Infection and Drug Resistance

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ORIGINAL RESEARCH

In vitro Evaluation of the Antibacterial Activity of the Combination of Avibactam and β-Lactams Against Highly Virulent Carbapenem-Resistant *Klebsiella pneumoniae*

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Purpose: To study the resistance of carbapenem-resistant *Klebsiella pneumoniae* to β-lactams combined with avibactam, as well as the distribution of resistance and virulence genes, and to analyze the clinical characteristics of infected patients.

Methods: Antibiotic susceptibility was examined using the trace broth dilution method. Carbapenem-resistance genes, porins, and virulence genes were identified using PCR. Strain adhesion was assessed through wire-drawing experiments, and clinical data from infected patients were collected.

Results: Among 80 CRKP strains, 93.8% harboured *bla*KPC-2, and 1.3% harboured both *bla*KPC-2 and *bla*NDM. Some strains lacked OMPK35 (6.2%) and OMPK36 (10%). Virulence genes *ycfM, entB, fimH, irp-1*, and *prmpA2* were prevalent. The combination of carbapenems, cephalosporins, and aztreonam with avibactam significantly lowered MIC values compared to single drugs ($P<0.01$). Significant differences in MIC were noted between low and high avibactam concentrations ($P<0.05$).

Conclusion: CRKP harbouring virulence genes poses significant risks. Combining carbapenems, cephalosporins, and avibactam enhances antibacterial activity against CRKP.

Keywords: *bla*KPC-2 gene, *bla*NDM gene, virulence gene, minimum inhibitory concentration, antibacterial activity, infection

Introduction

Over the past decade, infections caused by multidrug-resistant (MDR) pathogens have shown a significant and sustained upward trend worldwide, with carbapenem-resistant Enterobacteriaceae (CRE) playing a predominant role, particularly in infections caused by carbapenem-resistant *Klebsiella pneumoniae* (CRKP). The widespread distribution of this bacterium in natural environments directly influences its infection frequency in humans.[1] *Klebsiella pneumoniae* is a common opportunistic pathogen that primarily colonizes the human digestive and upper respiratory tracts. According to the statistics of the China Bacterial Resistance Monitoring Network (CHINET, www.chinets.com), the isolation rate of gram-negative bacteria was 21.2% in 2022, ranking second only to *Escherichia coli*. The widespread use of antibiotics has resulted in screening for *K. pneumoniae* strains that are resistant to a variety of antibiotics, including carbapenems. In 2017, the World Health Organization (WHO) released the first list of priority antibiotic-resistant bacterial pathogens and identified carbapenem resistance as a "key priority".[2] The global spread of CRKP is primarily attributed to the widespread dissemination of plasmid-encoded carbapenemase genes, particularly those encoding *K. pneumoniae* carbapenemase (KPC), Verona integron-encoded metallo-beta-lactamase (VIM), imipenemase (IMP), New Delhi metallo-β-lactamase (NDM), and oxacillinase-48 (OXA-48).[3,4] In different countries, distinct carbapenemase-producing CRE strains dominate, with blaKPC and blaNDM being the two primary carbapenemase genes identified in CRE strains from China.[5]

Received: 4 February 2025
Accepted: 3 April 2025
Published: 26 April 2025

The "permeability" of *K. pneumoniae* to mobile genetic elements is a critical factor in its transmission. This not only enables the potential acquisition of antibiotic resistance but also facilitates the uptake of genes that confer survival advantages, thereby driving the evolution of more virulent phenotypes.[6] According to their functions, virulence genes can be divided into the following categories: allantoin metabolism (alls), synthetic fimbriae types 1 and 3 (fimH, mrkD), synthetic lipopolysaccharide (ycfM, wabG), synthetic fucose (wcaG), high viscosity (prmpA, prmpA2, magA), iroN carrier (entB, kfu, iroB, iroN, irp-1, irp-2, iucA, iutA, ytbS), and transport metabolite (peg-344).[7–10] The pathogenic basis of *K. pneumoniae* infection includes the capsule, adhesin, enterocolin, and biofilm formation abilities.[9] This strain contains a wide variety of virulence genes, including fimH, kpn, mrkD, ycfM, entB, irp1, irp2, ybtS, and fyuA.[10] Based on its virulence characteristics, *K. pneumoniae* can be divided into classical *K. pneumoniae* (cKP) and highly virulent *K. pneumoniae* (hvKP). Unlike cKP, hvKP mainly infects healthy individuals in the community and is characterized by multiple infectious foci, rapid disease progression, and poor prognosis, which poses a significant challenge for clinical diagnosis and treatment.[11,12] Virulence and carbapenem-resistance genes are carried on different plasmids.[13] These plasmids carrying drug resistance or virulence genes can form conjugate plasmids with both drug resistance and virulence in bacteria through homologous recombination.[14,15] The presence of conjugated plasmids renders *Klebsiella pneumoniae* highly resistant and toxic and can cause serious infections. The WHO warns that highly virulent superbugs are spreading globally and that the risk of transmission in communities and hospitals will increase.[12] *Klebsiella pneumoniae*, with high virulence and high drug resistance, has successively appeared in different regions of the world, showing outbreak and epidemic trends. This bacterium has been reported in 16 countries and regions, including the United States, China, the United Kingdom, Canada, Japan, India, and Iran. The WHO states that this species is not easy to prevent, spreads easily, and causes infections that are difficult to treat.[12]

Avibactam, a novel beta-lactamase inhibitor, by binding to beta-lactamase, prevents these enzymes from destroying the beta-lactam drug, thereby maintaining its bactericidal effect.[16] Ceftazidime/avibactam has a narrow inhibition spectrum that only inhibits KPC and OXA-48 enzyme activity and is not effective for NDM enzymes.[17] Ceftazidime/avibactam is only suitable for non-metal enzyme-producing Enterobacteriaceae. Because some KPC enzyme-producing strains are also resistant to it, it has not been on the market for long, but its clinical application faces new challenges.[18] Little is known about the antibacterial activity of other β-lactams combined with avibactam against carbapenem-resistant bacteria, except for ceftazidime/avibactam.

This study focused on the evaluation of the antibacterial activity of β-lactam drugs (meropenem, imipenem, ertapenem, ceftazidime, ceftaroline fosamil, and aztreonam) combined with avibactam against CRKP with satisfactory results. The distribution of virulence genes in the strain and the clinical characteristics of infected patients were studied, providing data to support clinical treatment and prevention of disease.

Materials and Methods

Flowchart of This Experiment (Figure 1)

Strain Selection

This study strictly adhered to the ethical principles outlined in the Declaration of Helsinki. The *K. pneumoniae* strains were screened from the strain library of the First Affiliated Hospital of Hebei North University. All experimental procedures complied with the ethical review standards of the Ethics Committee of the First Affiliated Hospital of Hebei North University, which was preserved for non-repeated submission from January 1, 2022, to December 31, 2023. These strains showed resistance to carbapenem antibiotics after identification and susceptibility testing using a BD PhoenixTM 100 automated bacterial identification/susceptibility system (Becton Dickinson and Company, Lake Franklin, NJ, USA).

Patient Clinical Information Collection

Clinical data were collected from patients infected with the CRKP strains, including basic information (name, sex, age, and specimen type); basic diseases, such as chronic obstructive pulmonary disease, diabetes, and hypertension; treatment measures, including tracheotomy, tracheal intubation, central venous catheter insertion, and artificial nutrition; laboratory indicators, including leukocytes, neutrophils, lymphocytes, and C-reactive protein; and prognosis.

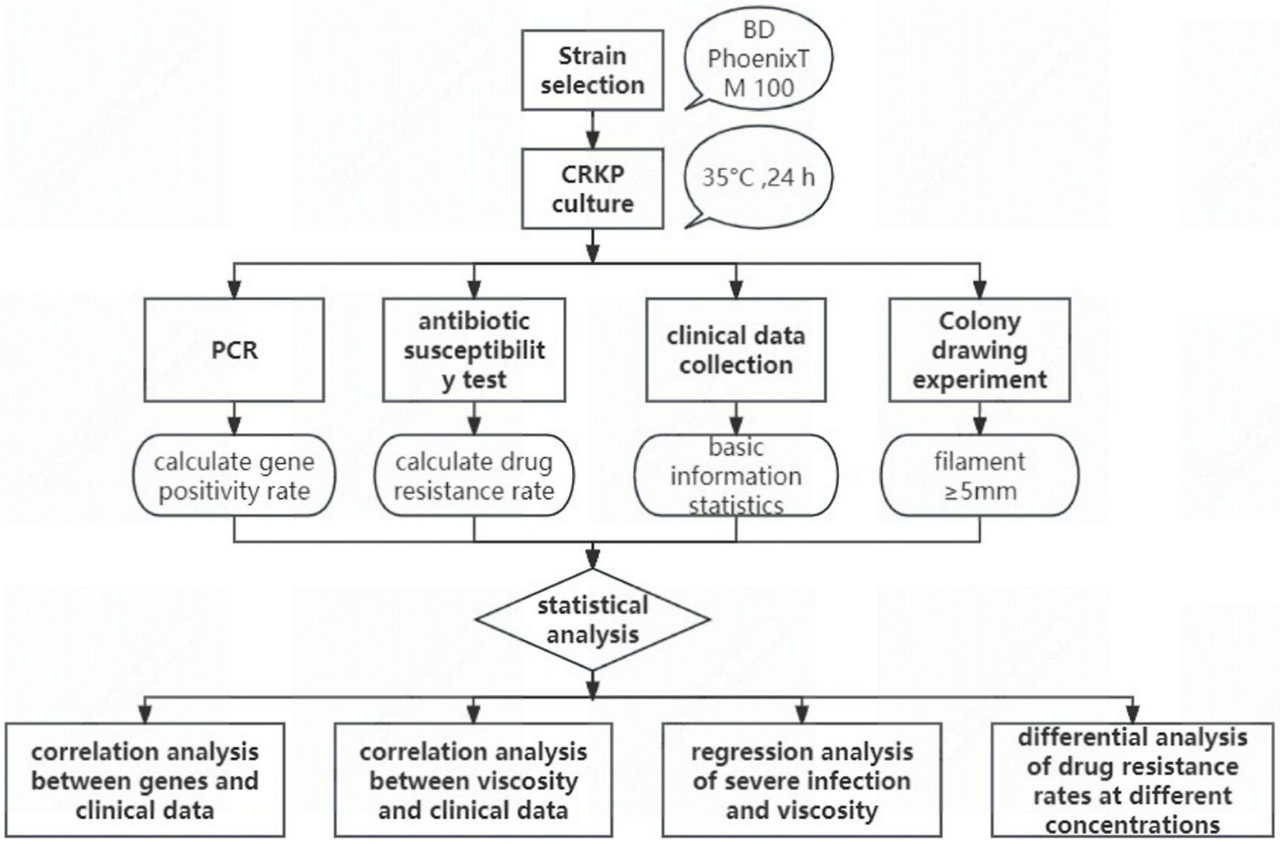

Figure 1 Experimental workflow for analyzing carbapenem-resistant Klebsiella pneumoniae (CRKP). The flowchart summarizes key steps: strain selection (BD Phoenix™ 100), CRKP culture (35°C, 24 h), PCR-based gene detection, antibiotic susceptibility testing, clinical data collection, colony morphology assessment (filamentation ≥5 mm), and statistical analyses (gene-clinical correlations, hypermucoviscosity outcomes, and resistance rate comparisons). Arrows indicate sequential progression.

String Test

The strains were inoculated onto Columbia blood agar plates and incubated at 35 °C for 24 h. Using a sterile inoculation loop, the surface of individual colonies was touched to perform the string test, and this procedure was repeated three times to ensure consistency. If the string formation length was ≥ 5 mm in each repetition, the strain was considered positive for the hypermucoviscous phenotype, indicating a high-mucus phenotype.

Amplification of Carbapenem-Resistance and Virulence Genes

A mung bean-sized colony was placed in a centrifuge tube containing 0.5 mL of ddH_2O and mixed thoroughly by vortexing. Subsequently, the centrifuge tube was placed in a water bath at 100°C and heated for 15 min. After removal, the tube was centrifuged at 6133 × *g* for 5 min using a high-speed centrifuge. The supernatant was collected to extract bacterial genomic DNA. PCR was performed to amplify carbapenem-resistance genes (blaKPC-2, blaVIM, blaNDM, blaIMP, and blaOXA-48), porin genes (OMPK35 and OMPK36), and virulence genes (prmpA, prmpA2, magA, fimH, ycfm, wcaG, entB, kfu, iroB, irp-1, iucA, iutA, and ytbS). The design and optimization of the experimental protocol were independently completed by our laboratory.All primers were designed and validated using the website "https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome" and synthesized by Beijing Ruibo Xingke Biotechnology Co., Ltd. The PCR reaction mixture (25 μL) consisted of 9.5 μL of deionized water (Solarbio, China), 12.5 μL of 2× Rapid Taq Master Mix (Vazyme, China), 1 μL of forward primer, 1 μL of reverse primer, and 1 μL of DNA template. These components were added sequentially into a PCR tube, mixed thoroughly by vortexing, and brought to a final volume of 25 μL. Amplification was carried out using a thermal cycler. The primer sequences, annealing temperatures, and other related information are presented in the tables (Tables 1 and 2).

Table 1 Primer Sequence, Product Length and Annealing Temperature of Drug Resistance Gene of CRKP Strain

Gene	Primer	Sequence (5′-3′)	Sequence Length (bp)	Annealing Temperature (°C)
KPC	KPC-F	ATGTCACTGTATCGCCGTCT	893	55.1
	KPC-R	TTTTCAGAGCCTTACTGCCC		
OXA-48	OXA-48-F	GCGTGGTTAAGGATGAACAC	438	55
	OXA-48-R	CATCAAGTTCAACCCAACCG		
NDM	NDM-F	GCAGCTTGTCGGCCATGCGGGC	782	72
	NDM-R	GGTCGCGAAGCTGAGCACCGCAT		
IMP	IMP-F	GAAGGCGTTTATGTTCATAC	587	50.1
	IMP-R	GTACGTTTCAAGAGTGATGC		
VIM	VIM-F	ATGGTGTTTGGTCGCATATC	510	55
	VIM-R	TGGGCCATTCAGCCAGATC		
Ompk36	OmpK36-F	GGGAAGAATCGCACGAAATA	582	57.9
	OmpK36-R	TCTTACCAGGGCGACAAGAG		
Ompk35	OmpK35-F	GGATGGAAAGATGCCTTCAG	516	57
	OmpK35-R	CATGACGAGGTTCCATTGTG		

Table 2 Primer Sequence, Product Length and Annealing Temperature of Virulence Gene of CRKP Strain

Gene	Primer	Sequence (5′-3′)	Product Size (bp)	Annealing Temperature (°C)
entB	entB-F	GTCAACTGGGCCTTTGAGCCGTC	Four hundred	57
	entB-R	TATGGGCGTAAACGCCGGTGAT		
fimH	fimH-F	TGCTGCTGGGCTGGTCGATG	550	55
	fimH-R	GGGAGGGTGACGGTGACATC		
iroB	iroB-F	ATCTCATCATCTACCCTCCGCTC	235	59
	iroB-R	GGTTCGCCGTCGTTTTCAA		
irp-1	irp-1-F	TGAATCGCGGGTGTCTTATGC	238	57
	irp-1-R	TCCCTCAATAAAGCCCACGCT		
iucA	iucA-F	GCTTATTTCTCCCCAACCC	583	59
	iucA-R	TCAGCCCTTTAGCGACAAG		
iutA	iutA-F	GGGAAAGGCTTCTCTGCCAT	920	63
	iutA-R	TTATTCGCCACCACGCTCTT		
kfu	kfu-F	GAAGTGACGCTGTTTCTGGC	797	58
	kfu-R	TTTCGTGTGGCCAGTGACTC		
magA	magA-F	GGTGCTCTTTACATCATTGC	1280	53
	magA-R	GCAATGGCCATTTGCGTTAG		
prmpA	prmpA-F	GAGTAGTTAATAAATCAATAGCAAT	332	50
	prmpA-R	CAGTAGGCATTGCAGCA		
prmpA2	prmpA2-F	GTGCAATAAGGATGTTACATTA	430	50
	prmpA2-R	GGATGCCCTCCTCCTG		
wcaG	wcaG-F	GGTTGGKTCAGCAATCGTA	169	65
	wcaG-R	ACTATTCCGCCAACTTTTGC		
ytbs	ytbS-F	GACGGAAACAGCACGGTAAA	242	50
	ytbS-R	GAGCATAATAAGGCGAAAGA		
ycfM	ycfM-F	ATCAGCAGTCGGGTCAGC	160	55
	ycfM-R	CTTCTCCAGCATTCAGCG		

In vitro Drug Sensitivity Test of Novel Compatibility

To conduct resistance experiments for new drugs, such as ceftazidime/avibactam, we prepared a drug-sensitive plate using the trace broth dilution method for additional experiments. A 96-well drug-sensitive plate was configured according to CLSI M07-A11. Ertapenem, imipenem, meropenem, ceftaroline fosamil, ceftazidime, and aztreonam alone, and their combinations with avibactam were prepared using the same drug concentration gradient (0.125–128 mg/mL). The concentrations of avibactam were low (4 μg/mL) and high (8 μg/mL). Single tigecycline and polymyxin medicines were prepared simultaneously.

The bacterial strains were inoculated onto Columbia agar and incubated at 35 °C for 24 h. The bacterial suspension was prepared as a 0.5 M turbidity bacterial suspension, diluted 100-fold in nutrient broth, mixed, and transferred to a 96-well drug-sensitive plate using a porous pipette; 100 μL was added to each well, and the results were read after incubation at 35 °C for 24 h. *Escherichia coli* ATCC25922 was used as the control strain. The sensitivity to polymyxin was determined according to the European Commission for Antimicrobial Susceptibility Testing (EUCAST) breakpoints, whereas that of tigecycline was determined according to the United States Food and Drug Administration (FDA) criteria. The other antibiotics followed the breakpoints of the Clinical and Laboratory Standards Institute.

Statistics

SPSS 27.0 statistical software was used for data analysis. Qualitative data were analyzed using the chi-square test and one-way logistic regression analysis, whereas quantitative data were analyzed using variance and correlation analysis. Differences were considered statistically significant at $P < 0.05$.

Results

Patient Clinical Information

Eighty patients with CRKP infection were included in this study. Among them, 73.5% were men and 26.5% were women. Patients aged 61–80 years accounted for 52.5% of the study population. Diabetes, hypertension, and COPD were comorbidities for 30%, 41.3%, and 8.8% of patients, respectively. Central venous catheterization and mechanical ventilation were performed in 56.3% and 66.3% of the patients, respectively. Intensive Care Unit (ICU) patients accounted for 51.8% of all patients, and patients with pneumonia accounted for 60.2%. The mortality rate was 38.8% (Figure 2 and Table 3).

An odds ratio (OR) of 5.299 (95% CI: 1.458–19.251) for severe infections (including bacteraemia, sepsis, liver abscess, intracranial infection, and septic shock) was an independent risk factor for infection with a highly viscous strain of CRKP. The male OR value of 0.229 (95% CI: 0.061–0.852) and artificial nutrition OR value of 0.049 (95% CI: 0.002–0.971) were protective factors against infection with highly viscous CRKP strains (Figure 3).

Drug Resistance and Virulence Genes

Of the 80 CRKP resistance-related genes, 75 (93.8%) were *bla*KPC-2 positive and 1 (1.3%) was *bla*NDM-positive. The *bla*VIM, *bla*IMP, and *bla*OXA-48 genes were not detected. Seventy-five strains (93.8%) were positive for the membrane pore protein gene OMPK35 and 72 strains (90%) were positive for OMPK36. The results of the virulence gene test showed that 75 strains were positive for ycfM (93.8%), 75 strains were positive for entB (93.8%), 74 strains were positive for fimH (92.5%), 71 strains were positive for irp-1 (88.8%), 38 strains were positive for prmpA2 (47.5%), 38 strains were positive for iucA (47.5%), 37 strains were positive for iuctA (46.3%), 28 strains were positive for kfu (35%), 16 strains were positive for prmpA (20%), and 5 strains were positive for iroB 6.3% (Figure 4).

Twenty-five strains (31.3%) harboured fimH, ycfM, entB, kfu, and irp-1 simultaneously; 17 (21.3%) harboured fimH, ycfM, prmpA2, iucA, iutA, entB, and irp-1 simultaneously; and 10 (12.5%) harboured ycfM, entB, fimH, irp-1, prmpA2, iucA, iuctA, and prmpA simultaneously (Figure 5).

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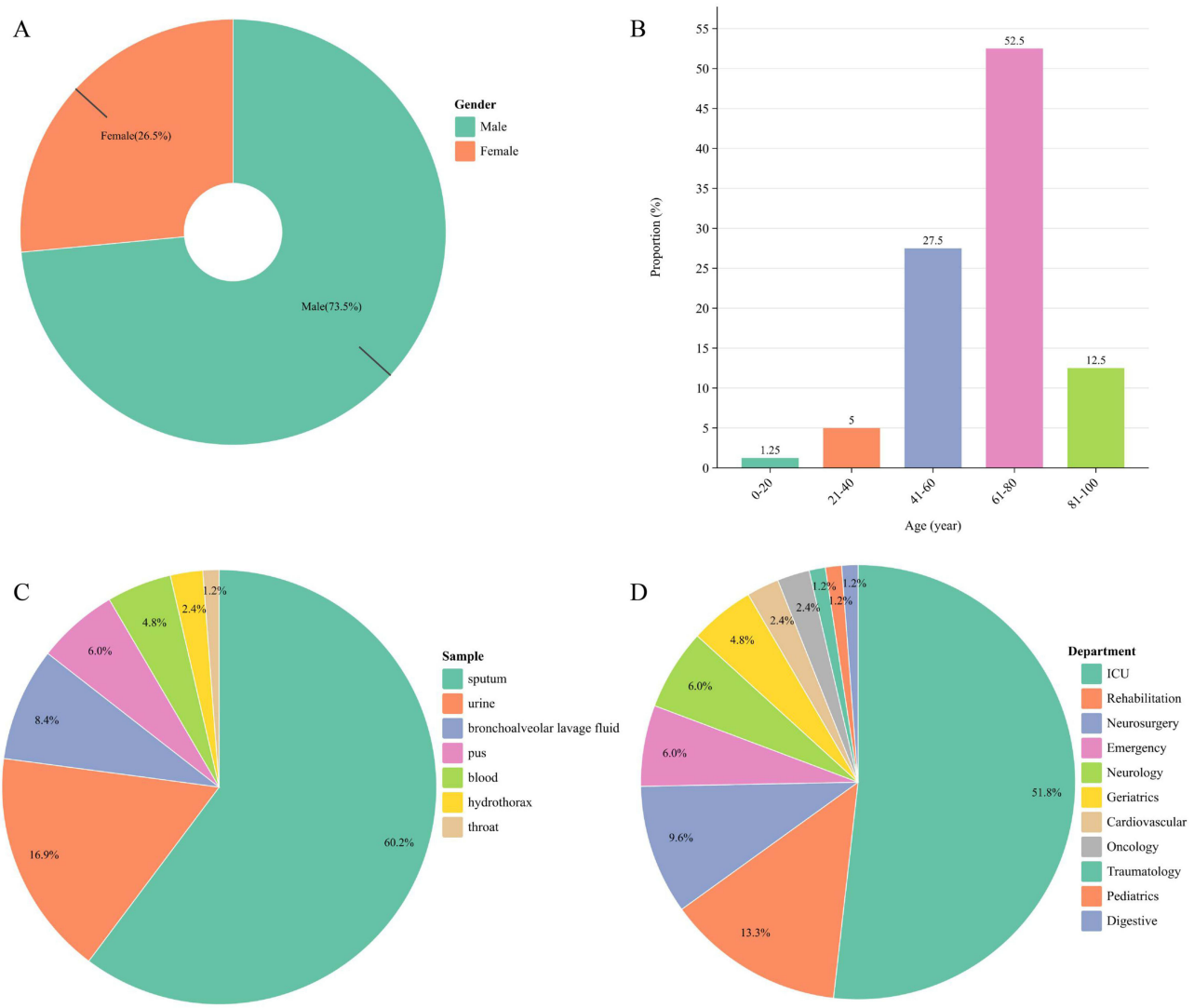

Figure 2 Clinical characteristics of patients with CRKP infection. Sex ratio (**A**), age distribution (**B**), sample type (**C**), and department (**D**).

Correlation Analysis Between Bacterial Strain Genes and Clinical Data of Patients

Through gene detection results and clinical data collection, the correlation analysis between the two parts of data showed that the *bla*KPC-2 gene was positively correlated with the percentage of neutrophils (NE) ($P<0.05$) and negatively correlated with artificial nutrition (ANH), central venous catheterization (CVC), and mechanical ventilation (MV) ($P<0.01$). The virulence gene prmpA was positively correlated with respiratory frequency ($P<0.05$); iroB was positively

Table 3 Basic Information of CRKP Infected Patients

Basic Information [Median (IQR)]		Basic Disease [n (%)]	
Age	69.5 (20)	Diabetes	24 (30)
Length of stay (day)	29 (38)	Hypertension	33 (41.3)
		COPD	7 (8.8)
Invasive medical treatment [n(%)]		Clinical outcome [n(%)]	
Central venous catheterization	45 (56.3)	Improve	49 (61.2)
Mechanical ventilation	53 (66.3)	Die	31 (38.8)

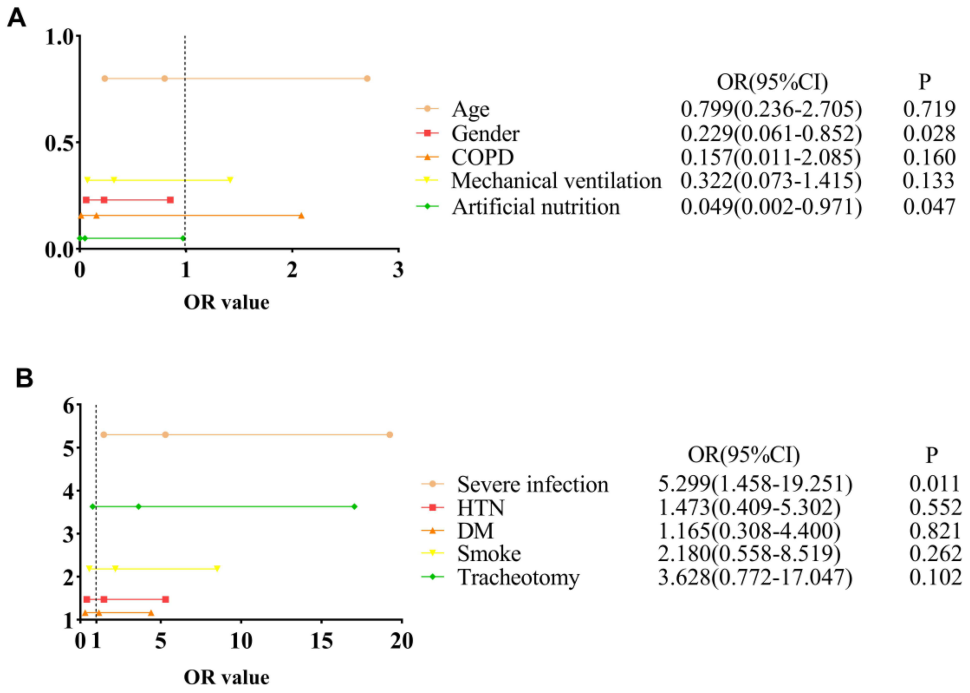

Figure 3 Univariate logistic regression analysis shows the relationship among age, sex, chronic obstructive pulmonary disease (COPD), mechanical ventilation, artificial nutrition, and viscosity of different strains (**A**), and the relationship between severe infection, hypertension (HTN), diabetes (DM), smoking, and tracheotomy, and viscosity of different strains (**B**). The left and right ends of the line represent the lower and upper limits of the 95% confidence interval, respectively; the middle point of the line represents the OR value, the line on the right side of 1 represents the risk factor for high viscosity of the strain, and the line on the left side of 1 represents the protective factor for high viscosity of the strain.

correlated with hypertension (HTN) and white blood cell count (WBC) (P<0.05); and iucA, prmpA2, and iutA were positively correlated with diabetes mellitus (DM) (P<0.05) (Figure 6).

Correlation Analysis Between Bacterial Adhesion and Clinical Data of Patients

Of the 80 CRKP strains, 25 (31.25%) showed high adhesion, and 55 (68.75%) showed low adhesion. Among the high-adhesion strains, 14 (56%) carried prmpA, 22 (88%) prmpA2, 21 (84%) iucA, 21 (84%) iutA, and 1 (4%) kfu. The adhesion performances of five genes—prmpA, prmpA2, iucA, iutA, and kfu—were significantly different (P<0.01) (Table 4).

In vitro Drug Sensitivity Test

Of the 80 CRKP strains, 39 (48.8%) were fully resistant to aztreonam, ceftazidime, meropenem, imipenem, ertapenem, polymyxin, and tigecycline. Fifteen strains (18.8%) were fully resistant to aztreonam, ceftazidime, meropenem, imipenem, ertapenem, and tigecycline. Fourteen strains (17.5%) were fully resistant to aztreonam, ceftazidime, meropenem, imipenem, ertapenem, and polymyxin. Seven strains (8.8%) were fully resistant to aztreonam, ceftazidime, meropenem, imipenem, and ertapenem. Furthermore, all strains were resistant to ceftaroline fosamil (Figure 7).

The in vitro antibacterial activity of β-lactam antibiotics (including aztreonam, ceftazidime, meropenem, imipenem, and ertapenem) against CRKP was significantly superior to that of the single drug after combination with avibactam (P<0.05) (Figure 8).

The minimum inhibitory concentration (MIC) of carbapenems (meropenem, imipenem, and ertapenem), cephalosporins (ceftazidime and ceftaroline fosamil), and aztreonam combined with avibactam were significantly lower than those of the individual drugs (P<0.01). MIC values were not significantly different between meropenem/avibactam 4 mg/mL and meropenem/avibactam 8 mg/mL (12.1 vs 10.0), imipenem/avibactam 4 mg/mL, and imipenem/avibactam 8 mg/mL (17.6 vs 12.6), and between ertapenem/avibactam 4 mg/mL and ertapenem/avibactam 8 mg/mL (22.5 vs 15.2; P>0.05). The MIC values of aztreonam/avibactam 4 mg/mL vs aztreonam/avibactam 8 mg/mL (31.1 vs 17.6), ceftazidime/

https://doi.org/10.2147/IDR.S515858

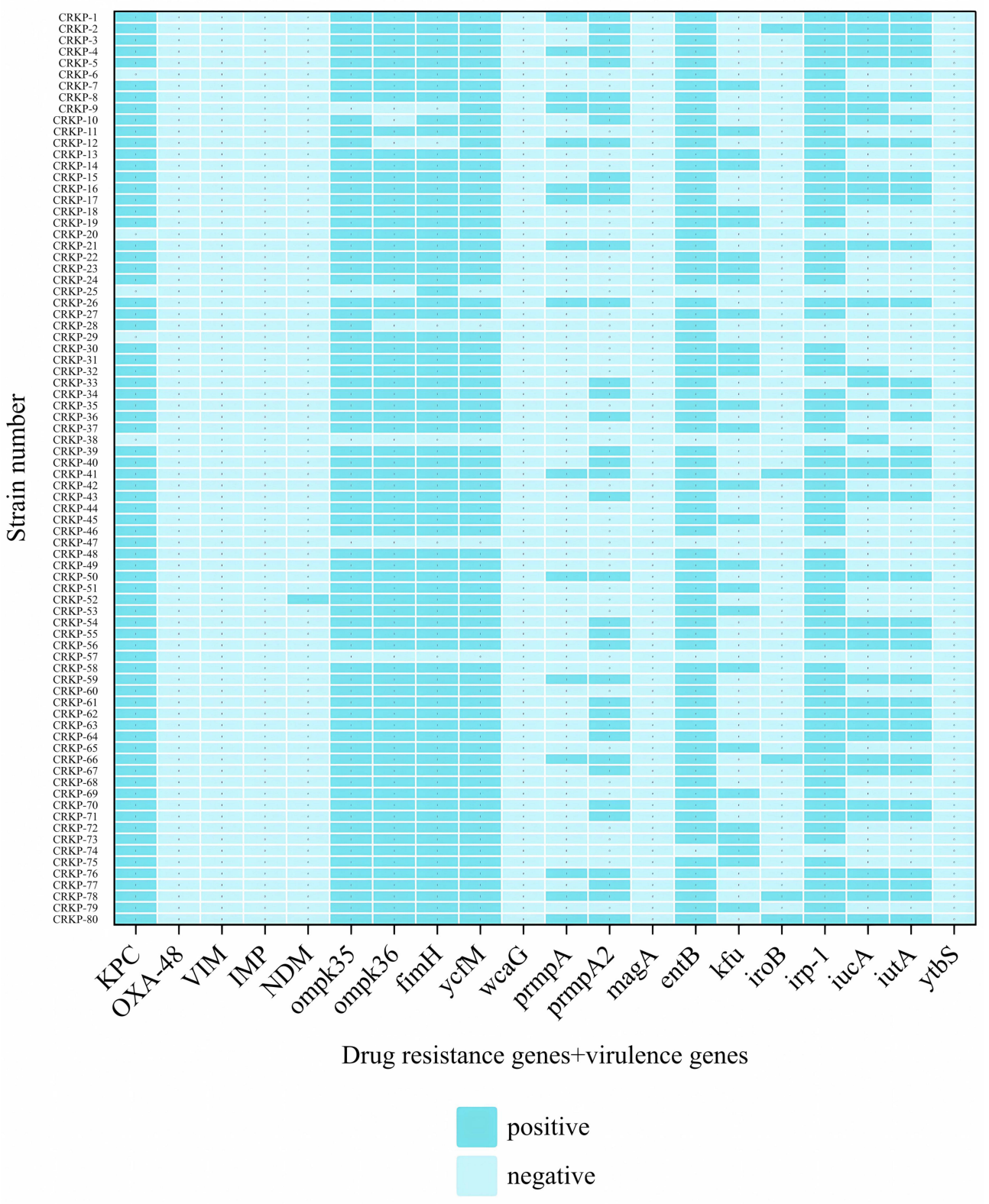

Figure 4 Drug resistance genes (*bla*KPC-2, *bla*VIM, *bla*NDM, *bla*IMP, *bla*OXA-48), porin genes (OMPK35, OMPK36), and virulence genes (prmpA, prmpA2, magA, fimH, ycfm, wcaG, entB, kfu, iroB).

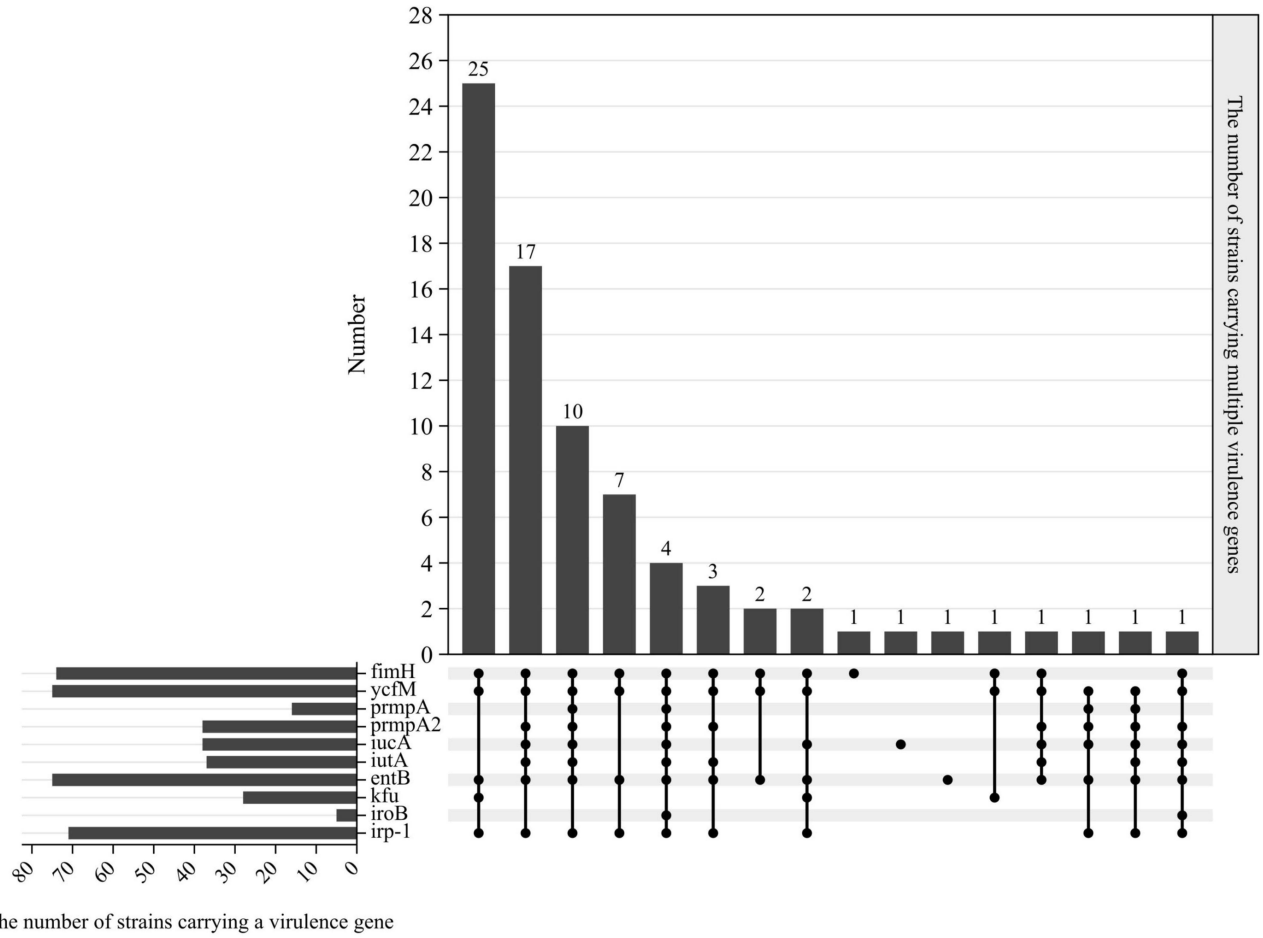

Figure 5 Virulence gene upset diagram shows 80 strains of CRKP carrying each virulence gene (bar on the left) and the number of strains carrying some virulence gene combinations simultaneously (bar on the right). The horizontal axis on the right shows the combination of virulence genes and the vertical axis shows the number of corresponding samples in each combination. Dotted lines represent the intersection of independent genes and histograms represent the frequency of each combination.

avibactam 4 mg/mL vs ceftazidime/avibactam 8 mg/mL (116 vs 104), ceftaroline fosamil/avibactam 4 mg/mL vs ceftaroline fosamil/avibactam 8 mg/mL (15.8 vs 7.3) were significantly different (P<0.05; Figure 9).

Discussion

CRKP is rapidly spreading worldwide, with 37% (16/43) of countries reporting its detection according to World Health Organization (WHO) statistics. The prevalence of CRKP is not limited to specific regions; its widespread distribution, coupled with limitations in detection techniques and challenges in tracking its spread, suggests that its actual prevalence may be significantly underestimated.[19,20] The high mortality rate associated with CRKP infections (38.8% in this study) and its resistance to multiple antibiotics make it a major threat to global public health. In this study, we conducted a comprehensive analysis of clinical data, strain genetic characteristics, and drug resistance profiles from 80 CRKP-infected patients, revealing the distribution of resistance genes, prevalence of virulence genes, and their correlation with clinical outcomes in this region. These findings provide a scientific basis for the development of clinical treatment and infection control strategies.

In our study, More than half of the enrolled patients were male, aged > 60 years, admitted to the ICU, or had undergone invasive medical procedures. Ultimately, 38.8% of the patients died. Mortality rates reported in studies on patients with CRKP infection in North America, South America, Europe, and Asia were 33.24%, 46.71%, 50.06%, and 44.82%, respectively.[19] The limited choice of antibiotics may be an important cause of the increased mortality in patients with CRKP infections.

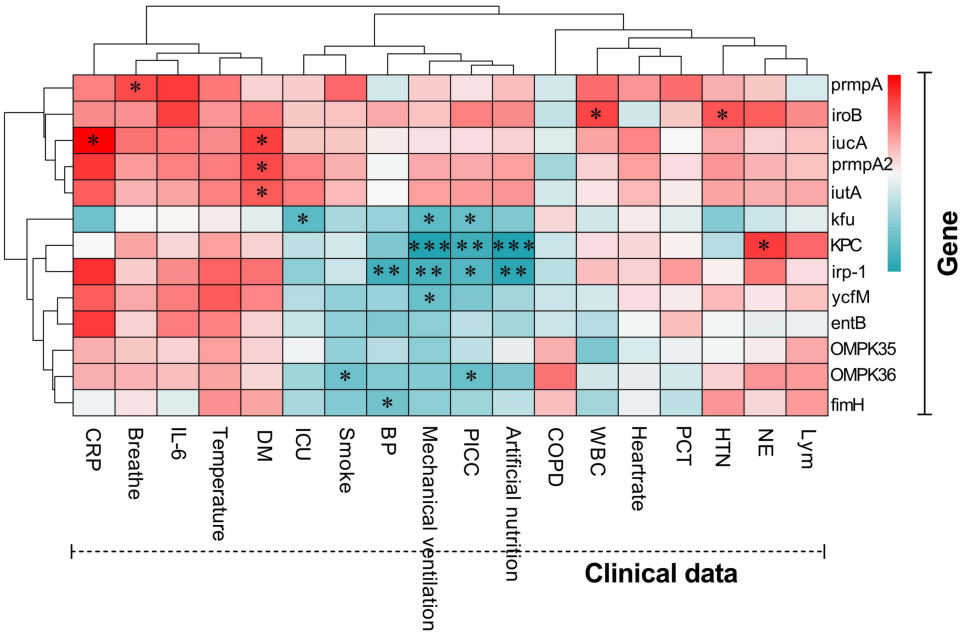

Figure 6 Correlation analysis of clinical indicators, basic diseases, invasive procedures, and genes carried by the CRKP strain. Red indicates a positive correlation, blue indicates a negative correlation, and darker colours indicate a stronger correlation. *P<0.05, **P<0.01, and ***P<0.001.

In the present study, 93.8% of the isolates harboured the *bla*KPC-2 gene, Confirming its dominance in this region. Notably, 1.3% of strains carried both *bla*KPC-2 and *bla*NDM resistance genes, indicating the potential for co-existence of multiple resistance mechanisms.[21,22] Such strains pose a significant challenge to clinical treatment, as they may exhibit

Table 4 χ^2 Test Results of CRKP Bacterial Strain Viscosity

Virulence Gene	Wire Drawing Experiment Result	CRKP Viscosity (%)		Total	χ^2	*p*
		H	L			
fimH	+	22(88.00)	52(94.55)	74(92.50)	0.328	0.567
	−	3(12.00)	3(5.45)	6(7.50)		
ycfM	+	24(96.00)	51(92.73)	75(93.75)	0.004	0.95
	−	1(4.00)	4(7.27)	5(6.25)		
prmpA	+	14(56.00)	2(3.64)	16(20.00)	29.455	<0.01
	−	11(44.00)	53(96.36)	64(80.00)		
prmpA2	+	22(88.00)	16(29.09)	38(47.50)	23.918	<0.01
	−	3(12.00)	39(70.91)	42(52.50)		
entB	+	24(96.00)	51(92.73)	75(93.75)	0.004	0.95
	−	1(4.00)	4(7.27)	5(6.25)		
kfu	+	1(4.00)	27(49.09)	28(35.00)	15.361	<0.01
	−	24(96.00)	28(50.91)	52(65.00)		
iroB	+	2(8.00)	3(5.45)	5(6.25)	0.004	0.95
	−	23(92.00)	52(94.55)	75(93.75)		
irp-1	+	23(92.00)	48(87.27)	71(88.75)	0.057	0.811
	−	2(8.00)	7(12.73)	9(11.25)		
iucA	+	21(84.00)	17(30.91)	38(47.50)	19.427	<0.01
	−	4(16.00)	38(69.09)	42(52.50)		
iutA	+	21(84.00)	16(29.09)	37(46.25)	20.845	<0.01
	−	4(16.00)	39(70.91)	43(53.75)		
Total		25	55	80		

Notes: H, High-viscosity; L, Low-viscosity; +, Positive; −, Negative.

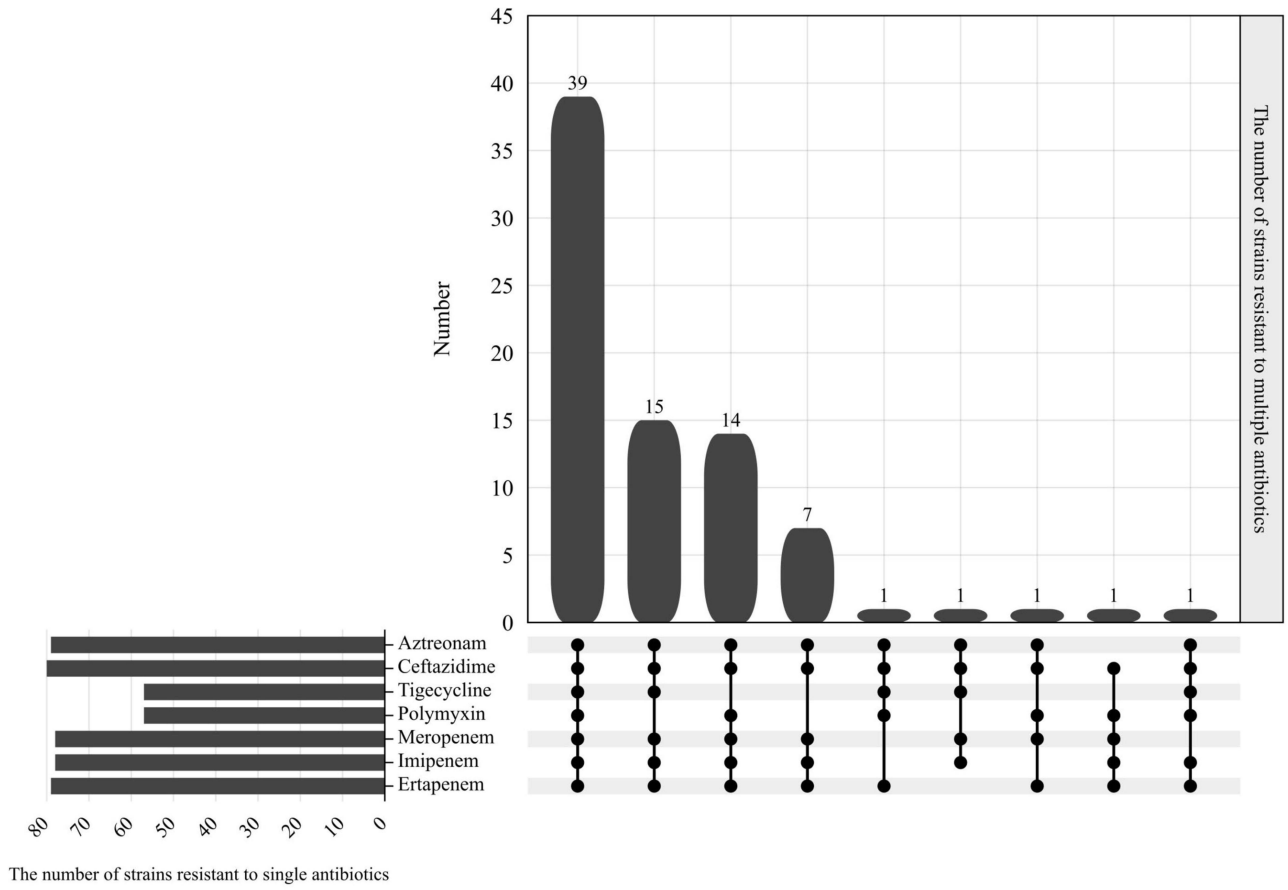

Figure 7 Upset diagram shows the number of single-drug-resistant strains (bar on the left) and multi-drug-resistant strains (bar on the right). The horizontal axis on the right represents each drug combination and the vertical axis represents the number of corresponding samples in each combination. Dotted lines indicate the intersection of drugs and bar charts indicate the frequency of each combination.

broader resistance profiles and higher transmission potential. Meanwhile, 6.3% (OMPK35) and 10% lacked membrane pore protein gene (OMPK36). This suggests that some CRKP strains may enhance their resistance through the loss of porin proteins, as the reduction or absence of porins can restrict the entry of antibiotics into bacterial cells, thereby reducing the effectiveness of antibiotics.[23] The percentages of the CRKP resistance genes *bla*KPC-2, *bla*NDM, *bla*OXA-48, and *bla*IMP in 36 hospitals in 24 provinces in China were 64.5%, 21.1%, 9.6%, and 1.2%, respectively.[24] The large sample size and geographical coverage of this study demonstrated the distribution of CRKP in different regions and populations. This study showed that the prevalent CRKP genotypes in our region were geographically concentrated and mainly originated in the ICU, which may be because of the nosocomial epidemic of the strain.

In this study, the positivity rate of ycfM, entB, and fimH was over 90%, and the co-existence probability of fimH, ycfM, entB, kfu, and irp-1 was over 30%, indicating that ycfM, entB, and fimH have epidemic advantages in this region. These virulence genes are carried by the IncHI1B plasmid,[13] which is a repository for genes transferred between animals and humans,[25] further increasing the risk of their spread. However, 10% of strains lacked these genes, potentially representing a distinct subgroup with different clinical characteristics. These strains may exhibit altered pathogenicity or resistance profiles, thereby influencing patient outcomes. Additionally, whether the high prevalence of virulence genes is associated with antibiotic resistance remains to be thoroughly investigated to fully understand the transmission mechanisms and pathogenicity of CRKP.

Avibactam is an enzyme inhibitor belonging to triethylenediamine (DABCOs) and does not have a β-amine ring structure; therefore, it is not easy to hydrolyze, has a broad-spectrum β-lactamase inhibition effect, a reversible enzyme inhibition effect, and can inhibit A-type and C-type β -lactamases, including carbapenemase. It also inhibits OXA-48 in

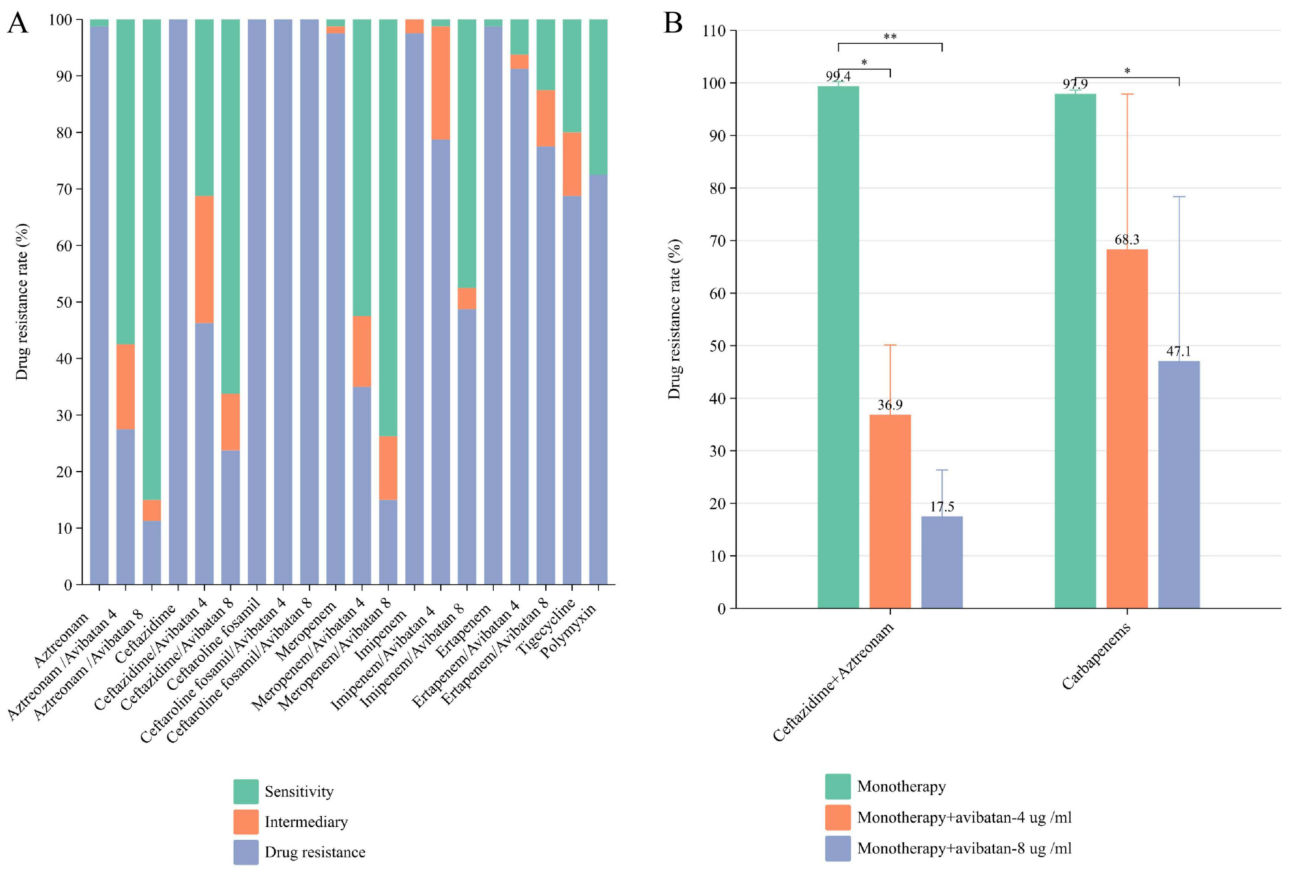

Figure 8 Sensitivity to various antibiotics (**A**). The difference in drug resistance rate before and after β-lactams combined with avibactam (**B**). *P<0.05, **P<0.01. The percentage bar stacking chart depicts the resistance, intermediary, and sensitivity rates of the 80 CRKP samples to each drug alone or in combination with different concentrations of avibactam. Each bar represents a drug, and the lengths of the different colours in each bar represent the percentages of drug-resistant, intermediate, and sensitive strains in the drug sensitivity test (**A**).

Class D enzymes. In this study, the combination of β-lactams and avibactam significantly reduced the MIC when compared to the individual agents. Furthermore, there was a significant difference ($p<0.05$) between the high concentration of avibactam (8 mg/mL) and the low concentration of avibactam (4 mg/mL). Ceftazidime/avibactam is approved for listing in the United States, Europe, Asia, and other countries. Ceftazidime-avibactam is an important tool against carbapenem-resistant Enterobacteriaceae infections. Most multidrug-resistant *Klebsiella* strains (96% of 2821) were susceptible to ceftazidime/avibactam.[26] The mechanism of CRKP resistance to ceftazidime/avibactam mainly involves the insertion and deletion of mutations in *bla*KPC-2.[27]

The MICs of aztreonam, aztreonam/avibactam −4 mg/mL, and aztreonam/avibactam −8 mg/mL against the *bla*NDM-carrying strains were 4 mg/mL, 0.25 mg/mL, and 0.25 mg/mL, respectively, and were determined to be "sensitive." Ceftazidime, ceftaroline fosamil, imipenem, meropenem, ertapenem, and abactam had no antibacterial activity (MIC > 128 mg/mL). Seven strains of CRKP found in China, harbouring both *bla*KPC-2 and *bla*NDM, were sensitive to aztreonam/avibactam.[28] The antibacterial activity of aztreonam/avibactam against the *bla*NDM genotype strain was as high as 80.95%.[29] It was deduced that aztreonam/avibactam is a good antibacterial agent against the *bla*NDM genotype, including strains carrying both *bla*NDM and *bla*KPC-2. The MIC of imipenem/avibactam (4 mg/L) versus imipenem alone against the MIC90 of CRKP was reduced to 1/16.[30] The synergistic rate of the combined application of meropenem/avibactam (4 mg/L) and polymyxin B against CRKP was 83.3%.[31] These experiments proved that avibactam is an effective inhibitor of carbapenemase and that its effect can be significantly enhanced by increasing its concentration.

The hypermucoviscous phenotype, as indicated by a positive string test, has been widely associated with enhanced biofilm formation and adhesion capabilities in *K. pneumoniae*.[32–34] These properties may facilitate bacterial colonization

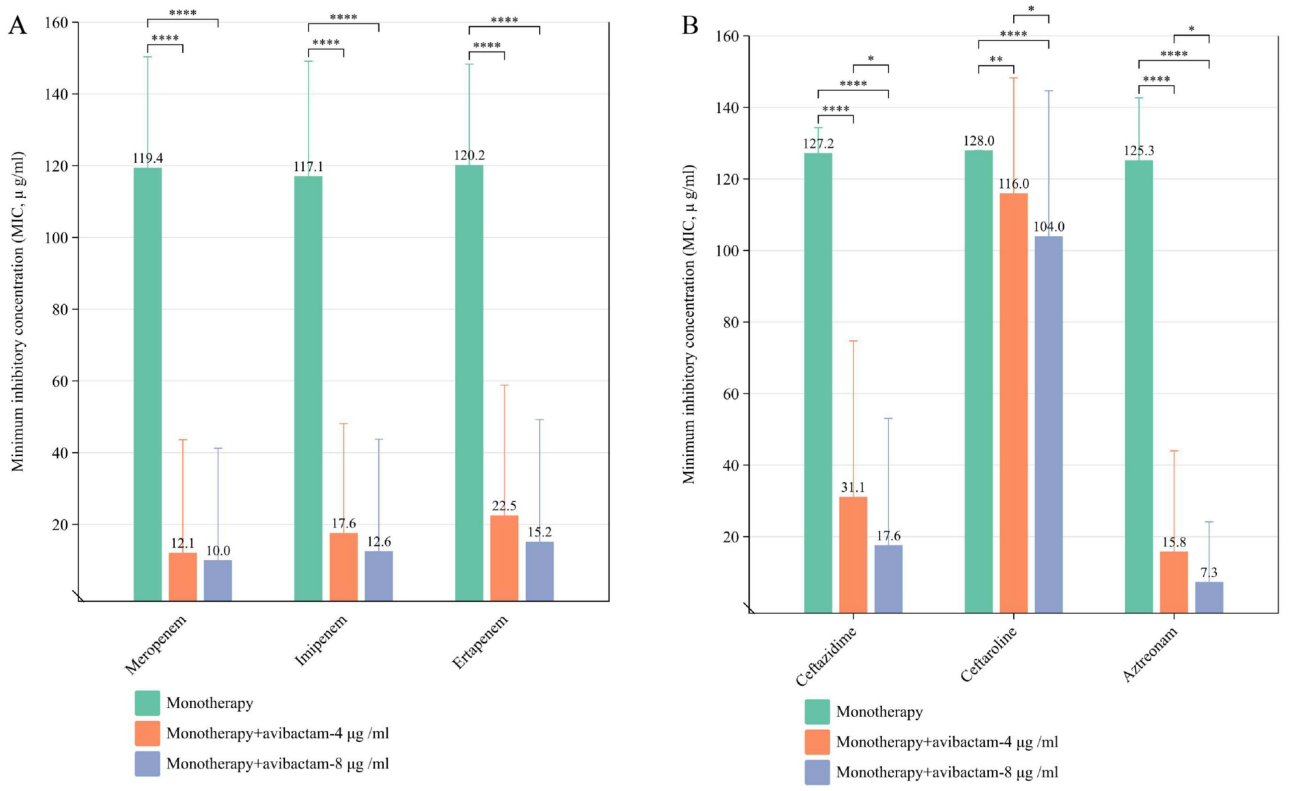

Figure 9 Changes in minimum inhibitory concentration of β-lactams combined with low concentration (4 mg/mL) and high concentration (8 mg/mL) of avibactam. Carbapenems (**A**). Ceftazidime, cefflorraine, and aztreonam (**B**). *P<0.05, **P<0.01, and ****P<0.0001.

and persistence in host tissues, contributing to the severity of infections. In this study, 31.25% (25/80) of the CRKP strains exhibited high adhesion, indicating that a significant proportion of these strains possess enhanced colonization and infection potential. This may contribute to their persistent presence in clinical settings and the challenges associated with their treatment.Previous studies have demonstrated that prmpA and prmpA2 are associated with the hypermucoviscous phenotype, which enhances bacterial adhesion and immune evasion capabilities.[35] In this study, among the high-adhesion strains, the prevalence of virulence genes prmpA, prmpA2, iucA, and iutA was significantly higher, with notable differences observed between strains of varying adhesion levels ($P < 0.05$). This suggests that these genes play a crucial role in bacterial adhesion. Additionally, iucA and iutA are involved in iron acquisition, a key factor for bacterial survival and proliferation in host tissues. The high prevalence of these genes in high-adhesion strains suggests a potential synergistic relationship between adhesion and virulence,[36] potentially leading to more severe clinical outcomes. Notably, the kfu gene was rarely detected in high-adhesion strains (4%), indicating its limited contribution to adhesion. This finding aligns with a previous study.[37] In our study, we found that severe infections are an independent risk factor for high-adhesion strains, and a strong correlation exists between high adhesion and specific virulence genes. This indirectly suggests an important link between high adhesion, high virulence, and specific genes, which may enhance bacterial pathogenicity through complex regulatory networks. However, due to technical limitations, this explanation remains incomplete. Additionally, the high-mucus phenotype has been suggested to play a role in antibiotic resistance by promoting the formation of biofilms, which can act as a physical barrier to drug penetration.[32] While our study did not directly investigate the relationship between viscosity and resistance, the presence of hypermucoviscous strains in our cohort suggests a potential link that warrants further exploration.

The limitations of this study include its restricted sample size and coverage, as it was a single-center study, and it did not fully elucidate the relationship between virulence genes and antibiotic resistance. In future research, we will expand the sample size and scope, incorporating multicenter collaborations. By integrating whole-genome sequencing

technology, we aim to analyze chromosomal and plasmid genetic information in depth, clarify the association between virulence genes and resistance, and uncover the molecular evolution and transmission patterns of CRKP.

Conclusion

This study demonstrates that the blaKPC-2 gene is the predominant resistance gene among CRKP strains in this region. Strains co-harboring blaKPC-2 and blaNDM resistance genes exhibit broad-spectrum resistance, complicating clinical treatment. Significant variations were observed in the presence of virulence genes—prmpA, prmpA2, iucA, iutA, and kfu—among strains with different mucoid phenotypes. The high prevalence of ycfM, entB, and fimH in local strains likely enhances bacterial colonization and pathogenicity, while the absence of these genes in a subset of strains may represent a distinct subgroup with different clinical characteristics. The use of avibactam significantly improved the antibacterial activity of β-lactam drugs, particularly at higher concentrations.

Abbreviations

CRKP, carbapenem-resistant *Klebsiella pneumoniae*; MIC, minimum inhibitory concentration.

Data Sharing Statement

Experimental data related to this study are available from the corresponding author upon reasonable request.

Ethics Approval and Consent to Participate

The study was reviewed and approved by the Ethics Committee of the First Affiliated Hospital of Hebei North University (Ethics Approval Number: K2019147, Approval Date: December 12, 2018). This research is a retrospective clinical study, and the data involved were sourced from previous medical records, which were de-identified during the processing. After review by the Ethics Committee of the First Affiliated Hospital of Hebei North University, it was confirmed that the study posed no additional risk to participants and complied with ethical standards; therefore, it was exempted from obtaining written informed consent.

Acknowledgments

We thank The Hebei North University for their approval of this study. We also appreciate the English language editing services provided by Editage (www.editage.cn).

Author Contributions

Jianliang Chang designed the research methodology and participated in the writing and editing of the manuscript. Xiaocui Peng and Xue Wang contributed to the execution of some experiments and were involved in data curation. Zhihua Zhang secured funding support. All authors made significant contributions to the reported work, whether in the conception, research design, execution, data acquisition, analysis, and interpretation, or in all of these areas; participated in the drafting, revision, or critical review of the article; approved the final version for publication; agreed to submit the manuscript to the journal; and agreed to be accountable for all aspects of the work.

Funding

This study was supported by the Natural Science Foundation of Hebei Province [grant number C2022405023]; 2024 hebei Province Master's Students Innovation Ability Training Program [grant number CXZZSS2024126]; and the government-funded Clinical Medicine Outstanding Talent Training Project [grant numbers ZF2024224 and ZF2025274].

Disclosure

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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