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Molecular epidemiological analysis and research on resistance and virulence of carbapenem-resistant *Klebsiella pneumoniae* in a tertiary hospital from 2016 to 2023

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Abstract

Purpose Carbapenem-resistant *Klebsiella pneumoniae* (CRKP) represents a significant global threat due to its high prevalence rates and limited therapeutic options. The primary objective of this study was to investigate the clinical distribution and molecular epidemiology of CRKP collected between 2016 and 2023 from a tertiary care hospital in northern China.

Methods Polymerase chain reaction (PCR) assays were used to identify resistance and virulence genes, while various assessments, including the string test and biofilm formation analysis, assessed CRKP's virulence. Multilocus sequence typing (MLST) and whole-genome sequencing were employed to elucidate strain classification and plasmid characteristics.

Results The study identified 100 unique CRKP strains, primarily isolated from neurosurgery and ICU, with sputum as the most common specimen type. The majority of strains harbored bla_{KPC-2} as the primary resistance mechanism. All CRKP strains harbored a minimum of four virulence genes, with *entB*, *mrkD*, *fimH*, and *ybtS* being most commonly detected across the isolates. Notably, 66 of 100 strains were classified as carbapenem-resistant hypervirulent *Klebsiella pneumoniae* (CR-hvKP). The prevailing sequence type (ST) observed was ST11, with serotype KL47 being most prevalent initially, subsequently supplanted by ST11-KL64. Specific strains harbored bla_{KPC-2} on IncFII-type plasmids, along with other resistance genes, such as bla_{TEM-1}. KP635_PlasmidB harbors multiple antibiotic resistance genes, and the sequence identity and coverage between KP635_PlasmidA and the NTUH-K2044 virulence plasmid are 99%, which contributes to the formation of a highly virulent and multidrug-resistant strain in KP635.

Conclusion The emergence of high resistance and hypervirulence in CRKP requires vigilance, enhanced surveillance, and stringent infection control measures to limit its spread.

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Keywords Carbapenem-resistant *Klebsiella pneumoniae*, Whole-genome sequencing, High virulence, Resistance, Plasmid

Introduce

Klebsiella pneumoniae is classified as one of the ESKAPE pathogens [1], together with *Enterococcus faecium*, *Staphylococcus aureus*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp. This bacterium is widely distributed in nature, thriving in environments such as soil and shallow water bodies. It asymptomatically colonizes mucosal surfaces in the human body, particularly the gastrointestinal tract. *Klebsiella pneumoniae*, known for its pathogenic potential, can cause a range of infections, including pneumonia, urinary tract infections, bacteremia, meningitis, and liver abscesses [2]. Furthermore, it demonstrates the ability to persist on abiotic surfaces such as medical devices. In the human body, *Klebsiella pneumoniae* primarily colonizes mucosal surfaces such as the oropharynx and gastrointestinal tract, with the potential to spreadto other tissues, increasing the risk of infection [3].

In recent years, clinical isolates of *Klebsiella pneumoniae* have diversified into multiple strains, exhibiting a wide spectrum of resistance and virulence profiles. The growing concern about CRKP has been fueled by advancements in diagnostic and therapeutic modalities, invasive medical procedures, and widespread antibiotic usage. Data from the China Antimicrobial Resistance Surveillance System (CARSS) reveal a concerning trend of increasing carbapenem resistance among *Klebsiella pneumoniae*, with meropenem resistance rising from 2.9% in 2005 to 27.5% in 2022. Despite their multidrug resistance, CRKP strains typically exhibit low levels of virulence.

Hypervirulent *Klebsiella pneumoniae* (hvKP) has attracted significant attention due to its highly mucoid and virulent nature, leading to severe complications like pyogenic liver abscesses and metastatic infections. Clinical hvKP isolates often exhibit invasiveness and a propensity for multi-site infections, typically accompanied by positive string test results, which are indicative of a hypermucoviscous phenotype [4]. However, defining hypervirulence remains a subject of debate. Recent studies aim to distinguish hvKP from classical *Klebsiella pneumoniae* (cKp) by identifying potential biomarkers such as *peg-344*, *iroB*, *iucA*, plasmid-borne *rmpA* and *rmpA2* genes, and high-iron carriers. Bioinformatic analyses suggest that these genes are frequently located on large virulence plasmids [5].

To date, research on CRKP and CR-hvKP in northern China has been limited. Therefore, a comprehensive understanding of the local clinical distribution, resistance patterns, virulence traits, and molecular characteristics of CRKP strains is crucial. Investigating the origins of resistant bacteria within wards of a tertiary hospital in Heilongjiang Province can provide valuable insights into their resistance profiles. This approach not only helps mitigate the rising CRKP detection rates within the hospital but also facilitates the scientific selection of empirical treatments for infectious diseases. By effectively preventing and controlling the emergence and dissemination of resistant bacteria, this research offers novel insights into averting potential outbreaks and serves as a pivotal foundation for infection control efforts.

Materials and methods

Sources and identification of clinical isolates

Non-duplicated CRKP strains obtained from clinical specimens collected between 2016 and 2023 at Daqing Oilfield General Hospital, Heilongjiang Province, China, were included. All strains underwent identification using the VITEK automated microbiology analyzer (BioMerieux, Lyons, France), supplemented by strain confirmation via the Kirby-Bauer (KB) method. Clinical data related to CRKP patients were acquired via the Laboratory Information System (LIS), encompassing an analysis of clinical details including departmental distribution, specimen origins, gender, and age demographics.

DNA extraction

Template DNA was extracted using the Bacterial DNA Kit (Tiangen Biotech, Beijing, China). Approximately 200 μL of DNA solution was obtained as the template for subsequent DNA reactions. The DNA specimens were stored at -80 °C to maintain integrity and facilitate subsequent research.

Detection of carbapenem-resistancegenes and virulence factors

PCR was used to detect five prevalent carbapenemase genes (bla_{KPC}, bla_{NDM}, bla_{VIM}, bla_{IMP}, bla_{OXA-48}). Additionally, the detection included siderophore virulence genes (*ybtS*, *iutA*, *iucA*, *iroN*, *entB*), mucoid regulator factors (*rmpA*, *rmpA2*), and fimbrial adhesion genes (*fimH* and *mrkD*). CR-hvKP was defined as CRKP strains harboring *rmpA* and/or r*mpA2* concurrently with *iucA*, *iroB*, or *peg-344*. The capsular serotypes KL1, KL2, KL5, KL20, KL54, and KL57 of CRKP strains were determined by sequence amplification analysis. For strains with negative serotypes, the *wzi* was PCR-amplified and subjected to first-generation sequencing to determine the serotype. Primer sequences for resistance genes, virulence genes

Table 1 Sequence of primers for resistance genes

Primers	Primer sequence(5′-3′)		Product length(bp)
bla_{KPC}	F	TGTCACTGTATCGCCGTC	1010
	R	CTCAGTGCTCTACAGAAAACC	
bla_{NDM}	F	GCAGCTTGTCGGCCATGCGGGC	782
	R	GGTCGCGAAGCTGCGCACCGCAT	
bla_{VIM}	F	GATGGTGTTTGGTCGCATA	365
	R	CGAATGCGCAGCACCAG	
bla_{IMP}	F	CATGGTTTGGTGGTTCTTG	448
	R	ATAATTTGGCGGACTTTGG	
bla_{OXA-48}	F	GCGTGGTTAAGGATGAACAC	438
	R	CATCAAGTTCAACCCAACCG	

Table 2 Sequence of primers for virulence genes

Primers	Primer sequence (5′-3′)		Product length (bp)
rmpA	F	GAGTAGTTAATAAATCAATAGCAAT	332
	R	CAGTAGGCATTGCAGCA	
mrkD	F	AAGCTATCGCTGTACTTCCGGCA	340
	R	GGCGTTGGCGCTCAGATAGG	
rmpA2	F	GTGCAATAAGGATGTTACATTA	430
	R	GGATGCCCTCCTCCTG	
peg-344	F	CTTGAAACTATCCCTCCAGTC	508
	R	CCAGCGAAAGAATAACCCC	
fimH	F	TGCTGCTGGGCTGGTCGATG	550
	R	GGGAGGGTGACGGTGACATC	
iroN	F	AAGTCAAAGCAGGGGTTGCCCG	668
	R	GACGCCGACATTAAGACGCAG	
iutA	F	GGGAAAGGCTTCTCTGCCAT	920
	R	TTATTCGCCACCACGCTCTT	
ybtS	F	GACGGAAACAGCACGGTAAA	242
	R	GAGCATAATAAGGCGAAAGA	
entB	F	GTCAACTGGGCCTTTGAGCCGTC	400
	R	TATGGGCGTAAACGCCGGTGAT	
iucA	F	AATCAATGGCTATTCCCGCTG	322
	R	CGCTTCACTTCTTTCACTGACAGG	
iroB	F	ATCTCATCATCTACCCTCCGCTC	322
	R	GGTTCGCCGTCGTTTTCAA	

Table 3 Sequence of primers for capsular serotypes

Primers	Primer sequence(5′-3′)		Product length(bp)
KL1	F	GTAGGTATTGCAAGCCATGC	1046
	R	GCCCAGGTTAATGAATCCGT	
KL2	F	GGAGCCATTTGAATTCGGTG	641
	R	TCCCTAGCACTGGCTTAAGT	
KL5	F	GCCACCTCTAAGCATATAGC	280
	R	CGCACCAGTAATTCCAACAG	
KL20	F	CCGATTCGGTCAACTAGCTT	741
	R	GCACCTCTATGAACTTTCAG	
KL54	F	CATTAGCTCAGTGGTTGGCT	881
	R	GCTTGACAAACACCATAGCAG	
KL57	F	CGACAAATCTCTCCTGACGA	1182
	R	CGCGACAAACATAACACTCG	
wzi	F	GTGCCGCGAGCGCTTTCTATCTTGGTATTCC	580
	R	GAGAGCCACTGGTTCCAGAATTACCGC	

and capsular serotypes are listed in Tables 1, 2 and 3 [5–8].

Primers targeting the seven housekeeping genes *mdh*, *rpoB*, *gapA*, *tonB*, *pgi*, *infB*, and *phoE* were custom-synthesized according to specifications provided by the website https://bigsdb.pasteur.fr/klebsiella/. Subsequently, the amplified products were subjected to sequencing, and the resulting sequences were uploaded to the MLST database for determination of the ST of the strains.

String test

Using a 1 μL inoculation loop, streak a single colony from an overnight culture in a vertical direction. Repeat this procedure three times to confirm the accuracy of the experiment. If a string ≥ 5 mm in length is formed when the colony is lifted, the result is considered positive.

Biofilm formation assay

This assay uses crystal violet staining to assess biofilm formation capacity [9], with each strain tested in triplicate. Bacterial colonies grown overnight in LB broth (Soleibao, Beijing, China) are inoculated into 5 mL of fresh LB broth and incubated at 37 °C with agitation at 120 rpm for 16–18 h. The bacterial suspension is then standardized to a 0.5 McFarland standard, and 10 μL is dispensed into each well of a 96-well plate, with negative controls set up concurrently. The plate is then sealed and incubated overnight at 37 °C. After incubation, the plate is retrieved, the bacterial suspension is aspirated, and the wells rinsed with PBS buffer. Formaldehyde fixation solution is added to each well and incubated for 15 min, followed by removal, rinsing with distilled water, and air-drying. Then, 1% crystal violet staining solution is added to each well for 15 min, followed by aspiration, washing with sterile distilled water until colorless, and air-drying. Finally, absolute ethanol is added to each well, gently agitated for 10 min, and absorbance at 570 nm is measured using a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA).

Serum killing assay

Serum bactericidal activity was determined as previously described [10]. Before the experiment, blood samples are collected from 10 healthy volunteers, centrifuged to isolate the serum, pooled, and stored appropriately. Then, single bacterial colonies are cultured in 5 mL of LB broth at 35 °C with shaking at 120 rpm for 18 h. The bacterial suspension is adjusted to a 0.5 McFarland standard and then further diluted to 10^6 colony-forming units per

milliliter (cfu/mL). Then, 25 μL of the bacterial suspension is mixed with 75 μL of serum in sterile EP tubes, and incubated on a constant-temperature shaker at 37 °C for 0, 60, 120, and 180 min. At each time point, bacterial colony counting is performed using the gradient dilution method to determine viable bacterial counts, which are then compared to the initial count to calculate the percentage survival rate. The grading criteria are as follows: grades 1–2 indicate sensitivity, grades 3–4 are categorized as intermediate, and grades 5–6 indicate resistance. Each strain undergoes at least three trials at the same concentrations, and the average survival rate is calculated .

Whole Gene Sequencing

Whole genome sequencing involves three main stages: DNA extraction, library construction, and sequencing. During library construction, genomic DNA is fragmented, and libraries are generated using the NovaSeq6000 (Illumina, USA) and Sequel IIe platforms (PacBio, USA). For Illumina libraries, DNA is fragmented to approximately 400 bp, and library preparation is performed using the NEXTFLEX Rapid DNA-Seq Kit. In contrast, for PacBio libraries, DNA is fragmented to approximately 10 kb and constructed using the SMRT Bell method. During sequencing, Illumina libraries undergo paired-end sequencing on the NovaSeq6000, with each cycle incorporating a single base and recording template DNA sequences through fluorescence signals. In contrast, PacBio sequencing uses single-molecule real-time (SMRT) detection on the Sequel IIe sequencing platform. The generated data undergo bioinformatics analysis on the Shanghai Majorbio Cloud Platform, including genome assembly, gene annotation, and prediction of virulence and resistance genes. Assembly is performed using SOAPdenovo2 and Unicycler, with annotation carried out using various software and databases for functional prediction. Gene prediction and annotation involve the use of Glimmer, GeneMarkS, and Prodigal software for coding sequence prediction, as well as the prediction of virulence and resistance genes using the VFDB and ResFinder databases. tRNA and rRNA prediction is performed using tRNAscan-SE and Barrnap software.

Results

Clinical distribution of strains and specimen types

The majority of isolates were obtained from the neurosurgery department (39/100, 39%), followed by the ICU (32/100, 32%), respiratory medicine (7/100, 7%), NICU (4/100, 4%), urology (4/100, 4%), rehabilitation medicine (3/100, 3%), general surgery (3/100, 3%), respiratory and critical care (2/100, 2%), neonatology (1/100, 1%), nephrology (1/100, 1%), gastroenterology (1/100, 1%), vascular surgery (1/100, 1%), ophthalmology (1/100, 1%), and the internal medicine ward (1/100, 1%). The patient population consisted of 70 males and 30 females, with an mean age of 65.11 ± 17.28 years. The predominant specimen types were sputum (72/100, 72%), followed by clean urine (11/100, 11%), blood (9/100, 9%), and others (See Fig. 1).

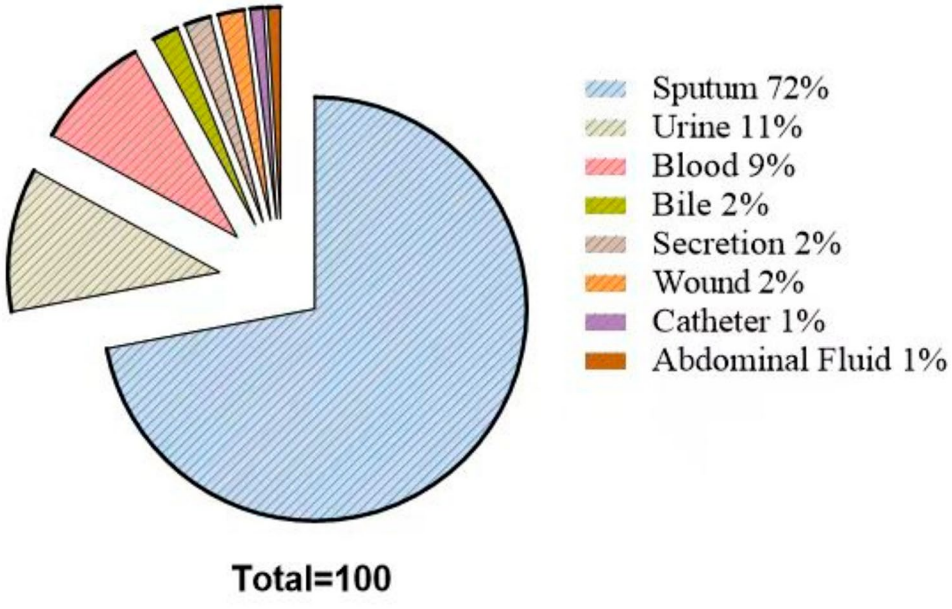


Fig. 1 Sample type scale map

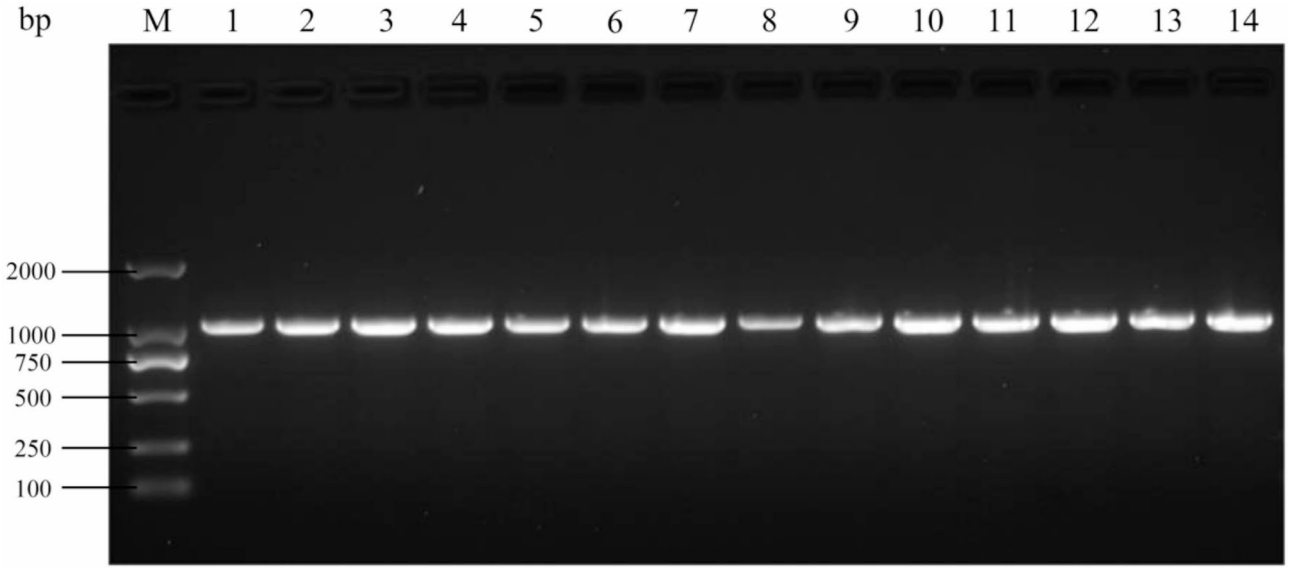


Fig. 2 Sequencing map of bla_{KPC}

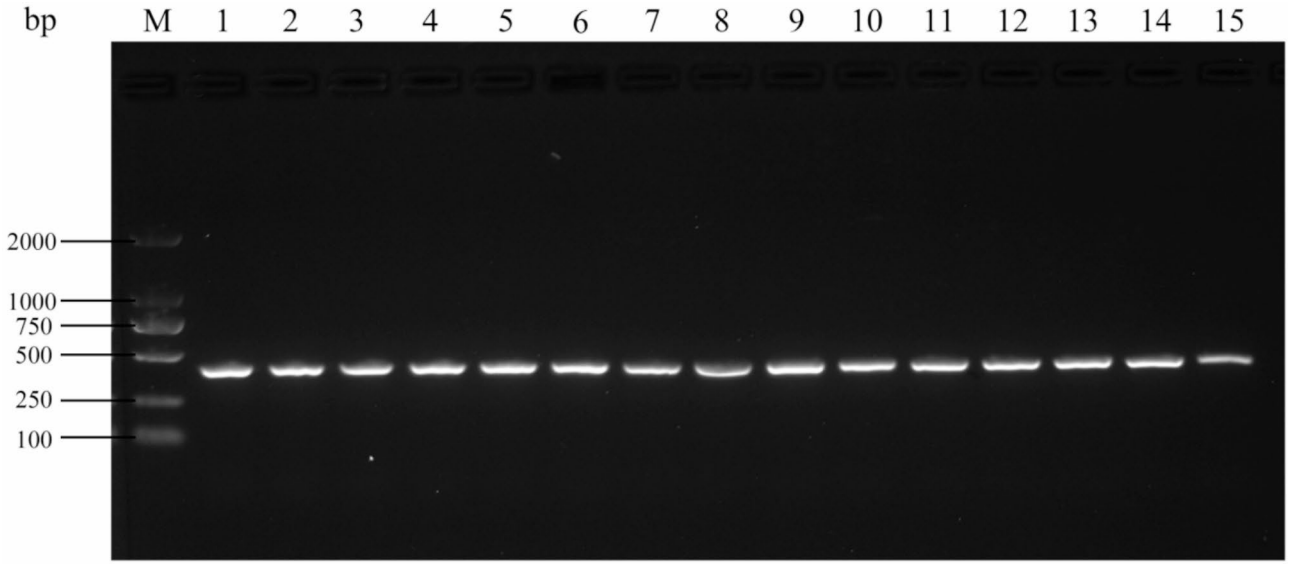


Fig. 3 Sequencing map of *entB*

Carbapenemase gene

Based on the results of carbapenemase gene detection, KPC is identified as the predominant resistance gene, accounting for 93% of the cases. Sequencing analysis and BLAST alignment confirmed that all instances corresponded to bla_{KPC-2}. One strain harbored bla_{NDM-5}, two strains carrying both bla_{KPC-2} and bla_{NDM-5}, and one carrying bla_{KPC-2} and bla_{IMP-38}. Partial positive gene sequencing results and the corresponding electrophoresis images are shown in Figs. 2 and 3, and 4. None of the isolates tested positive for bla_{VIM} or bla_{OXA-48}. PCR amplification yielded negative results for all three CRKP strains.

Capsular serotype and Virulence-Associated genes

The presence of *entB* was detected in (100/100, 100%) all strains, while *mrkD* was detected in (97/100, 97%) of the strains, *fimH* in (96/100, 96%) of the strains, *ybtS* in (83/100, 83%) of the strains, *iutA* in (70/100, 70%) of the strains, *iucA* in (69/100, 69%) of the strains, *rmpA2* in (67/100, 67%) of the strains, *peg-344* in (28/100, 28%) of the strains, *iroB* in (26/100, 26%) of the strains, *iroN* in (23/100, 23%) of the strains, and *rmpA* in (17/100, 17%) of the strains. The majority of strains harbored virulence genes, including *entB*, *mrkD*, *fimH*, and *ybtS*. The combination of *entB*, *fimH*, *mrkD*, *rmpA2*, *ybtS*, *iutA*, *iucA*, and *iroB* was the most prevalent (18/100, 18%). Of the 100 CRKP strains, (66/100, 66%) were classified as CR-hvKP.

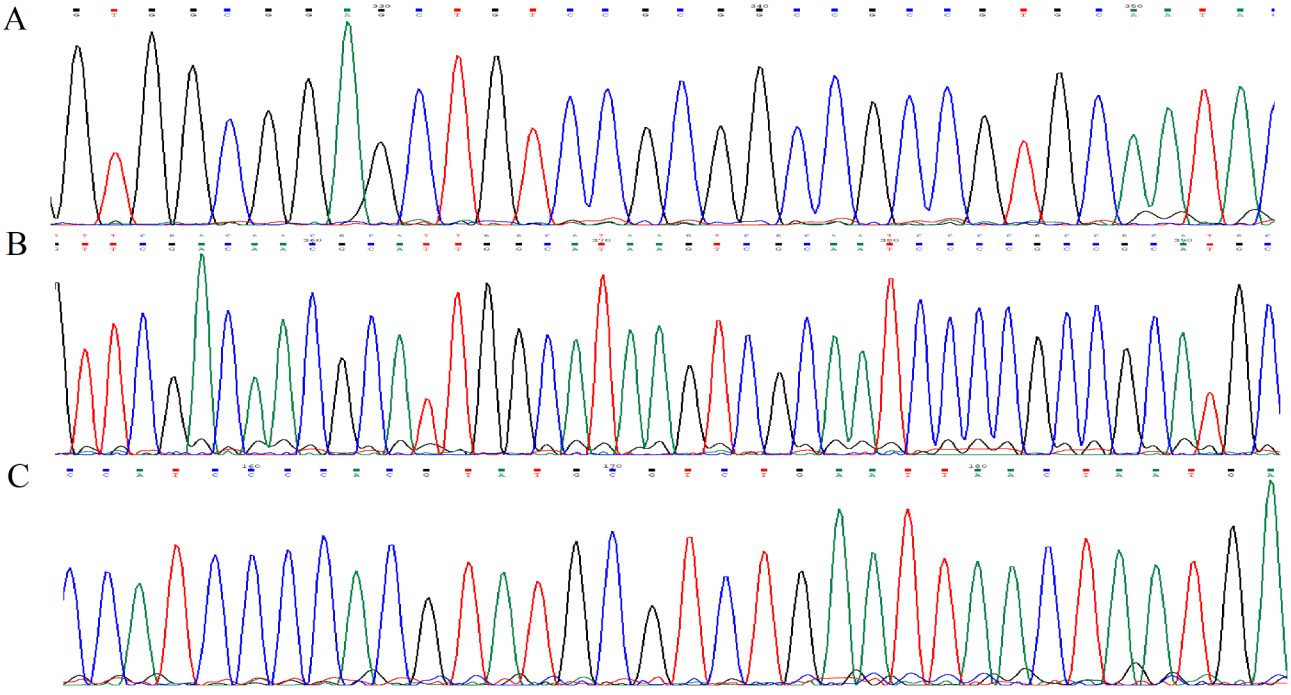


Fig. 4 Sequencing of drug resistance genes. (**A**) Sequencing map of bla_{KPC}. (**B**) Sequencing map of bla_{NDM}. (**C**) Sequencing map of bla_{IMP}

Among them, KP635 and KP329 were identified as serotype KL1, while KP663, KP704, and KP18064 were classified as serotype KL2. The remaining strains could not be assigned to serotypes KL5, KL20, or KL57. PCR-based *wzi* sequencing revealed that (44/100, 44%) strains belonged to KL47, (18/100, 18%) to KL64, (4/100, 4%) to KL10, (2/100, 2%) each to KL19 and KL30, and (1/100, 1%) to KL21, KL27, KL28, and KL45.

String test and MLST typing

A string test was conducted on 100 CRKP isolates, which revealed that only six strains exhibited positive string formation on blood agar plates (Oxoid, Hampshire, UK), indicative of the hypermucoviscous phenotype. MLST analysis revealed that the CRKP isolates could be categorized into 13 distinct STs, with ST11 predominating (68/100, 68%), followed by other STs. The other STs identified were ST37 (11/100, 11%), ST76 (5/100, 5%), ST65 (3/100, 3%), ST1 (2/100, 2%), ST20 (2/100, 2%), ST23 (2/100, 2%), ST3157 (2/100, 2%), ST211 (1/100, 1%), ST323 (1/100, 1%), ST526 (1/100, 1%), ST595 (1/100, 1%), and ST2294 (1/100, 1%)(Fig. 5).

Biofilm formation and serum killing

Of the 100 CRKP strains analyzed, the majority exhibited the ability to form biofilms, with variations in the extent of formation. Among these, 6 strains (6/100, 6%) showed no biofilm formation, 35 strains (35/100, 35%) exhibited weak biofilm formation, 32 strains (32/100, 32%) displayed moderate biofilm formation, and 27 strains (27/100, 27%) showed strong biofilm formation. Despite variations in biofilm formation capabilities among isolates, this trait contributes to the pathogenicity and resistance of *Klebsiella pneumoniae*. Serum resistance was assessed in a total of 100 strains. Of these, 57% (57/100) were sensitive to serum, 24% (24/100) exhibited moderate resistance, and 19% (19/100) demonstrated full serum resistance. Notably, all strains displaying serum resistance were identified as CR-hvKP. These findings suggest that CR-hvKP strains exhibit a distinct and significantly higher level of serum resistance compared to other strains. The results of the serum killing assay and biofilm formation assay are shown in Fig. 6. The key characteristics of CR-hvKP strains, such as capsular serotypes, string test results, and sequence types, are summarized in Fig. 7.

Isolate analysis based on whole gene sequencing

Whole-genome sequencing was performed on two carbapenem-resistant hypervirulent *Klebsiella pneumoniae*strains, KP635 and KP26. Subsequently, the whole-genome sequencing data were deposited in NCBI, with strain KP26 assigned the SRA accession number SRP482864, and strain KP635 assigned the SRA accession number SRP483050. The raw data were filtered, and sequencing was performed using Illumina-HiSeq 6000 and PacBio Sequel IIe, yielding the genome sequences of KP26, which total 5,748 kb in size with an average GC content of 57.07%. The genome comprises three plasmids and one circular chromosome, with its chromosomal

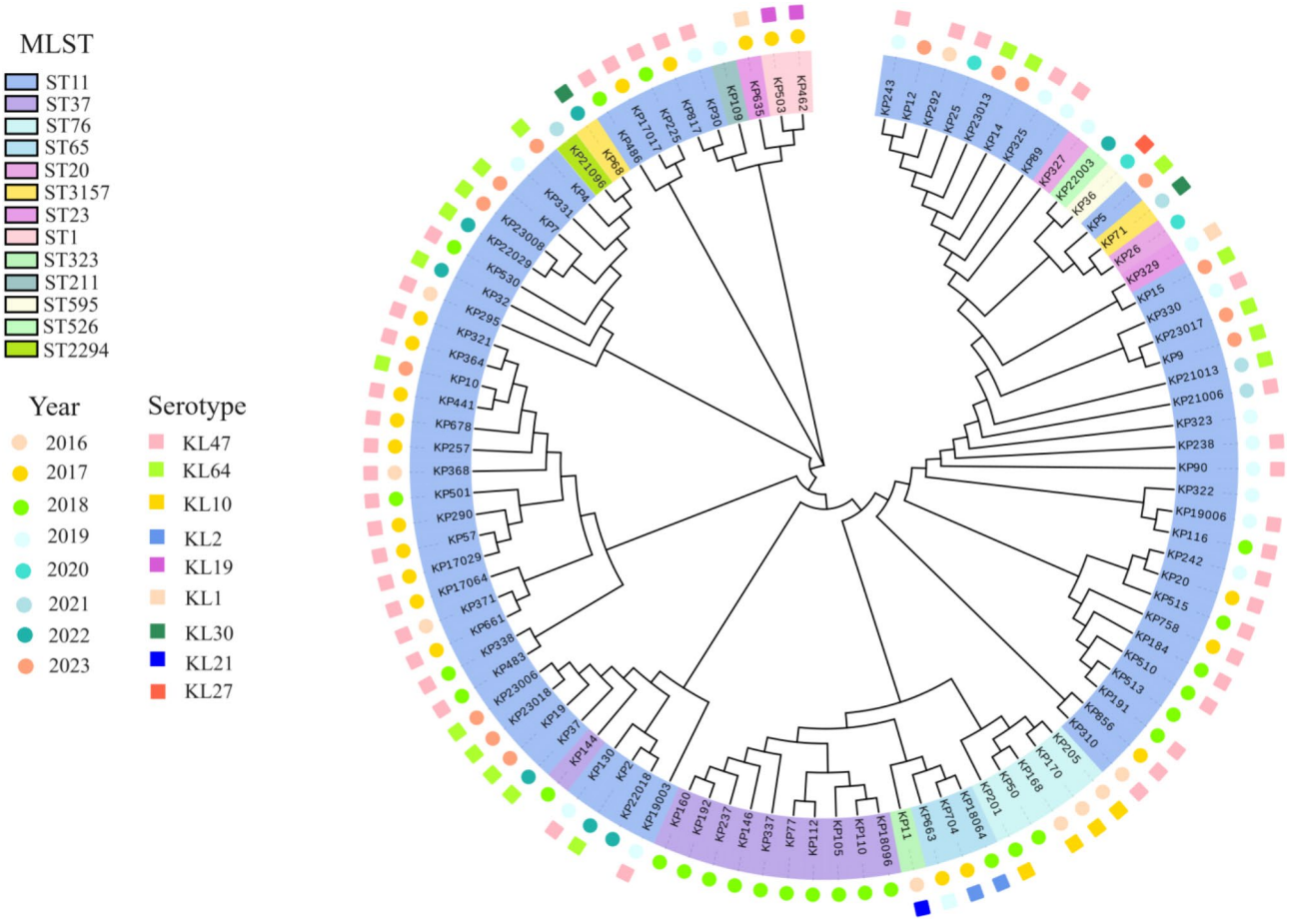


Fig. 5 Phylogenetic tree

structure shown in Fig. 8. Notably, the chromosome also harbors various virulence genes, including those associated with the iron acquisition system (*irp-1*, *irp-2*, *fyuA*, *ybtAEPQSTUX*, and *entACES*), as well as genes implicated in fimbrial colonization and adhesion (*ureA*, *ureB*, *ureG*, *allS*, *wcaJ*, *mrkABCDFJIH*, and *fimABCDEFGHIK*), among others (Supplementary Fig. 1). The carbapenem resistance gene $bla_{\text{NDM-5}}$ is located in a plasmid with a size of 50,989 bp, in which a stable protein (parA) was present in addition to the resistance gene. The pivotal $bla_{\text{KPC-2}}$ is located on a larger Inc FII type plasmid with a size of 108.41 kb. In addition to the carbapenem resistance genes, the plasmid carries a β-lactamase gene, $bla_{\text{TEM-1}}$, along with a plasmid-segregating protein, parM. Furthermore, the plasmid also contains a type IV secretion system (T4SS) that facilitates plasmid conjugation. A detailed depiction of the plasmid's circular structure is shown in Fig. 9. BLAST analyses of the genomic region surrounding $bla_{\text{KPC-2}}$ revealed a diverse array of insertion elements encoded by the plasmid, including ISKpn27, IS6, IS1182, IS26, and others. The inclusion of these elements may facilitate the integration or dissemination of drug resistance genes. A comparative analysis between KP26_PlasmidB and the chromosome of *Klebsiella pneumoniae* (accession no. CP110688.1) revealed 71% gene coverage and 99.50% sequence identity. The $bla_{\text{KPC-2}}$ gene is located within the transposon Tn4401 of Tn3 and encompasses two insertion sequences: ISKpn26 and ISKpn7. The environmental landscape, along with comparative linear analysis, is depicted in Fig. 10.

The total length of the gene sequences of KP635 is 5.7 Mb, with an average GC content of 57.16%. GeneMarkS software identified 5,259 coding sequences (CDSs), while Barrnap and tRNAscan-SE v2.0 software predicted 87 tRNAs and 25 rRNAs, respectively. The complete genome of KP635 genome consists of one chromosome and two plasmids. The chromosome spans 5.4 Mb, with a G + C content of 57.51%, and contains 3,264 CDSs (Supplementary Fig. 2).

KP635_PlasmidB is an IncN plasmid carrying the sulfonamide resistance gene dfrA14, the carbapenemase gene $bla_{\text{KPC-2}}$, two extended-spectrum β-lactamase (ESBL) genes ($bla_{\text{CTX-M-3}}$ and $bla_{\text{TEM-213}}$), and the quinolone resistance gene QnrS8. KP635_PlasmidA, a 226.35 kb plasmid of the IncFIB(K) classification, encompasses virulence genes such as *iroABCDN*, *iucABCD*,

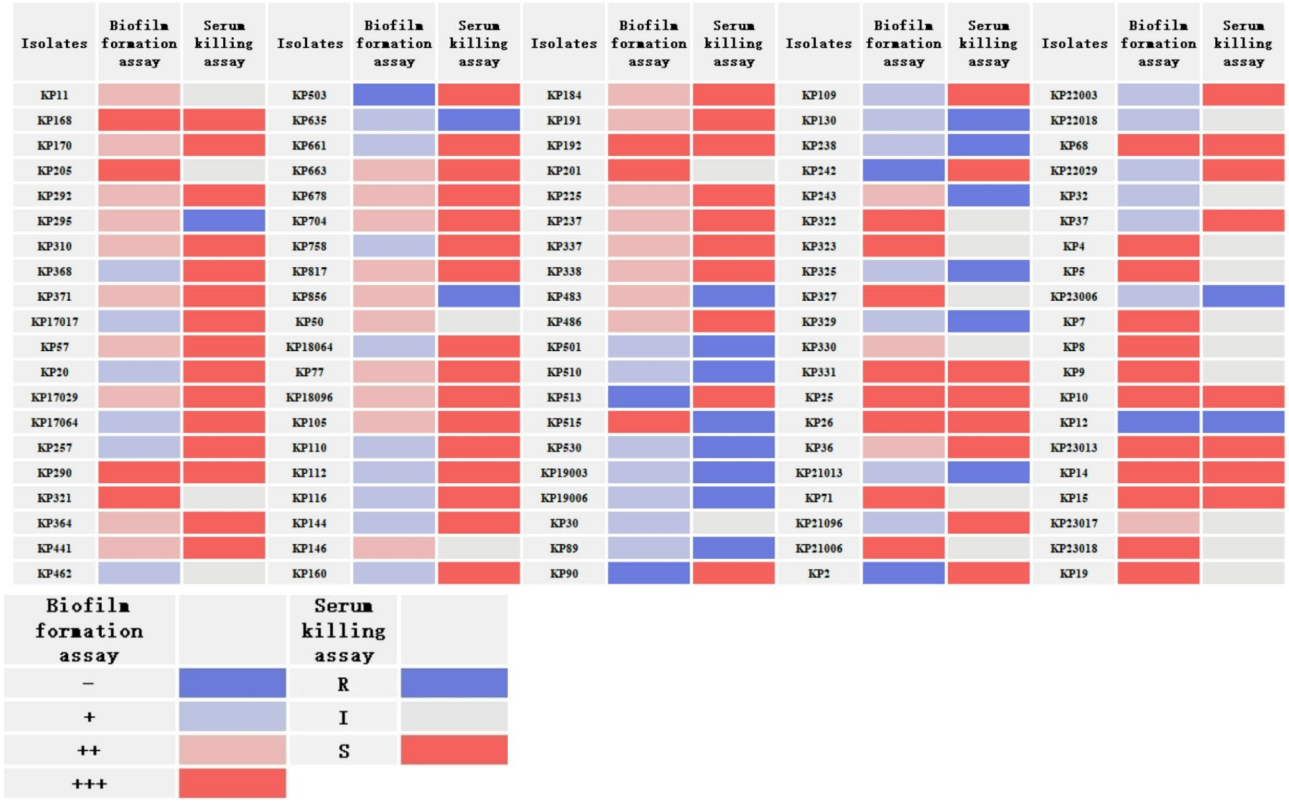


Fig. 6 Serum killing test and biofilm formation test

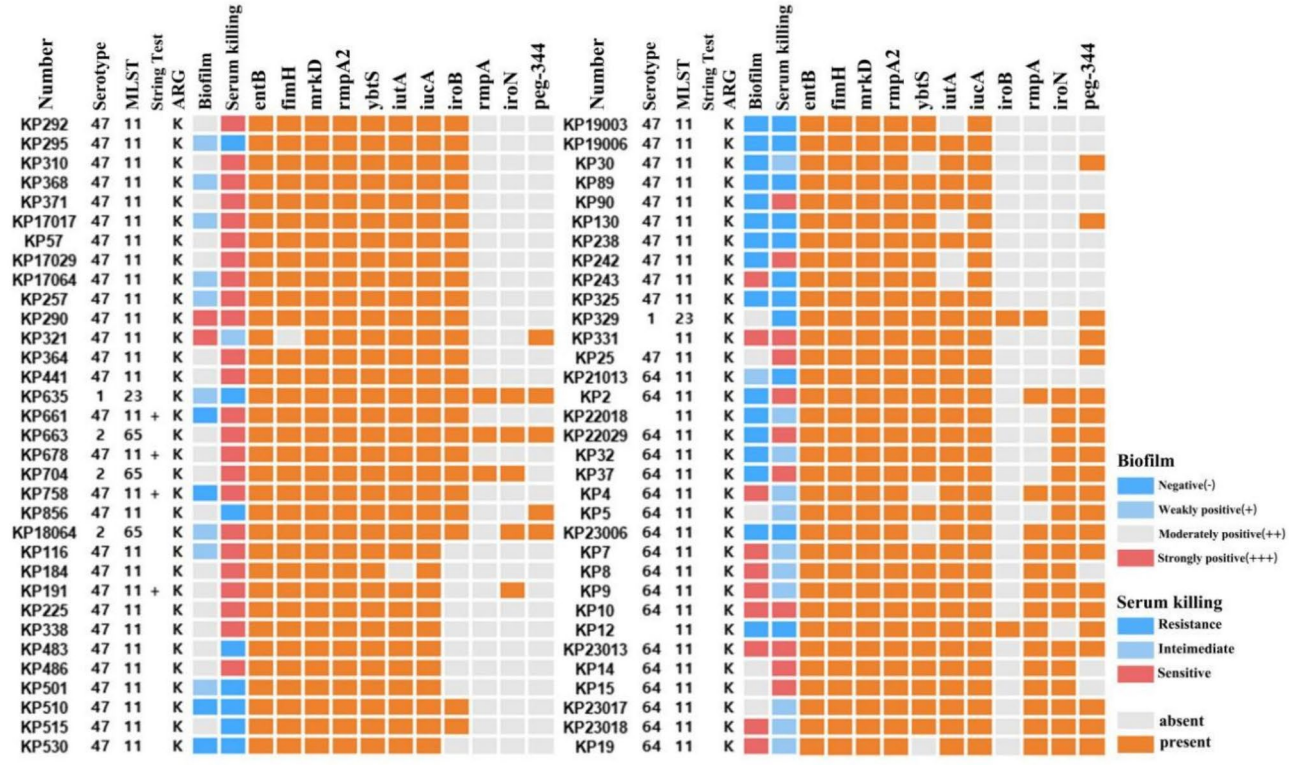


Fig. 7 Summary of CR-hvKP Strains' characteristics

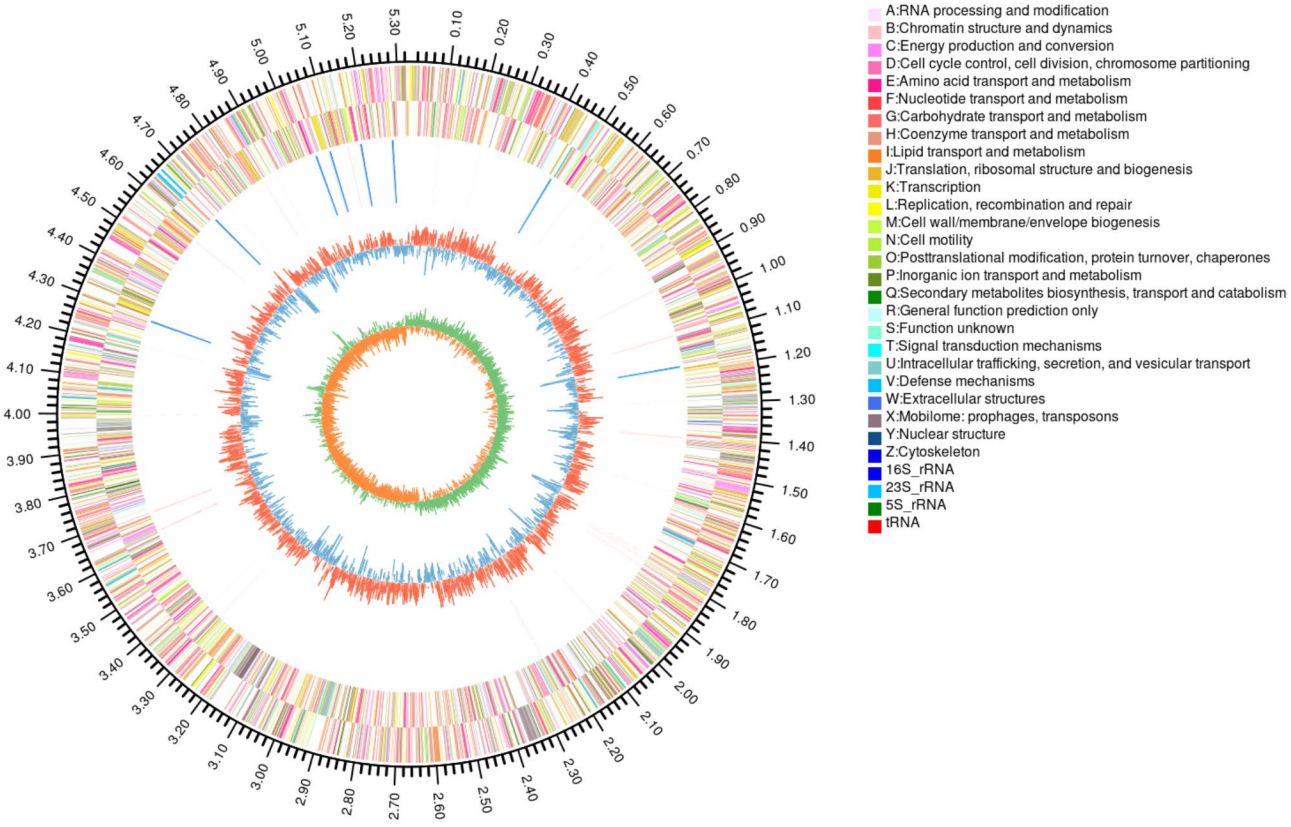


Fig. 8 Chromosome structure map of KP26. The outermost layer of the circle diagram shows the genome size identification; the second and third circles represent the CDSs on the positive and negative strands, respectively; the fourth circle shows the rRNAs and tRNAs; the fifth circle represents the GC content; and the innermost circle displays the GC-skew value. Different colors represent the functional classification of different COGs of CDSs

and *iutA*. KP635_PlasmidA demonstrates 99% sequence coverage and 99.99% sequence identity with the virulence plasmid pK2044 derived from the NTUH-K2044 strain. An in-depth comparative analysis was performed on KP635_PlasmidA and the top five closely related virulence plasmids, identified by their GenBank accession numbers: AP006726.1, CP089599.1, CP096235.1, CP103513.1, and CP125085.1 (Fig. 11).

Conclusion

This study examined 100 CRKP isolates obtained between February 2016 and May 2023. The average age of the patients was 65.11 ± 17.28 years, with males outnumbering females at approximately 2:1. Neurosurgery emerged as the predominant source of isolates, presumably due to the extended hospital stays of trauma patients in this department, which often necessitate prolonged bed rest, surgical interventions, or various invasive procedures. The ICU followed closely, characterized by severe patient conditions, compromised immune function, and frequent administration of high-dose combination therapies. The primary specimen type was sputum, potentially linked to oral colonization and subsequent pneumonia through microaspiration or macroaspiration. This suggests that CRKP strains in this hospital primarily manifest as respiratory tract infections, with transmission likely facilitated by respiratory droplets or colonization.

The carbapenem gene carriage profile observed in our clinical CRKP isolates is primarily characterized by single-gene carriage, predominantly by bla_{KPC-2}, in line with prevailing national trends [11]. KPC enzyme-producing *Klebsiella pneumoniae* is typically associated with invasive, high-risk clonal lineages. When patients are infected or colonized by KPC-producing *Klebsiella pneumoniae* in healthcare settings, these bacteria pose a significant transmission risk, both within and between healthcare facilities [12]. Notably bla_{VIM} and bla_{OXA-48} were absent in this study. bla_{OXA-48}-producing *Klebsiella pneumoniae* predominates in regions such as Turkey, North Africa, and Europe, whereas its variant, $bla_{OXA-181}$, is more prevalent in the Indian subcontinent [13].

As reported by a notable study [14], 36% of screened CRKP isolates harbor significant virulence factors. In our study, PCR analysis was employed to identify 11 virulence genes, including siderophore virulence genes (*ybtS*, *iutA*, *iucA*, *iroN*, *entB*), mucoid regulators (*rmpA* and *rmpA2*), iron acquisition transporter (*peg-344*), and pilus-related genes (*fimH* and *mrkD*). Virtually all CRKP strains exhibit

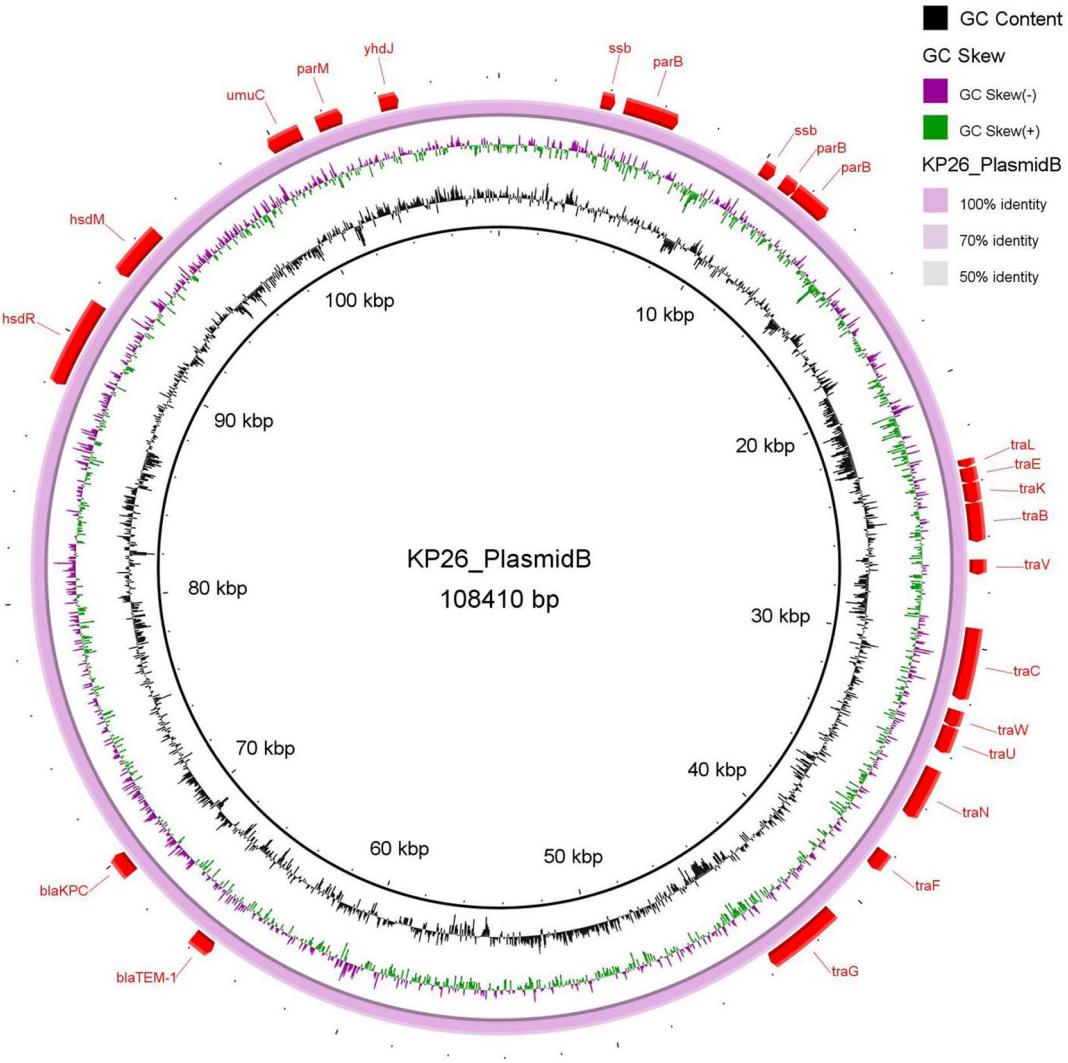


Fig. 9 Circle diagram of KP26_PlasmidB. GC content, GC Skew + and GC Skew- are shown in black, green and purple, respectively

four or more virulence genes, with *entB*, *mrkD*, and *fimH* being the three most commonly carried genes. The *fimH* and *mrkD* genes encode Type I and Type III pili, respectively, commonly associated with respiratory and urinary tract infections, which are prevalent among the majority of patients in our hospital with pulmonary diseases. The *entB* demonstrates a 100% detection rate, and the activity of the enzyme it encodes is crucial for the bacterium's survival within the host By synthesizing enterobactin, *Klebsiella pneumoniae* effectively competes for limited iron resources in the host environment, thereby ensuring its proliferation and survival. In a study by Mulani et al. [15] investigated *entB*, revealing its role in synthesizing abundant enterobactin, consequently enhancing the virulence of highly virulent *Klebsiella pneumoniae* strains, promoting iron acquisition and biofilm formation, thereby augmenting the strain's pathogenicity.

A pivotal virulence trait of *Klebsiella pneumoniae* is its ability to form biofilms, intricate bacterial communities composed of one or more species embedded within an extracellular matrix rich in polysaccharides, proteins, and DNA. Biofilm formation confers increased resistance to exogenous stressors and antimicrobial agents. To further understand CRKP's virulence traits, we conducted additional assays to evaluate biofilm formation and serum killing resistance in a cohort of 100 CRKP strains [16]. A study of 68 CRKP isolates [17] revealed that 77.9% exhibited robust biofilm formation, while 22% displayed moderate biofilm formation, underscoring a direct correlation between biofilm formation capacity and CRKP. However, contradictory findings also exist. Cusumano et

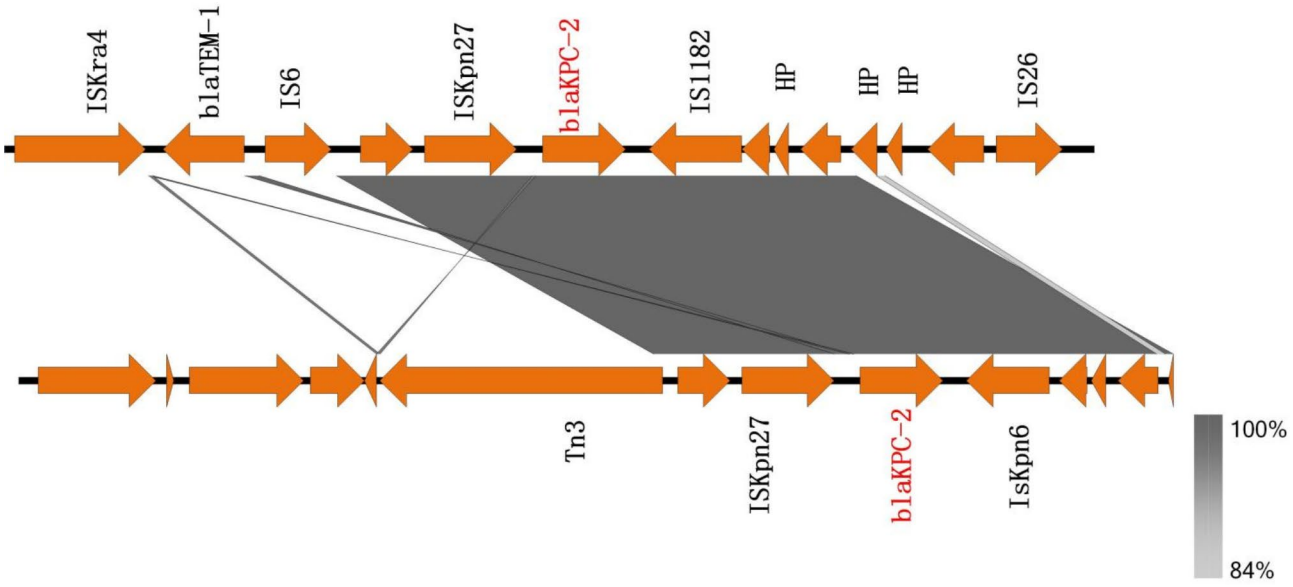


Fig. 10 Environmental maps of bla_{KPC-2} with comparative linear analyses

al. [18] found a 91% decrease in the likelihood of CRKP forming robust biofilms, suggesting an inverse relationship between biofilm formation and carbapenem resistance. In our study, variations in biofilm formation were observed, with 41% exhibiting weak or negative biofilm formation, 32% exhibiting moderate biofilm formation, and only 27% exhibiting strong biofilm formation. This experimental outcome is consistent with the findings of Cusumano et al.

Because antibodies and complement components in human whole blood can elicit an immune response against bacteria, leading to bacterial eradication, the serum killing resistance assay is a valuable tool for evaluating bacterial bactericidal efficacy. Isolate KP26 was obtained from a 58-year-old female patient admitted to the emergency department with acute onset of severe headache and altered mental status lasting approximately 3 h. After a series of diagnostic tests, she was diagnosed with a subarachnoid hemorrhage due to an intracranial aneurysm. The isolate exhibited resistance to cephalothin, ampicillin, meropenem, piperacillin-tazobactam, cefepime, and imipenem. After surgical intervention, therapeutic regimen included tranexamic acid, piracetam, and furosemide to control hemorrhage and reduce intracranial pressure, along with adjunctive measures for neuroprotection. Considering the critical importance of cerebral function, a drainage catheter was inserted to maintain intracranial pressure equilibrium, along with prophylactic ceftriaxone administration for infection prevention. Throughout the 24-day hospitalization, the patient showed clinical improvement without requiring intensive antibiotic therapy, suggesting the potential susceptibility of KP26 to serum complement-mediated killing.

Among the 100 CRKP strains investigated in this study, only 6 exhibited positivity in the string test. Initial studies considered a positive string test (≥ 5 mm) as a method for identifying hvKP strains. However, the validity of this method has been challenged by numerous subsequent studies. While not all hvKP strains exhibit high mucoviscosity, some cKP strains also possess this trait. Consequently, relying solely on the string test to determine the virulence status of a strain may result in false negatives.

Serotyping has become a widely used method for the taxonomic classification of Gram-negative bacteria. *Klebsiella pneumoniae* is classified by its capsular (K) types, which include at least 79 serotypes, and lipopolysaccharide (LPS) O types, which consist of 9 serotypes [19]. K typing is currently used for the rapid and accurate identification of capsular types, relying on conserved loci within cps genes, particularly *wzi*.

hvKp strains are associated with six prevalent serotypes, namely K1, K2, K5, K20, K54, and K57. These variants are distinguished by their ability to synthesize capsular polysaccharides, facilitating bacterial evasion and undermining the human immune response, including complement and neutrophils [20]. In contrast to cKP strains, hvKp strains of K1 and K2 serotypes exhibit pronounced resistance to both intracellular and extracellular neutrophil-mediated killing mechanisms. Currently, K1 and K2 serotypes account for 70-80% of the documented hvKP strains [21]. Therefore, serotyping emerges as a potential virulence determinant. Based on PCR analysis of the *wzi*, the majority of the 44 KL47 serotype strains in this investigation were obtained prior to

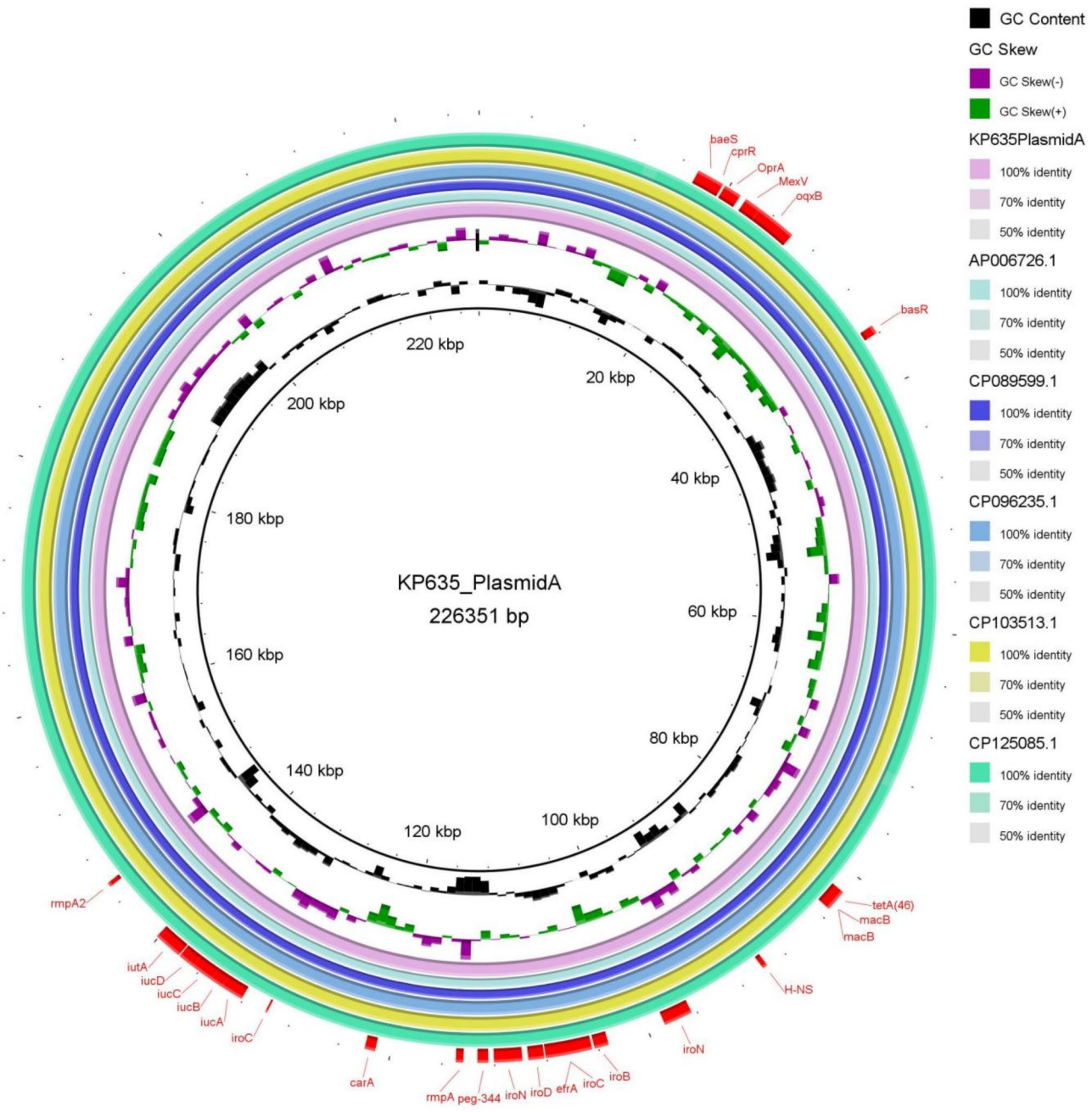


Fig. 11 Comparison and analysis of KP635_PlasmidA circle graph

2021. After 2021, KL47 strains vanished from detection, with ST11-KL64 emerging as the predominant archetype. This implies a gradual replacement of ST11-KL47 by ST11-KL64 strains as the principal clone lineage [22]. Within the ST11-KL64 CRKP lineage, certain plasmids incur moderate fitness burdens. Upon acquiring virulence plasmids akin to pLVPK, these CRKP lineages are poised to disseminate swiftly and cause hospital outbreaks. Zhou et al. observed similarities between ST11-KL64 CR-hvKP strains and hvKP K1 and K2 lineages in specific traits including mucoidy, siderophore synthesis, and biofilm development [23]. However, substantial differences were observed in resistance determinants and levels of antibiotic resistance. K1/K2 lineages exhibited a marked decrease in the number of resistance determinants and showed low-grade antibiotic resistance. In our investigation, strain KP635, of serotype K1, carries one chromosome and two plasmids. The resistant plasmid KP635_PlasmidB, classified as IncN type, contains resistance genes not only for carbapenemases but also

for sulfonamides, aminoglycosides, quinolones, and extended-spectrum β-lactamases. Contrary to the findings of Zhou et al., the cumulative effect of numerous resistance genes and plasmids confers multidrug resistance (MDR) upon KP635, making it resistant to all antibiotics except gentamicin, amikacin, meropenem, and tetracycline.

It is noteworthy that the dissemination of these resistance genes may occur concurrently with the transmission of the KP635_PlasmidB resistance plasmid, thereby facilitating the emergence of additional MDR strains. KP635_PlasmidA, the virulence plasmid, belongs to the IncFIB(K) plasmid category, harboring a cluster of virulence genes, including *iucABCDiutA* and *iroBCDN*, which encode the biosynthesis of aerobactin and salmochelin. Aerobactin primarily contributes to the heightened virulence, accounting for approximately 90% of the total iron carrier synthesis [24].

In the present investigation, we have identified a strain of ST20 CRKP harboring both bla_{KPC-2} and bla_{NDM-5}. Among the three of plasmids observed within KP26, the sole possession of carbapenem resistance genes is attributed to plasmid KP26_PlasmidB. The plasmid, classified as an IncFIB type, contains both the carbapenemase gene bla_{KPC-2} and the β-lactamase gene bla_{TEM-1}. Insertion sequences (IS) situated at the terminal regions of bla_{KPC-2} and bla_{TEM-1} facilitate the transfer of resistance genes. With 71% gene coverage and 99.50% identity to the chromosome of *Klebsiella pneumoniae* (accession number: CP110688.1), bla_{KPC-2} on KP26_PlasmidB is located within the Tn4401 transposon of Tn3, housing two insertion sequences, namely ISKpn26 and ISKpn7. Additionally, the presence of the T4SS on plasmid KP26_PlasmidB, which facilitates gene exchange and the transfer of effector proteins to target cells, was identified. The complexity and diversity of mobile genetic elements carrying bla_{KPC-2} in plasmids indicate their role not only in facilitating the transposition of bla_{KPC-2} to diverse genetic loci within the bacterial genome but also in enhancing the genetic adaptability of plasmids, thereby enhancing their capacity to adjust to various bacterial hosts. bla_{NDM-5} is harbored on the IncX3 type plasmid KP26_PlasmidC. The co-occurrence of these two carbapenemases enhances antibiotic resistance, complicating treatment protocols and facilitating the horizontal dissemination of infections. Consequently, the development of novel diagnostic strategies is imperative to track these resistant strains and mitigate the risk of large-scale outbreaks globally scale.

There are significant regional variations in antimicrobial resistance and CRKP prevalence across China. Notably, the limited number of CRKP reports in northern China presents considerable challenges to antimicrobial therapy and hospital infection control efforts. This study aims to elucidate the trends and genetic traits of CRKP through genetic analysis, providing a basis for more targeted treatment strategies. The definition of CR-hvKP lacks a unified standard, and classifying CR-hvKP strains solely based on genotypic criteria introduces certain limitations.

Supplementary Information

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Supplementary Material 1

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Author contributions
Xindi Wang wrote the main manuscript text. Lina Zhang, Jinwen Wang and Xudong Jiang conceived of and designedthe study. Liji Huang, Zhuoyan Huang and Qing Wei performed the assays and collected data. All authors read and approved the finalmanuscript.

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Data availability
The whole-genome sequencing data were archived in the NCBI, with strain KP26 designated with the SRA accession number SRP482864, and strain KP635 designated with the SRA accession number SRP483050.

Declarations

Ethics approval and consent to participate
The study was approved by the Ethics Committee of the Daqing Oilfield General Hospital (Document number: ZYAF/SC-07/02.0) and was conducted in compliance with the principles of the Declaration of Helsinki. As this study was retrospective and did not involve clinical intervention in patients, the Ethics Committee of the Daqing Oilfield General Hospital waived the requirement for informed consent.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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