474
NEUT (%)	82.04 ± 12.42	82.42 ± 12.46	81.51 ± 12.43	0.196
LYMPH (%)	10.91 ± 9.40	10.67 ± 10.05	11.26 ± 8.44	0.098
MONO (%)	5.74 ± 4.11	5.69 ± 3.28	5.81 ± 5.08	0.739
NEUT # (10 ⁹ /L)	8.96 ± 5.26	9.18 ± 5.49	8.64 ± 4.92	0.427
LYMPH # (10 ⁹ /L)	1.03 ± 1.12	1.02 ± 1.26	1.05 ± 0.88	0.176
MONO # (10 ⁹ /L)	0.59 ± 0.49	0.62 ± 0.54	0.54 ± 0.41	0.471
RBC # (10 ¹² /L)	3.89 ± 10.60	4.58 ± 13.80	2.91 ± 0.77	0.000
Hb (g/L)	96.31 ± 23.16	101.52 ± 23.48	88.90 ± 20.67	0.000
Hct (%)	29.40 ± 8.50	31.04 ± 9.05	27.06 ± 7.08	0.000
PLT # (10 ⁹ /L)	161.54 ± 108.91	160.63 ± 101.91	162.84 ± 118.78	0.770
PT (sec)	15.89 ± 4.05	15.40 ± 2.72	16.60 ± 5.34	0.082
APTT (sec)	43.75 ± 14.34	41.97 ± 9.68	46.29 ± 18.88	0.017
FIB (g/L)	4.39 ± 2.01	4.57 ± 1.94	4.15 ± 2.10	0.152
TP (g/L)	56.39 ± 8.75	56.98 ± 9.48	55.56 ± 7.57	0.241
ALB (g/L)	33.60 ± 5.98	33.88 ± 6.37	33.20 ± 5.39	0.418
GLO (g/L)	22.80 ± 5.66	23.10 ± 6.25	22.36 ± 4.70	0.963
A/G	1.56 ± 0.50	1.57 ± 0.55	1.55 ± 0.44	0.843

(Continued)

Table 3 (Continued).

Laboratory Parameters from Blood ^a , mean ± SD	Total (n = 201)	CSKP (n = 118)	CRKP (n = 83)	P ^b
TBIL (μmol/L)	27.99 ± 51.12	21.80 ± 28.43	36.79 ± 71.32	0.317
ALT (U/L)	99.02 ± 266.21	92.00 ± 290.90	109.00 ± 227.90	0.116
AST (U/L)	182.02 ± 662.51	168.87 ± 702.35	200.71 ± 605.05	0.254
ALP (U/L)	101.31 ± 81.45	99.51 ± 84.13	103.88 ± 77.91	0.117
GGT (U/L)	82.99 ± 106.51	82.07 ± 106.08	84.30 ± 107.75	0.366

Notes: ^aWBC #, (3.50–9.50) × 10⁹/L; NEUT, (40.00–75.00) %; LYMPH, (20.00–50.00) %; MONO, (3.00–10.00) %; NEUT #, (1.80–6.30) × 10⁹/L; LYMPH #, (1.10–3.20) × 10⁹/L; MONO #, (0.10–0.60) × 10⁹/L; RBC #, male (4.30–5.80) × 10¹²/L and female (3.80–5.10) × 10¹²/L; Hb, male (130.00–175.00) g/L and female (115.00–150.00) g/L; Hct, male (40.00–50.00) % and female (35.00–45.00) %; PLT #, (125.00–350.00) × 10⁹/L; PT, (11–16) s; APTT, (28–42) s; FIB, (2.00–4.00) g/L; TP, (65.00–85.00) g/L; ALB, (40.00–55.00) g/L; GLO, (20.00–40.00) g/L; A/G, 1.20–2.40; TBIL, (0–23.00) μmol/L; ALT, male (9.00–50.00) U/L and female (7.00–40.00) U/L; AST, male (15.00–40.00) U/L and female (13.00–35.00) U/L; ALP, male (45.00–125.00) U/L female (35.00–100.00) U/L, GGT, male (10.00–60.00) U/L and female (7.00–45.00) U/L. ^bP values were from the comparisons between groups of CSKP and CRKP.

Abbreviations: CSKP, carbapenem-sensitive *Klebsiella pneumoniae*; CRKP, carbapenem-resistant *Klebsiella pneumoniae*; SD, standard deviation; WBC #, white blood cell count; NEUT #, neutrophil count; LYMPH #, lymphocyte count; MONO #, monocyte count; RBC #, red blood cell count; Hb, hemoglobin; Hct, hematocrit; PLT #, platelet count; PT, prothrombin time; APTT, activated partial thromboplastin time; FIB, fibrinogen; TP, total protein; ALB, albumin; GLO, globulin; A/G, albumin/globulin ratio; TBIL, total bilirubin; ALT, aspartate aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; GGT, γ-glutamyl transpeptidase.

a statistically significant difference in APTT ($P < 0.05$) rather than other coagulation-related indicators (eg, platelet or PLT, PT and FIB). The remaining laboratory parameters tested were not statistically different between the two groups.

Antimicrobial Resistance Patterns of *K. pneumoniae*

After classifying all the *K. pneumoniae* isolates into CRKP and CSKP groups, the overall results of in vitro susceptibility tests for these isolates were comprehensively analyzed. As shown in Figure 3, generally, CRKP from the ICU patients exhibited significantly lowered levels or even no any of susceptibility to all the tested antimicrobial agents except ceftazidime–avibactam (CZA), tigecycline and minocycline. Specifically, the data pointed out that the CRKP cohort presented remarkably higher levels of resistance against multiple antibiotics, particularly including the third generation of cephalosporin, carbapenem, the β-lactam/β-lactamase inhibitor combination, ampicillin, ciprofloxacin, gentamicin, amikacin, trimethoprim/sulfamethoxazole, when compared to the group of CSKP ($P < 0.001$). To be noted, both CRKP and CSKP showed comparable and nearly excellent in vitro susceptibility to CZA, tigecycline and minocycline.

Carbapenemase-Encoding Genes, Virulence-Associated Genes and Capsular Polysaccharide Serotypes Among CRKP

Twenty-one carbapenemase-encoding genes were selected for identification of the potential molecular mechanisms of the CRKP-infected cases. Among the 79 CRKP isolates that were successfully recovered from cryopreserved strains for subcultures, 77 isolates (97.47%) were positive for *bla*_{KPC}, 6 (7.59%) for *bla*_{OXA-23}, and 5 (6.33%) for *bla*_{NDM}, and the remaining carbapenemase-encoding genes tested were absent in all the CRKP strains of this study. The typical carbapenemase-encoding gene fragments from *Klebsiella pneumoniae* strains were sequenced for validation of PCR products (data not shown). Notably, to our knowledge, the vast majority of the *bla*_{KPC}-harboring CRKP strains ($n = 69$, 87.34%) simply carried *bla*_{KPC}, while there were two strains (2.53%) of CRKP expressing *bla*_{NDM} alone, in the present study. Whereas, there existed eight strains (10.13%) of CRKP possessing a combination of the carbapenemase-encoding genes for which the combined pattern of *bla*_{KPC} and *bla*_{OXA-23} was the predominant (6.33%), followed by the pattern of *bla*_{KPC} and *bla*_{NDM} (2.53%), and the pattern of *bla*_{KPC}, *bla*_{OXA-23} and *bla*_{NDM} (1.27%). In comparison of the frequencies of carbapenemase-encoding genes in CRKP, there were no statistically significant differences among the three cohorts with different infection outcomes, despite the fact that nearly all the *bla*_{OXA-23} and *bla*_{NDM} genes were barely found in the cohort with worse outcome of infection (Figure 4).

As for the virulence-associated genes presented in CRKP of the local area, the dominant candidates were *irp2* ($n = 77$, 97.47%), *rmpA2* ($n = 57$, 72.15%), *peg589* ($n = 55$, 69.62%), *iucA* ($n = 54$, 68.35%), *terB* ($n = 54$, 68.35%) and *peg1631*

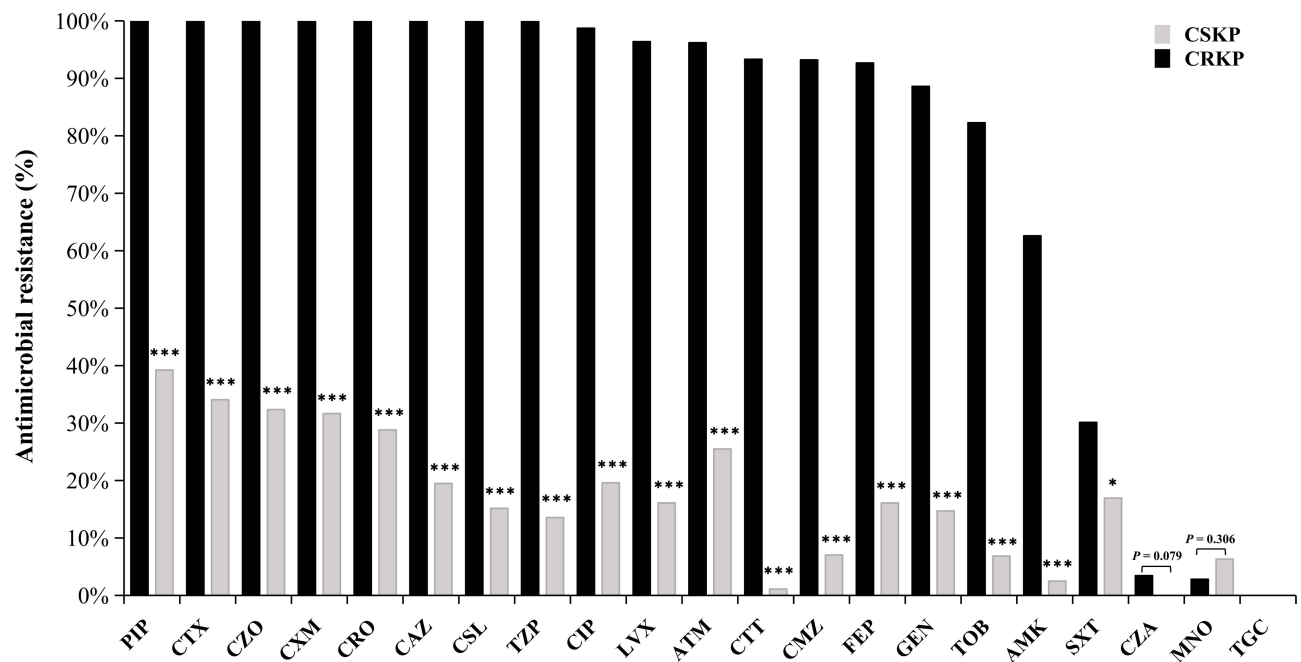


Figure 3 Antimicrobial resistance patterns of CSKP (n = 118) and CRKP (n = 83) from the patients with *K. pneumoniae* infections. * $P < 0.05$, *** $P < 0.001$; all comparisons were performed between CSKP and CRKP for each antimicrobial agent as indicated.

Abbreviations: CSKP, carbapenem-sensitive *Klebsiella pneumoniae*; CRKP, carbapenem-resistant *Klebsiella pneumoniae*; PIP, piperacillin; CTX, cefotaxime; CZO, ceftazolin; CXM, cefuroxime; CRO, ceftriaxone; CAZ, ceftazidime; CSL, cefoperazone/sulbactam; TZP, piperacillin/tazobactam; CIP, ciprofloxacin; LVX, levofloxacin; ATM, aztreonam; CTT, cefotaxime; CMZ, cefmetazole; FEP, cefepime; GEN, gentamicin; TOB, tobramycin; AMK, amikacin; SXT, trimethoprim/sulfamethoxazole; CZA, ceftazidime-avibactam; MNO, minocycline; TGC, tigecycline.

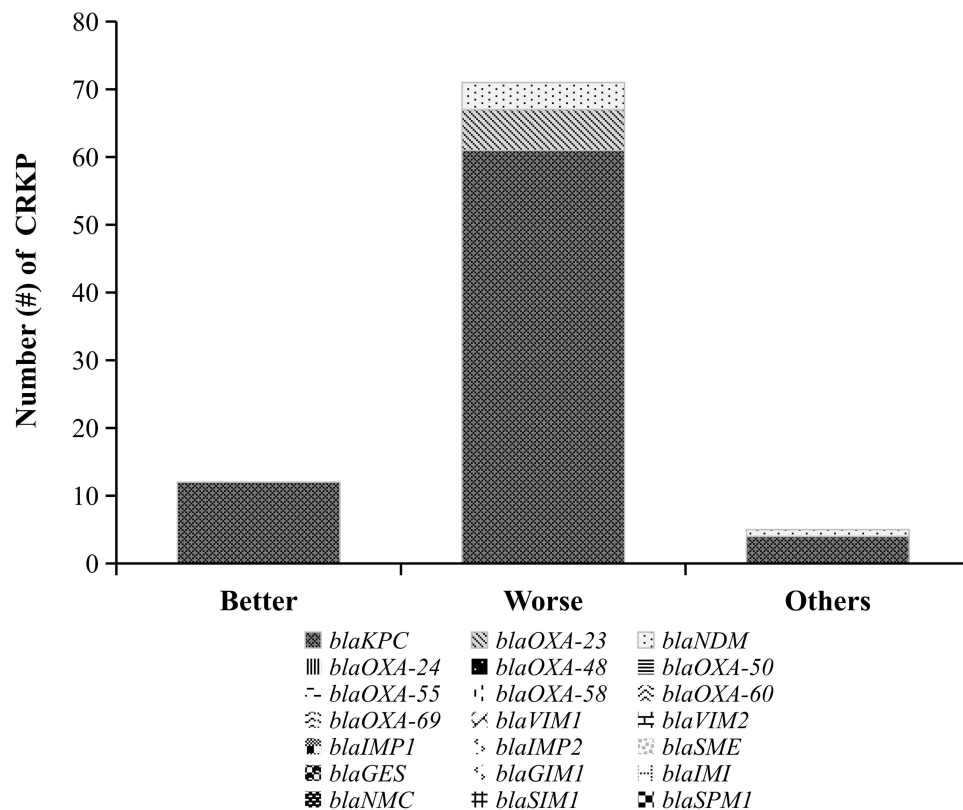


Figure 4 Identification and comparison of carbapenemase-encoding genes in CRKP strains from the ICU patients (n = 79) with different infectious outcomes. Better, the cohort with better infectious outcomes in which CRKP infections were effectively controlled. Worse, the cohort with worse infectious outcomes in which CRKP infections worsened progressively. Others, the cohort with unknown outcomes as the infected patients were discharged with voluntary withdrawal of treatment with unknown reasons.

Abbreviation: CRKP, carbapenem-resistant *Klebsiella pneumoniae*.

($n = 49$, 62.03%). The presence of the other genes were all below 50.00% in this study. Likewise, no significant differences were observed among the three cohorts with different outcomes of infection when comparing the frequencies of CRKP virulence-associated genes, though *terB* and *peg1631* exhibited considerably higher percentages in the cohort with worse outcome of infection than those in the other two cohorts (Table 4). Additionally, the capsular polysaccharide serotypes were further assessed for CRKP strains through PCR and sequencing of the *wzi* allele genes. Within these 79 CRKP isolates from the local region, three major capsular polysaccharide serotypes were identified as K14.K64 (*wzi*-64), KL47 (*wzi*-209) and K19 (*wzi*-19), accounting for 44.30% ($n = 35$), 27.85% ($n = 22$) and 16.46% ($n = 13$), respectively. The remaining serotypes involved were all less than 4.00% in proportion. Moreover, few significant differences were found in cohorts with different infection outcomes in comparison of the incidence of CRKP capsular polysaccharide serotypes, except the serotype of K28 (*wzi*-84) with $P < 0.05$. Interestingly, the KL47 (*wzi*-209) serotype appeared to prevail in the cohort with better outcome of infection, whereas the K14.K64 (*wzi*-64) dominated the cohort with worse one (Table 5). In addition, the overall data were summarized together to reveal potential correlation characteristics among the carbapenemase-encoding genes, polysaccharide serotyping (*wzi* allele) and virulence-associated genes from all the recovered CRKP strains, and meanwhile their MDR features were individually determined (>50.00% of CRKP strains were MDR) according to the criteria previously described (Supplementary Table S1).³⁰

Table 4 Virulence-Associated Genes in CRKP with Different Clinical Outcomes

Virulence-Associated Genes	Total $n = 79$ (%)	Better Outcome ^a $n = 12$ (%)	Worse Outcome $n = 63$ (%)	Others ^b $n = 4$ (%)	P^c
<i>irp2</i>	77 (97.47)	12 (100.00)	61 (96.83)	4 (100.00)	1.000
<i>rmpA2</i>	57 (72.15)	9 (75.00)	46 (73.02)	2 (50.00)	0.632
<i>peg589</i>	55 (69.62)	9 (75.00)	44 (69.84)	2 (50.00)	0.721
<i>iucA</i>	54 (68.35)	9 (75.00)	43 (68.25)	2 (50.00)	0.641
<i>terB</i>	54 (68.35)	6 (50.00)	46 (73.02)	2 (50.00)	0.193
<i>peg1631</i>	49 (62.03)	6 (50.00)	41 (65.08)	2 (50.00)	0.484
<i>rmpA</i>	33 (41.77)	4 (33.33)	28 (44.44)	1 (25.00)	0.611
<i>peg344</i>	27 (34.18)	4 (33.33)	22 (34.92)	1 (25.00)	1.000
<i>iroB</i>	3 (3.80)	0 (0)	3 (4.76)	0 (0)	1.000
<i>crmpA</i>	1 (1.27)	0 (0)	1 (1.59)	0 (0)	1.000

Notes: ^aBetter outcomes meant the carbapenem-resistant *Klebsiella pneumoniae* infections of patients were effectively controlled, otherwise became progressively worse. ^bThese patients were discharged with voluntary withdrawal of treatment with unknown reasons, and without the data of infectious outcomes. ^c P values were from the comparisons between groups with better or worse outcomes.

Table 5 Capsular Polysaccharide Serotypes of CRKP with Different Clinical Outcomes

Capsular Polysaccharide Serotypes (<i>wzi</i> Alleles)	Total $n = 79$ (%)	Better Outcome ^a $n = 12$ (%)	Worse Outcome $n = 63$ (%)	Others ^b $n = 4$ (%)	P^c
K14.K64 (<i>wzi</i> -64)	35 (44.30)	5 (41.67)	29 (46.03)	1 (25.00)	0.823
KL47 (<i>wzi</i> -209)	22 (27.85)	6 (50.00)	16 (25.40)	0 (0)	0.129
K19 (<i>wzi</i> -19)	13 (16.46)	1 (8.33)	11 (17.46)	1 (25.00)	0.599
KL102/KL149/KL155 (<i>wzi</i> -173)	3 (3.80)	0 (0)	2 (3.17)	1 (25.00)	0.206
K35 (<i>wzi</i> -162)	2 (2.53)	0 (0)	2 (3.17)	0 (0)	1.000
K2 (<i>wzi</i> -72)	1 (1.27)	0 (0)	1 (1.59)	0 (0)	1.000
KL57 (<i>wzi</i> -206)	1 (1.27)	0 (0)	1 (1.59)	0 (0)	1.000
K46 (<i>wzi</i> -223)	1 (1.27)	0 (0)	1 (1.59)	0 (0)	1.000
K28 (<i>wzi</i> -84)	1 (1.27)	0 (0)	0 (0)	1 (25.00)	0.049

Notes: ^aBetter outcomes meant the carbapenem-resistant *Klebsiella pneumoniae* infections of patients were effectively controlled, otherwise became progressively worse. ^bThese patients were discharged with voluntary withdrawal of treatment with unknown reasons, and without the data of infectious outcomes. ^c P values were from the comparisons between groups with better or worse outcomes.

Discussion

CRKP is considered as a public health threat worldwide as it is one of the leading causes of death among patients with hospital-acquired infections. The ICUs have been described as a factory for creating, disseminating, and amplifying antimicrobial resistance due to their extremely vulnerable population of critically ill patients, heavy use of invasive procedures, and frequent application of antimicrobials. Thus, regularly monitoring of CRKP would facilitate the choice and efficacy of empirical therapy, especially in the ICU settings.³¹ The present study assessed the local distribution features, antimicrobial resistance pattern, clinical risk factors, prevalence of the carbapenem-resistant genes, virulence-linked variant genes and capsular polysaccharide serotype-associated genes of CRKP strains isolated from clinical specimens of ICU patients in one of the largest tertiary comprehensive teaching hospitals in Central China. This study demonstrated a high prevalence of CRKP that carried a variety of carbapenem-resistant genes, virulence-linked variant genes and capsular polysaccharide serotypes.

CRKP infections threaten ICU patients because of the ability to contaminate environmental surfaces including hospital wards, increasing its transmission potentials among the environment, patients and medical staff.³² Previous researchers reported that the incidence of CRKP infections was connected with the switching of cold/hot seasons.³³ Here we observed a relatively higher local prevalence of CRKP in spring and autumn. Although the underlying links between the seasons and the CRKP infection incidence remain unclear, understanding their potential relationship would contribute to the prophylaxis of such infections in future. Studies revealed that undergoing invasive procedures would increase such infection risks.³⁴ Intubation or tracheotomy might disrupt normal body barriers and facilitate contact between the interior of body and the external environment, leading to easy invasion and attachment of opportunistic pathogens to the intubation lining, creating a biofilm cover that would be difficult to eradicate. As a result, pathogenic bacteria might enter deeper tissues of host and increase the chance of CRKP infections.^{35–37} Our ICU patients with multiple clinical invasive interventions had significant risks for CRKP infections. Moreover, prior treatment with certain antimicrobials substantially increased the emergence of CRKP. Some investigators suggested that frequent exposure to one antimicrobial class would augment the impact of exposure to another antimicrobial class on the CRKP infection risks.^{38,39} Hence, antimicrobial combination therapy and long-term high-dose treatment with carbapenems likely increase antimicrobial selection pressure and allow carbapenem-resistant bacteria to further develop novel resistance mechanisms. It is worth noting that MDR bacteria have been increasingly emerging worldwide.²⁰ Our study indicated over half of the CRKP population with MDR phenotype. The control and prevention of MDR pathogens, particularly including MDR *K. pneumoniae* strains from the ICU settings, urgently need rational utilization of antimicrobials and rapid screening strategies.^{19–24} Based on our laboratory data, we speculated that CRKP-infected ICU patients were more likely accompanied by symptoms of anemia and bleeding, due to relatively lowered RBC/Hb/Hct and extended APTT, which might aggravate the severity of diseases and *K. pneumoniae* infections.

K. pneumoniae is able to resist antimicrobial actions through multiple mechanisms including alteration of targets, inactivation of drugs, enhanced activity of efflux pumps, and attenuated permeability of cells.¹⁹ *K. pneumoniae* resistance to carbapenems is mainly attributable to the presence of carbapenemases, often assisted by other resistance pathways such as extended-spectrum β -lactamases (ESBLs).⁴⁰ Our findings illustrated that CRKP in ICU patients exhibited significantly lower levels of susceptibility to all antibiotics except CZA, tigecycline and minocycline. It reported that administration of tigecycline in patients with CRKP infections contributed to rapid development of in vitro resistance.⁴¹ The novel FDA-approved CZA has been verified to mount a good activity against CRKP, especially against the KPC-producing, but not NDM-producing CRKP.^{42,43} We also noticed that merely 3.45% of CRKP were resistant to CZA. Therefore, to prevent the development of new CZA-resistant strains in the local area of China, identification of resistance mechanisms ought to be an essential prerequisite for development of a rational antibiotic regimen.

Recent CRKP prevalence rates of Chinese major provinces ranged from 0.80% to 28.10%, with an average rate of 11.30%, according to China Antimicrobial Resistance Surveillance System (CARSS, <http://www.carss.cn>), and the dominant carbapenem-resistant genotype of CRKP was *bla*_{KPC}, accounting for approximately 65.00%.⁴⁴ Based on the findings in the present study, the local incidence of CRKP infections among the ICU patients was higher than the average level of China, which should draw special attention for our clinicians to focusing on the current situation. With the first

KPC-producing CRKP isolate emerging in Hangzhou City, KPC-type enzymes have constantly been the predominant carbapenemases of CRKP in China.^{45,46} Similarly, such type of enzymes were the most prevalent amidst our tested CRKP isolates, followed by OXA-23 and NDM. Interestingly, eight of these isolates were carrying combinations of *bla*_{KPC}, *bla*_{OXA-23} and/or *bla*_{NDM} genes. Further studies on the combined carbapenemase-associated genes might help to understand the evolution of CRKP. Previous studies reported several defining factors in pathogenic agents for virulence and toxicity, mainly including capsules, pili and siderophores, which fundamentally affect both classic and virulent strains of *K. pneumoniae* causing severe bacteremia, pneumonia and even meningitis in vulnerable population with compromised immunity such as the ICU patients.^{10,47,48} Acquisition of virulence-associated genes in CRKP is one of the main strategies to produce highly virulent CRKP.⁴⁹ It is proposed that MDR clones would be more likely to subsequently acquire virulence genes than highly pathogenic clones that, however, would be prone to get access to resistance genes, suggesting that CRKP strains with pre-existing resistance genes might take an advantage of subsequent acquisition of virulence-associated genes, when compared to CSKP strains.⁵⁰ In the present study, nearly all of CRKP strains from ICU patients possessed a variety of virulence-associated genes tested, indicating that these gene members might be ubiquitously spread among CRKP isolates in the local region. Finally, according to previous epidemiology studies, the capsular polysaccharide serotype of K14.K64 (*wzi*-64) in *K. pneumoniae* was identified as a common type within CRKP strains in China as well as other countries.^{51,52} Here, our observation pointed out that such serotype of K14.K64 (*wzi*-64) was certainly the main type for local CRKP strains. In addition, the serotypes of KL47 (*wzi*-209) and K19 (*wzi*-19) also occupied relatively high proportions in the local CRKP population. Further investigations will be needed to consolidate the understanding of potential connections between the capsular polysaccharide serotypes and treatment of *K. pneumoniae* infections.

Conclusion

The current study reported epidemiological and antimicrobial resistance patterns, clinical characteristics and risk factors of CRKP in critically ill patients. Importantly, carbapenemase-encoding genes, virulence- and capsular polysaccharide serotype-associated genes of CRKP were well documented and evaluated. Our data revealed seasonal bias in the prevalence of CRKP infections for ICU patients. The CRKP strains showed significantly attenuated susceptibility to most of commonly used antimicrobials and were subject to MDR phenotype. Recent exposure to certain antibiotics and prior treatment with invasive interventions were prone to increase CRKP infection risk with worsened infectious outcomes. In the local area, the top three carbapenemase-encoding genes of CRKP were *bla*_{KPC}, *bla*_{OXA-23} and *bla*_{NDM}. The virulence-associated gene *irp2* dominated the regional CRKP population. Moreover, nearly half of the CRKP isolates presented the capsular polysaccharide serotype of K14.K64 (*wzi*-64) which preferentially emerged in the cohort with worse infectious outcome at the bedside. In short, our findings supported the strict policies for antibiotic administration, cautious implementation of invasive procedures, and careful management of critically ill patients infected with virulent CRKP in the ICUs.

Abbreviations

K. pneumoniae, *Klebsiella pneumoniae*; ICU, intensive care unit; CSKP, carbapenem-sensitive *Klebsiella pneumoniae*; CRKP, carbapenem-resistant *Klebsiella pneumoniae*; MDR, multidrug-resistant; MALDI-TOF MS, matrix-assisted laser desorption ionization–time-of-flight mass spectrometry; CLSI, Clinical and Laboratory Standards Institute; PCR, polymerase chain reaction; CRRT, continuous renal replacement therapy; ECMO, extracorporeal membrane oxygenation; WBC, white blood cell; PT, prothrombin time; APTT, activated partial thromboplastin time; FIB, fibrinogen; Hb, hemoglobin; Hct, hematocrit; TP, total protein; ALB, albumin; RBC, red blood cell; PLT, platelet; CZA, ceftazidime–avibactam; ESBL, extended-spectrum β -lactamase; KPC, *Klebsiella pneumoniae* carbapenemase; NDM, New Delhi metallo- β -lactamase; OXA, oxacillinase.

Ethics Approval

The study protocol was approved by the Ethics Committee of Clinical Medicine Research of the First Affiliated Hospital of Anhui Medical University. All the subjects provided written informed consents according to the Helsinki statement.

Author Contributions

Fanbo Lu and Luwen Zhang share first authorship. All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

All the authors declared no competing interests for this work.

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