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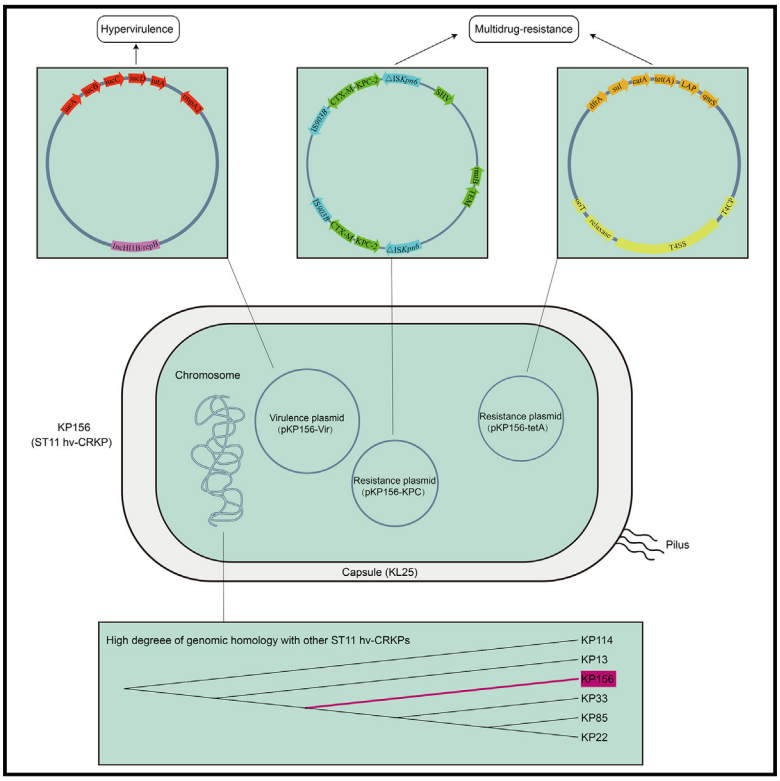
Genomic characterization of ST11-KL25 hypervirulent KPC-2-producing multidrug-resistant *Klebsiella pneumoniae* from China

Graphical abstract



Highlights

- KP156, belonging to ST11-KL25 hv-CRKP, exhibits resistance and high virulence traits
- Virulence factors in the pKP156-Vir are essential contributors to its high virulence
- Resistance genes are situated within multiple transposons or conjugative elements
- KP156 shares high degree of genomic homology with other ST11 hv-CRKPs

Authors

Yuting Kang, Chao Xu, Wanting Ma, Qiujie Li, Wei Jia, Pengtao Wang

Correspondence

jiawei6365@126.com (W.J.), wangpengtao2021@163.com (P.W.)

In brief

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Genomic characterization of ST11-KL25 hypervirulent KPC-2-producing multidrug-resistant *Klebsiella pneumoniae* from China

Yuting Kang,[1,4] Chao Xu,[3,4] Wanting Ma,[3] Qiujie Li,[3] Wei Jia,[1,2,*] and Pengtao Wang[1,5,*]

[1]Ningxia Key Laboratory of Clinical and Pathogenic Microbiology, Institute of Medical Sciences, General Hospital of Ningxia Medical University, Yinchuan, Ningxia 750004, China

[2]Center of Medical Laboratory, General Hospital of Ningxia Medical University, Yinchuan, Ningxia 750004, China

[3]First Clinical Medical College, Ningxia Medical University, Yinchuan, Ningxia 750004, China

[4]These authors contributed equally

[5]Lead contact

*Correspondence: jiawei6365@126.com (W.J.), wangpengtao2021@163.com (P.W.)

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SUMMARY

The global prevalence of ST11 hypervirulent carbapenem-resistant *Klebsiella pneumoniae* (hv-CRKP) isolates has been increasingly documented, yet genomic characterization of this clone remains insufficiently explored. Here, we report a clinical ST11-KL25 hv-CRKP strain (KP156) that exhibited resistance to multiple antibiotics and demonstrated hypervirulence in a mouse infection model. Whole-genome sequencing revealed that KP156 harbored one virulence plasmid (pKP156-Vir) and two resistance plasmids (pKP156-KPC and pKP156-tetA). The pKP156-Vir contains several virulence factors, including *rmpA2* and *iucABCD*, which are critical contributors to its hypervirulence. The bla_{KPC-2} and $bla_{CTX-M-65}$ genes, located within the Tn*6296* transposon of pKP156-KPC, along with a multidrug-resistant (MDR) region containing multiple transposons and conjugative elements in pKP156-tetA, are associated with the transfer of resistance genes. Phylogenetic analysis indicates that KP156 shares high homology with other ST11 hv-CRKPs, suggesting potential transmission of this clone. Our study informs the development of genomic surveillance and control strategies for this strain.

INTRODUCTION

Klebsiella pneumoniae, an opportunistic nosocomial pathogen, is known to cause various infectious syndromes in both adults and newborns, including pneumonia, urinary tract infections, abdominal infections, and bacteremia.[1] This organism has emerged as a significant pathogen in global nosocomial infections.[2,3] Carbapenem-resistant *Klebsiella pneumoniae* (CRKP) poses a serious threat to public health worldwide, with high rates of morbidity and mortality.[4] There are four main types of carbapenemases identified globally: bla_{KPC}, bla_{OXA-48}, bla_{NDM}, and bla_{VIM}.[5] Among these, bla_{KPC} is prevalent in the United States, South America, and China, with bla_{KPC-2} being the most prevalent variant of KPC-like in China.[5] In Asia, the ST11 clone is the predominant strain of CRKP, accounting for 60% of carbapenem-resistant cases.[4] Infections caused by KPC-2-ST11 CRKP are increasingly challenging to treat due to their extensive antibiotic resistance and rapid dissemination.[6]

Hypervirulent *Klebsiella pneumoniae* (hvKP) was initially discovered in Chinese Taiwan in the late 1980s among patients with severe liver abscesses and is known to cause serious community-acquired infections in young and healthy individuals.[7] Its prevalence has increased in the Asia-Pacific region and is increasingly reported in other parts of the world.[8] Previous studies initially classified hvKP based on clinical severity or a hypermucoviscous phenotype, but subsequent research has shown that the high virulence of *K. pneumoniae* may not always be linked to hypermucoviscosity.[1] hvKP has been characterized by virulence markers located on specific plasmids, including *rmpA*, *rmpA2*, *iro*, *iuc*, and/or *peg-344*, which are essential for a high virulence phenotype.[1] Apart from virulence factors, the capsule type of *K. pneumoniae* also plays a role in hvKP. Over 100 capsule serotypes have been identified to date,[9] with reports indicating that the KL1 and KL2 serotypes are closely associated with hvKP.[9] The most notable virulence plasmids include the pLVPK plasmid from the KL2 hvKP strain CG43 and the pK2044 plasmid from the KL1 hvKP strain NTUH-2044, showing 96% coverage and 99% homology.[3] Although most hvKP are intrinsically resistant to ampicillin, they are susceptible to other antimicrobial agents.[6]

The coexistence of resistance and virulence plasmids within the same isolate has led to the emergence of hypervirulent CRKP (hv-CRKP) strains, which is a major concern in clinical settings. The emergence of the ST11 hv-CRKP strain in China, attributed to the acquisition of a pLVPK-like virulence plasmid by the ST11 CRKP, poses a significant threat.[10] This strain

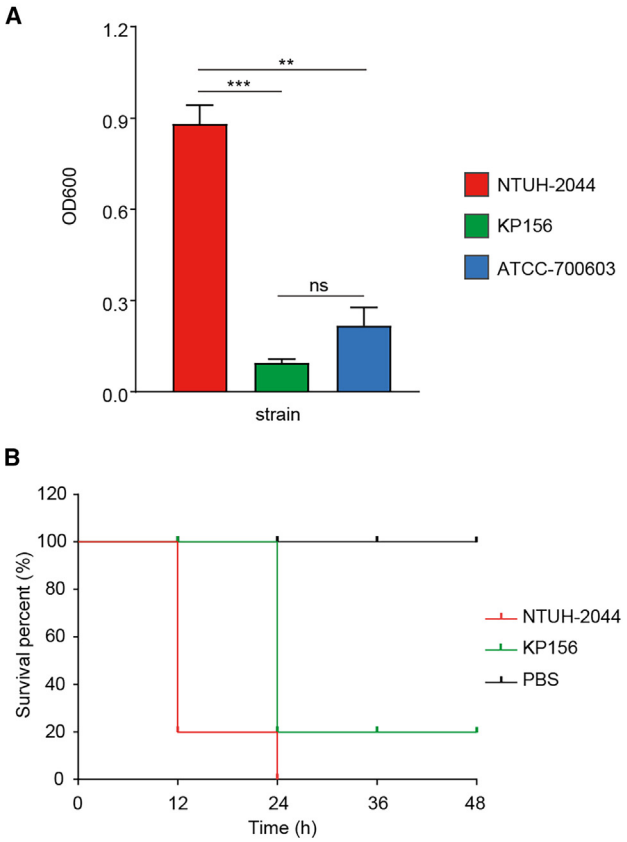


Figure 1. The viscosity and virulence phenotype of KP156 were measured

(A) The mucoviscosity experiment was conducted to determine the mucoviscosity levels of the strains. NTUH-2044 and ATCC-700603 served as controls for high mucoviscosity and low mucoviscosity, respectively.

(B) The mouse infection model was utilized to assess the virulence of KP156. NTUH-2044 was used as a positive control, and PBS was used as a negative control. Each group comprised 10 mice, and each mouse was injected with 5×10^7 CFU bacteria. The data are analyzed for differences between multiple groups by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons tests and presented as the mean ± SD of three independent experiments. The significance levels were defined as: ns: not significant, $^{**}p < 0.01$, $^{***}p < 0.001$.

has the capability to cause severe infections in otherwise healthy individuals and is difficult to treat with existing antibiotics, raising global alarm. Cases of ST11-hv-CRKP infections have been reported in various regions of China.[11] Chen et al. documented five fatal cases of KPC-2-bearing hypervirulent ST11 *K. pneumoniae* infections at the Second Affiliated Hospital of Zhejiang University.[12] In a separate study conducted by Fangyou Yu at a hospital in Shanghai, it was observed that ST11-KL64 hv-CRKP strains displayed increased multidrug resistance and virulence, posing a significant challenge to healthcare systems. Since 2016, the predominant ST11-KL64-CRKP strain in China has exhibited enhanced resistance to harsh conditions and heightened virulence. Evidence suggests that the pLVPK-like virulence plasmid is more frequently found in ST11-KL64 clones.[8] However, other capsular serotypes of ST11 hv-CRKP are rarely reported. In our investigation, we isolated a bla_{KPC-2}-producing ST11 hv-CRKP strain with KL25 serotype from Ningxia, China. This strain exhibited high virulence and multi-drug resistance characteristics. Whole-genome sequencing (WGS) was utilized to analyze the genetic information and transmission route, which highlights the importance of public awareness regarding the rapid spread of hv-CRKP strains.

RESULTS

Case report

A 65-year-old female patient was admitted to the General Hospital of Ningxia Medical University on September 25, 2022, with symptoms of hemoptysis and cough. Computed tomography (CT) scans revealed pulmonary nodules and patchy exudates, leading to a diagnosis that included pulmonary infection, bronchiectasis, bronchial artery malformations, and pulmonary embolism. The patient's total white blood cell count was 10.9×10^9 cells/L, with a neutrophil percentage of 76.1%, a lymphocyte relative value of 12.2%, and a C-reactive protein level of 5.4 mg/L, indicating lung infection. Sputum culture identified the presence of *K. pneumoniae* (KP156). The patient underwent arteriovenous fistula embolization, transcatheter bronchial artery embolization, and percutaneous internal thoracic artery embolization, followed by a 2-day course of ceftazidime. Following the treatment, her condition improved, and she was discharged on September 27.

Characteristics of resistance and virulence of KP156

Drug sensitivity testing revealed that KP156 displayed resistance to carbapenems (imipenem and meropenem), cephalosporins (cefepime), aminoglycosides (amikacin and gentamicin), quinolones (ciprofloxacin and levofloxacin), and tetracycline (tigecycline) among the tested antibiotics. The strain was only susceptible to colistin and ceftazidime/avibactam. These findings suggest that KP156 is a multi-drug resistant strain, severely limiting the options for antibiotic therapy. The results are summarized in Table S1. To assess the mucoviscosity and capsular polysaccharide (CPS) levels of KP156, we performed a mucoviscosity assay. Previous studies have reported that NTUH-2044 demonstrates hypervirulence and hypermucoviscosity.[13] The results of the mucoviscosity assay revealed that KP156 displays low mucoviscosity, which is comparable to that of the low-mucoviscosity strain ATCC-700603 and is lower than that observed in the hypermucoviscosity strain NTUH-2044 (Figure 1A). To evaluate the virulence of KP156, a mouse model of intraperitoneal infection was utilized, with NTUH-2044 serving as the hypervirulent control strain and PBS as the negative control. The survival rate of mice infected with KP156 was 20% compared to 0% in the NTUH-2044 group, whereas those treated with PBS demonstrated a 100% survival rate at 24 h. These results suggest that KP156 exhibits a virulence profile similar to that of strain NTUH-2044 (Figure 1B). As a result, we conclude that KP156 is a hypervirulent CRKP strain.

Overview of genomic information of KP156

To investigate the genetic basis of multidrug resistance (MDR) and high virulence in KP156, we conducted whole genome

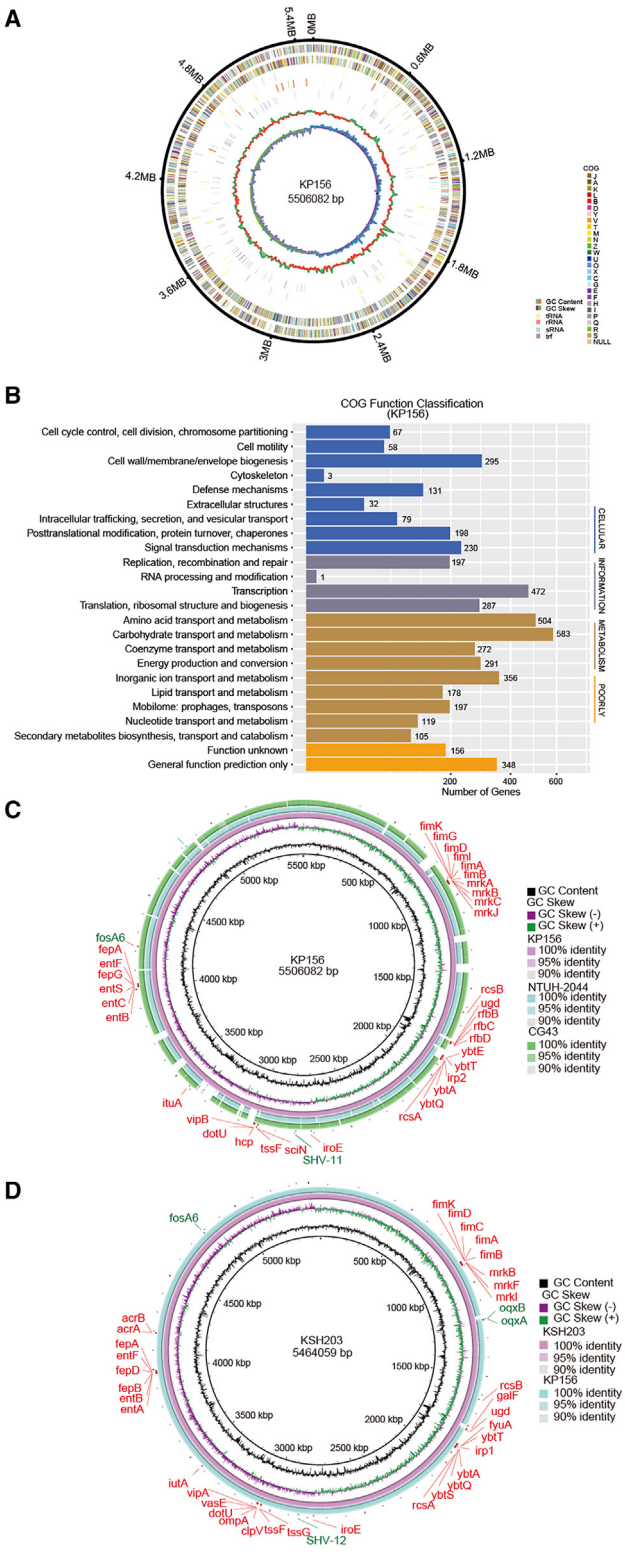


Figure 2. WGS was utilized to acquire the genome data of KP156

(A) The chromosome genomic information of KP156 was depicted.

(B) The COG functional categories for KP156 were identified using the NCBI COG database along with its respective function descriptions.

sequencing (WGS) and obtained relevant genetic details. The average coverage depth of KP156 is 183X, with completeness encompassing one bacterial chromosome and three plasmids, achieving a coverage of 100%. A joint analysis of GC-Depth indicates that the sequencing is free from contamination. The genome of KP156 comprises a 5.51 Mb chromosome (KP156-C) and three plasmids: a 216 kb plasmid (pKP156-Vir), a 121 kb plasmid (pKP156-KPC), and an 87 kb plasmid (pKP156-tetA). Bioinformatics analysis provided an overview of the chromosome sequences, including a GC content of 57.31%, 4,728 predicted protein-coding genes, an average gene length of 876 bp, and a coding region proportion of 89.1%. Analysis of COG functional data highlighted that the main proteins were involved in carbohydrate transport and metabolism (11.2%), amino acid transport and metabolism (9.7%), transcription (9.1%), and inorganic ion transport and metabolism (6.8%) (Figures 2A and 2B).

Sequence comparison results revealed that KP156-C and NTUH-2044-chromosome (GenBank accession number NC_012731) shared a high level of similarity, with 99.42% identity and 88% coverage. Additionally, KP156-C exhibited 99.40% identity and 88% coverage with CG43-chromosome (GenBank accession number NC_022566) (Figure 2C). Sequence type (ST) and serotype analysis identified KP156 as belonging to ST11 and KL25. A comparison between KP156-C and KSH203 chromosome, which belongs to another ST11-KL25 CRKP strain, revealed a 99.99% identity and 99% coverage, suggesting a close homology between the two strains (Figure 2D).

A total of 71 virulence genes were identified in KP156, including those from the *ent*, *fep*, *ytb*, *rfb*, *mrk*, and *fim* families, as well as *iutA* and *iroE*, with 65 of these located within chromosomes (Table S2). Additionally, six virulence genes were found on plasmid pKP156-Vir, consisting of five virulence marker genes (*rmpA2* and *iucABCD*) along with *iutA*. Notably, no virulence genes were detected on the other two resistance plasmids. Regarding resistance genes, KP156 harbored a total of 15, with 2 located on chromosomes (bla_{SHV-11} and *fosA6*) in addition to some efflux pump genes. Plasmid pKP156-KPC harbored five drug resistance genes, including the carbapenemase gene bla_{KPC-2}, three β-lactamase genes ($bla_{CTX-M-65}$, bla_{SHV-12}, and bla_{TEM-1}), and an aminoglycoside resistance gene *rmtB*, with duplications of bla_{KPC-2} and $bla_{CTX-M-65}$. Furthermore, plasmid pKP156-tetA contained six drug resistance genes: *tet(A)*, *qnrS1*, bla_{LAP-2}, *catA2*, *sul2*, and *dfrA14* (Table S1).

Genome characteristics of virulence plasmid of KP156 (pKP156-Vir)

The pKP156-Vir plasmid has a total nucleotide sequence length of 216 kb, with an average GC content of 50.27% and 241 open reading frames identified (Table S1). This plasmid

(C) A comparative genomic analysis was performed between the KP156 strain and hypervirulent *Klebsiella pneumoniae* strains, including NTUH-2044 and CG43, where red indicates virulence genes and green indicates resistance genes.

(D) A comparative genomic analysis was conducted between the KP156 strain and KSH203, a KL25 ST11 CRKP strain, with red denoting virulence genes and green denoting resistance genes.

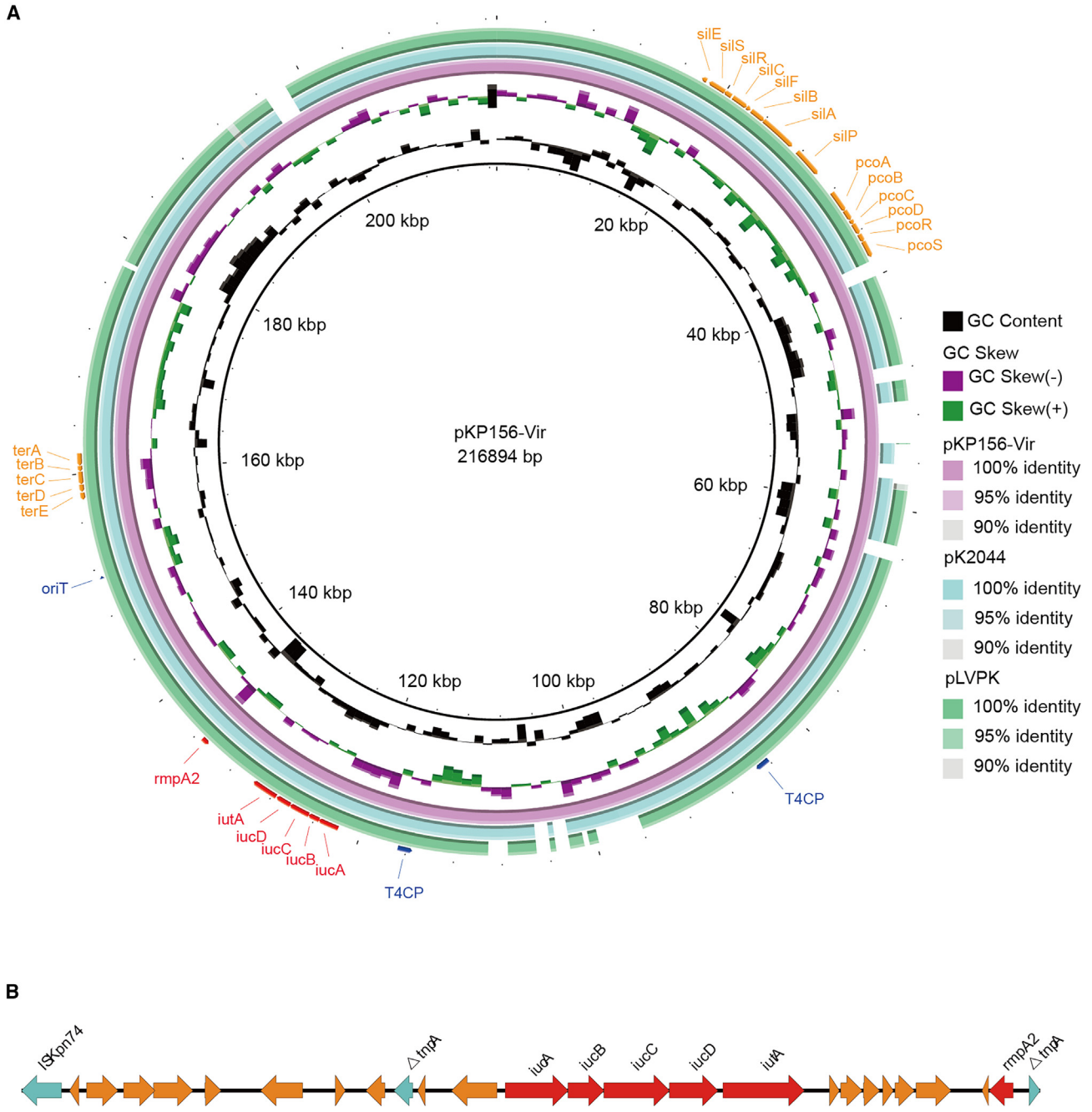


Figure 3. Analysis of virulence factors and transposable elements of pKP156-Vir was performed

(A) Alignment of pKP1156-Vir was performed against two known virulence plasmids, namely pLVPK (Accession No. AY378100) and pK2044 (Accession No. NC_006625).

(B) Analysis of upstream and downstream transposable elements associated with virulence factors was conducted. In the alignment, red indicates virulence genes, blue represents conjugation elements, and orange denotes metal resistance genes.

harbors two types of plasmid replication initiation genes: IncHI1B (pNDM-Mar) and repB. The virulence factors (*rmpA2*, *iucABCD*, and *iutA*) are located within this virulence plasmid. Comparative genomics analysis of plasmids revealed that pKP156-Vir and pK2044 exhibit 99% identity and 93% coverage, while also showing 99% identity and 90% coverage with pLVPK (Figure 3A). It is noteworthy that pKP156-Vir does not possess certain virulence factors present in pK2044 and pLVPK, such as *rmpA*, *peg-344*, and *iroBCDN*, despite demonstrating significant virulence characteristics. This finding

emphasizes the importance of other existing virulence factors in influencing the overall virulence observed. Several mobile elements, including Δ*tnpA*-IS*630*, Δ*tnpA*-IS*Ec16*, and IS*Kpn74*, were identified within the virulence region (Figure 3B). Although the plasmid includes an *oriT* gene and T4CP, it lacks other conjugative elements like relaxase and T4SS system, indicating a passive transfer mechanism aided by other auxiliary plasmids. Furthermore, the plasmid carries genes associated with heavy metal resistance, including the *ter*, *pco*, and *sil* families, which allow bacteria to survive in harsh environment.

Genome characteristics of *bla*$_{KPC-2}$ plasmid (pKP156-KPC)

The drug-resistance plasmid pKP156-KPC, possessing the IncFII(pHN7A8)/IncR replicons, harbors multiple drug-resistant genes. It contains two copies of *bla*$_{KPC-2}$, surrounded by the *tnpR*-IS*Kpn27*-*bla*$_{KPC-2}$-ΔIS*Kpn6*-*korC*-*klca*-Δ*repB*-*tnpR*-*tnpA* transposon, a component of transposon Tn*6296*, suggesting possible transposable replication of *bla*$_{KPC-2}$. The *bla*$_{CTX-M-65}$ gene is flanked upstream by the IS*26* sequence and downstream by the IS*Kpn6* insertion sequence of Tn*6296* (Figure 4A). Additionally, the IS*26* sequence and transposase gene *tnpA* are located around the *rmtB* and *bla*$_{TEM-1}$ genes. Linear comparative genomics analysis of the plasmids indicated that the pKP156-KPC shares high homology with other resistance plasmids that co-produce *bla*$_{KPC-2}$ and *bla*$_{SHV-12}$ in ST11 CRKP strains. In particular, the pKP156-KPC showed 100% sequence identity and 99% coverage when compared to hvKp-C789, a known resistance plasmid from a KPC-2-ST11 CRKP recovered in China[14] (Figure 4B).

Genome characteristics of *tet(A)* plasmid (pKP156-tetA)

The drug-resistance plasmid pKP156-tetA is 87 kb in length and shares 99.99% identity and 100% coverage with p3. It carries the MDR region between approximately 60 kb and 80 kb, which severely limits the availability of effective antibiotics (Figure 5A). Within this region, various transposon sequences have been identified: *sul2* in the truncated Tn*6302*, *catA2* in the truncated Tn*6383*, *tet(A)* in the truncated Tn*1721*, and *qnrS1* in the truncated Tn*6361* (Figure 5B). These transposons are crucial for facilitating the replication and transfer of resistance genes. An integron system is formed by the upstream *intI* and downstream *attC* of *dfrA14*, allowing *dfrA14* to be integrated into other chromosomes or plasmids. Furthermore, there are numerous transfer elements ranging from approximately 10 kb–50 kb, including *oriT*, relaxase, T4CP, and the T4SS system, suggesting that pKP156-tetA could be transferred via conjugative elements (Figure 5A).

Phylogenetic analysis of KP156 in relation to other ST11 hv-CRKPs

To investigate the evolutionary prevalence of KP156, we screened four additional strains of ST11 hv-CRKPs (KP13, KP22, KP33, KP85) and one strain of ST65 hv-CRKP (KP114), followed by WGS. Using KP114 as the reference genome, we constructed an evolutionary tree based on single nucleotide polymorphisms (SNPs). The high virulence characteristics of hv-CRKPs were assessed using a mouse infection model (Figure S1). Similar to KP156, all other ST11 strains possess at least the virulence genes *rmpA2*, *iucABCD-iutA*, and the carbapenemase gene *bla*$_{KPC-2}$, exhibiting a close evolutionary relationship (Figure 6A). This finding suggests a potential risk of clonal transmission of ST11 hv-CRKPs within our hospital. Furthermore, the presence of the Tn*6296* transposon within the KPC gene region of various ST11 hv-CRKPs implies that the dissemination of KPC-2 may significantly contribute to the widespread occurrence of carbapenem resistance in hospital settings (Figure 6B).

DISCUSSION

In China, CRKP poses a significant threat to public health, particularly the ST11 clone strain that produces *bla*$_{KPC-2}$.[15,16] Recently, the convergence of high virulence and drug resistance characteristics has led to the emergence of hv-CRKP, further endangering public health. From Ningxia, we isolated a *bla*$_{KPC-2}$-producing ST11 CRKP strain, KP156, which possesses a KL25 capsular serotype, a novel and rarely reported type. The strain exhibited high virulence traits, as evidenced by a significant mortality rate in a mouse infection model. Susceptibility tests indicated its sensitivity to colistin and ceftazidime/avibactam but resistance to 23 other tested antibiotics. The patient was successfully treated with ceftazidime and discharged. WGS and bioinformatics analysis revealed that the drug resistance and virulence traits of KP156 are associated with the presence of multiple resistance and virulence genes, along with diverse mobile genetic elements. This indicates the possibility of these factors being mobile within and between bacterial species, underscoring the significance of genomic surveillance for emerging pathogens and the pressing requirement for improved infection prevention and control strategies.

Genome sequencing analysis revealed that KP156 belongs to sequence type 11 (ST11) and serotype KL25. Serotypes KL1 and KL2 were found to have superior capsular characteristics among hvKP.[17–19] Furthermore, hvKP has been associated with serotypes KL5, KL20, KL54, KL57, KL47, and KL64.[1,5] Among these, the ST11-KL64 strain carrying the *bla*$_{KPC-2}$ gene is identified as the most prevalent hv-CRKP strain in China.[20] It has been hypothesized that KL64 isolates may have evolved from KL47 ancestors, with ST11-KL64 isolates showing an increased fitness over their KL47 counterparts.[21,22] Although KL25 is rarely reported, in 2020, a strain designated KSH203, classified as KL25 ST11-CRKP, was discovered in Henan Province, China.[23] Whole-genome sequence comparison indicated that chromosome KP156 shared higher homology with KSH203 than with the hypervirulent strains CG23 and NTUH-2044, suggesting a common origin between KP156 and KSH203. Hv-CRKP may evolve from various pathways, including the acquisition of a pLVPK-like virulence plasmid by CRKP[24] or the acquisition of drug resistance genes by hvKP.[25] It has been reported that hv-CRKP primarily evolved from CRKP through the acquisition of a virulence plasmid.[26] Given that the prevalent CRKP strain in China is ST11-KPC2, we speculate that KP156 evolved from ST11-KL25-KPC2 to acquire the virulence plasmid. These findings imply ongoing evolution and dissemination of hvKP.

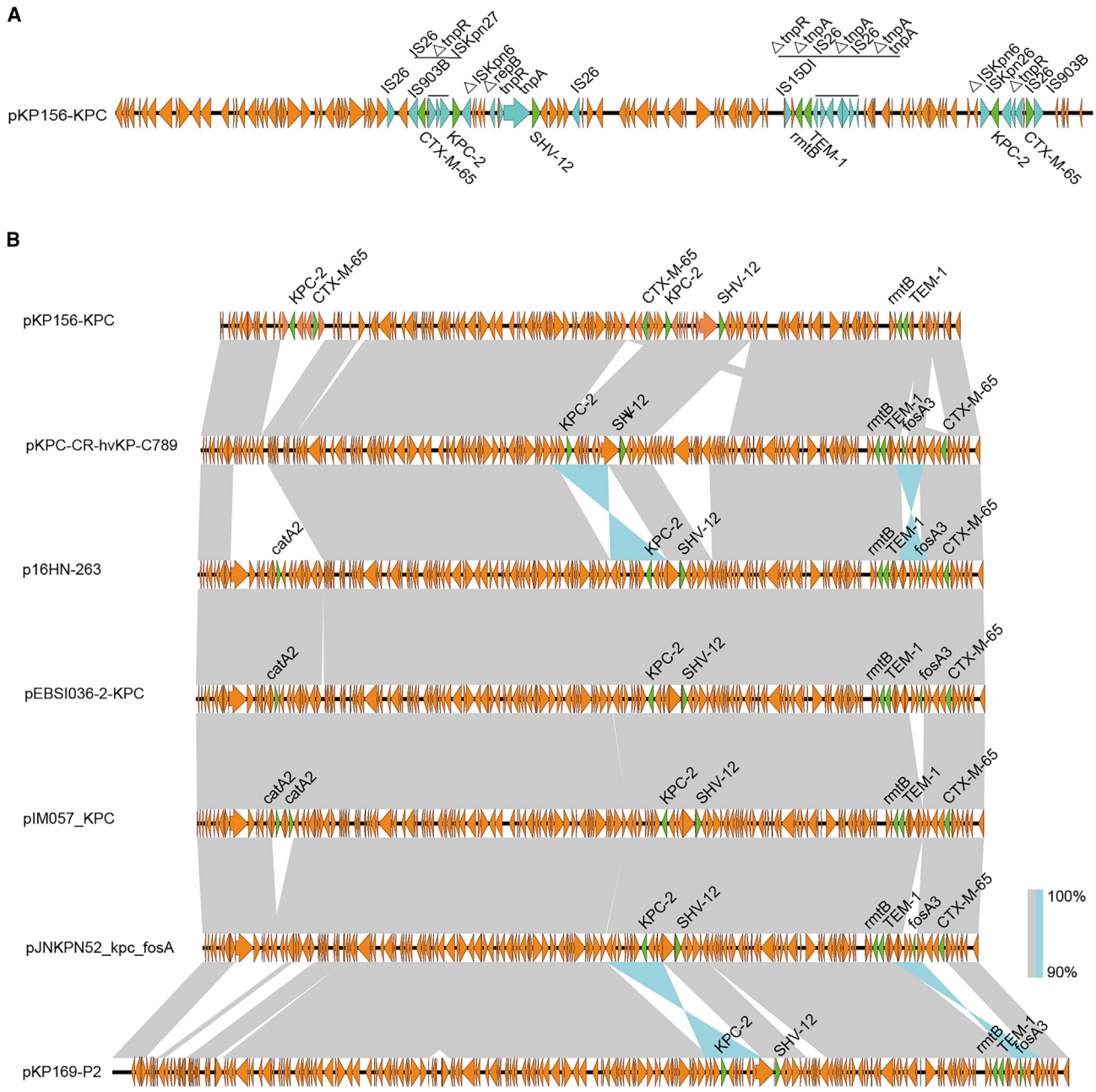
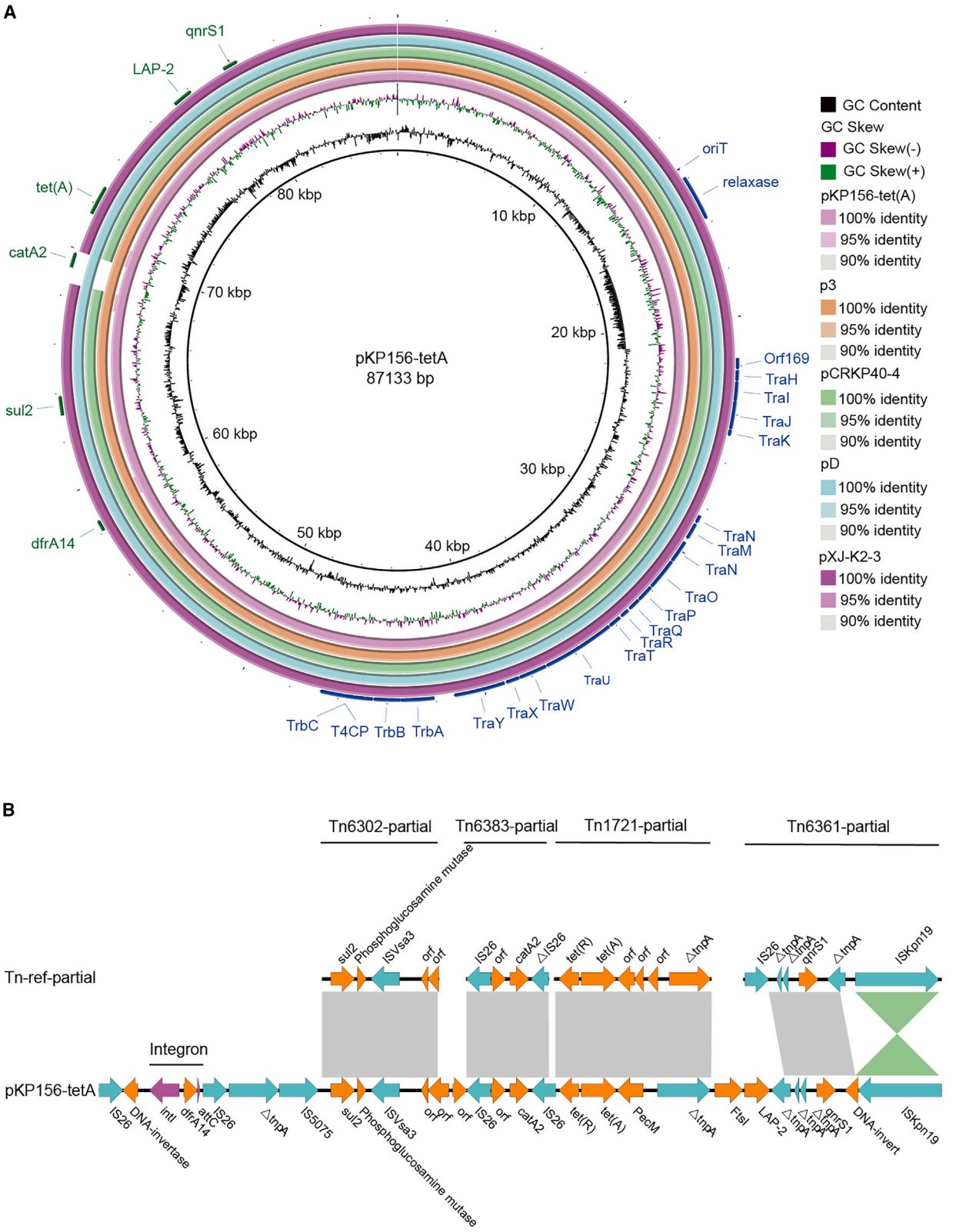


Figure 4. Analysis of virulence factors and transposable elements of pKP156-KPC was performed

(A) Analysis of upstream and downstream transposable elements associated with resistance factors was conducted.

(B) Alignment of pKP156-KPC was performed against six known resistance plasmids in KPC-2-ST11 CRKP strains from different regions of China. Green indicates resistance genes, and baby blue represents transposable elements.

Among the prevalent carbapenemase coding genes, such as bla_{KPC}, bla_{NDM}, bla_{OXA}, bla_{IMP}, and bla_{VIM}, bla_{KPC-2} is showing a rapid increase in China, raising concerns.[21] The bla_{KPC-2} genes in ST11 CRKP strains in the USA are predominantly located in Tn*4401* transposon (NTE_{KPC}), whereas non-Tn*4401* elements are common transfer vehicles for bla_{KPC-2} genes in Chinese isolates. The Tn*1721* transposon plays a crucial role in facilitating the horizontal transfer of the bla_{KPC-2} gene, contributing to the molecular diversification of ST11 CRKP strains.[21] Furthermore, the Tn*6296* transposon is identified as a significant mobile platform carrying bla_{KPC-2} in China,[27] aligning with our findings in the pKP156-KPC plasmid. In addition to bla_{KPC-2}, the extended-spectrum β-lactam resistance gene $bla_{CTX-M-65}$ is also located within the Tn*6296* and is present in duplicate on the pKP156-KPC plasmid along with bla_{KPC-2}. The MIC value of KP156 for imipenem and meropenem is ≥ 32.0 μg/mL, which does not



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align with the results of three copies of bla_{KPC-2} presented in pJCL-4, showing the MIC value for imipenem and meropenem to be $\geq$ 128 μg/mL.[28] Although the increase in bla_{KPC-2} copy number on the plasmid did not enhance the resistance of KP156 to β-lactam antibiotics, its presence may have heightened the likelihood of bla_{KPC-2} mutations, leading to the generation of new bla_{KPC} variants that can adapt to the pressure of new antibiotics, as demonstrated in previous studies.[28,29] Major bla_{SHV} variants in ST11 CRKP, including bla_{SHV-12}, bla_{TEM-1},[21] and the aminoglycoside resistance gene *rmtB*, were also identified in the pKP156-KPC with IncFII(pHN7A8)/IncR replicon. Similar multidrug-resistant ST11 CRKP strains with the same replicon have also been reported in Chinese Taiwan and Egypt.[27] Plasmids carrying the bla_{KPC} or bla_{NDM} often contain the IncFII replicon, congruent with other replicons like IncFIB, IncR, and repBR1701, expanding the range of host strains.[27] The spread of bla_{KPC} in ST11 CRKP is frequently associated with the IncFII(pHN7A8) plasmid, which often carries additional replicons such as IncR replicons. These plasmids have been predominantly reported in China, aligning with the high prevalence of ST11 CRKP in the area.[27] Further genomic analysis uncovered a significant similarity between the pKP156-KPC and pKPC-CR-hvKP-C789,[20] both carrying bla_{KPC-2} and $bla_{CTX-M-65}$ genes, showing 99% query coverage and 100% identity. This suggests an epidemiologic link between these two isolates and that plasmid acquisition was involved.

Another resistance plasmid, pKP156-tetA, belonging to an unknown Inc-type, harbors a multidrug-resistant (MDR) region with a variety of drug-resistant genes, such as *dfrA14*, *sul2*, *catA2*, *tet(A)*, bla_{LAP-2}, and *qnrS1*. These genes confer resistance to trimethoprim, sulfonamides, chloramphenicol, tetracycline, β-lactamase, and quinolones, respectively. This observation explains the *in vitro* resistance of KP156 to tigecycline, despite the patient not being treated with tigecycline. Furthermore, these genes are integrated into an integron (*intI-attC*) and various transposable elements (Tn*6302*, Tn*6383*, Tn*1721*, Tn*6361*), aiding in the spread of these resistance genes. The pKP156-tetA plasmid shows significant similarity to previously characterized plasmids with the same MDR region, such as p3, pD, pCRKP40-4, and pXJ-K2-p3, which were isolated from hospitals in Hengyang, Wuxi, Wuhan, and Chengdu, respectively.[5,30–32] The presence of conjugation transfer elements (*oriT*, relaxase, T4CP, T4SS) in these plasmids may contribute to their dissemination across China. The chromosome of KP156 also contains resistance genes (bla_{SHV-11}, *fosA6*) that enhance resistance to antibiotics, which must be carefully considered. Mobile genetic elements (MGEs), including transposons, insertion sequences, and conjugative mobile genes, play a significant role in the spread of resistance genes among plasmids and chromosomes. These elements can also lead to plasmid reorganization, ultimately increasing resistance levels.[27]

The mouse infection experiment with KP156 demonstrated its high virulence characteristics, as supported by the presence of several virulence factors in the pKP156-Vir, including the aerobactin synthetic operon (*iucABCD*), the aerobactin receptor of the outer membrane (*iutA*), and the regulator of mucoid phenotype A2 (*rmpA2*).[12] A low level of mucoid production was observed in KP156, which is consistent with findings from other studies.[33] Notably, the majority of ST11-KL64 hvKP strains that possess *rmpA* or *rmpA2* are non-mucoid.[17] Current research indicates that hvKP and high-mucus-producing *K. pneumoniae* should not be regarded as synonymous.[34] It is plausible that the expression of a highly viscous phenotype involves a complex interplay of multiple genes beyond *rmpA2*.[35,36] Vaccines targeting surface antigens, such as the K antigen, are considered a promising alternative for the future treatment of MDR bacteria. Research has demonstrated that anti-capsular vaccines provide resistance to *K. pneumoniae*; however, their implementation in clinical practice remains lacking.[18,37–39] Hu et al. identified that the prevalent CRKP strains in China are primarily ST11-KL64 and ST11-KL47.[26] In this context, we identified a strain of ST11-KL25 CRKP, which has been shown to exhibit high virulence in a mouse infection model. This finding presents new challenges for clinical treatment and vaccine development. The chromosome of KP156 contains virulence factors such as yersiniabactin, enterobactin, salmochelin gene *iroE*, and other iron chelator genes (*fyuA*, *fepA*, *irp1*, *irp2*), which significantly enhance the virulence phenotype of the strain. Both pKP156-Vir and pLVPK, as well as pK2044, share the same replicon IncHI1B(pNDM-Mar)/repB, *oriT*, and T4CP genes, but they lack both the relaxase and type IV secretion systems.[27] The mechanism by which the IncHI1B plasmid is transferred into ST11 CRKP is both interesting and complex. The homologous regions identified between the non-conjugative virulence plasmid and conjugative KPC-2-bearing MDR plasmid indicate a potential mechanism for the transmission of the virulence plasmid from ST23 *K. pneumoniae* to ST11 CRKP.[40] It is hypothesized that the conjugative plasmid pKP156-tetA may facilitate the binding and transfer of pKP156-Vir to ST11 CRKP, although experimental validation is required to confirm this hypothesis. In comparison to the ST65-KL2 strain, KP156 exhibits a closer relationship with other ST11-KL47 and ST11-KL64 hvKP isolates from our hospital. Neutrophil phagocytosis and Galleria mellonella lethality experiments have demonstrated that certain ST11-CRKP strains carrying the *iucA* and *rmpA2* genes are classified as hv-CRKP.[24] Our sample of ST11-hv-CRKP also harbors at least the *rmpA2* and *iucA* genes, all of which carry the bla_{KPC-2} gene, suggesting the potential for nosocomial transmission of ST11-KPC2-hv-CRKP. The conjugation assay demonstrates that the bla_{KPC-2} gene cannot be transferred from KP156 to *Escherichia coli* EC600 or DH5α. The pKP156-KPC plasmid lacks essential components of the transfer system, including the *traO*, relaxase, and T4CP genes, which may account for its inability to facilitate conjugation and transfer..[41] Nevertheless, the prevention and control of ST11-KL25-CRKP transmission should not be underestimated, as the IncFII-like plasmid

Figure 5. Analysis of virulence factors and transposable elements of pKP156-tetA was performed

(A) Alignment of pKP156-tetA was performed against four known resistance plasmids carrying *tet(A)* in ST11 CRKP strains from various regions of China.

(B) Analysis of upstream and downstream transposable elements associated with resistance factors was conducted. Green indicates resistance genes, and orange represents transposable elements.

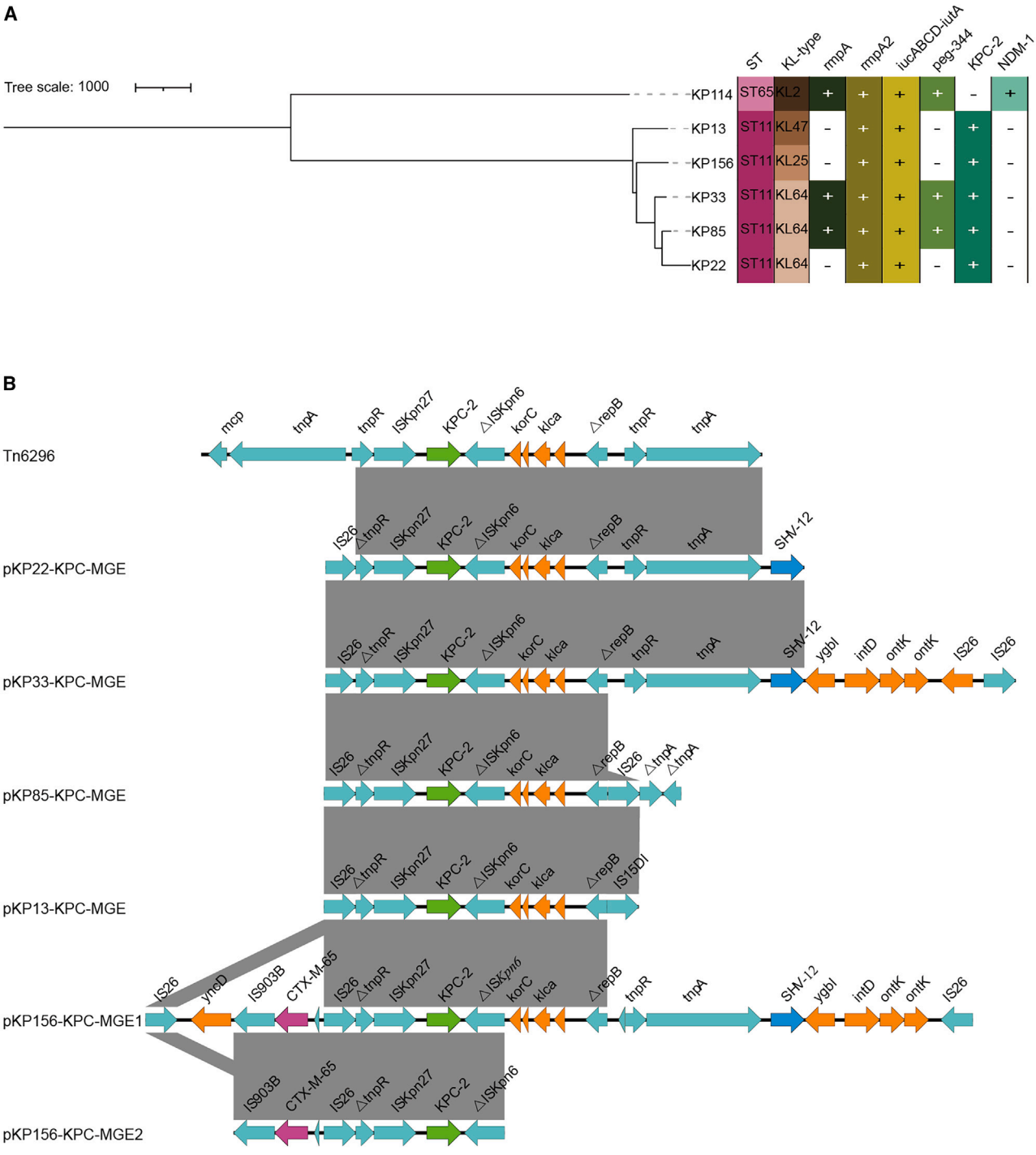

Figure 6. Phylogenetic tree and KPC-related MGEs comparative genomic analysis were performed

(A) An NJ tree is constructed based on single nucleotide polymorphisms, using ST65 KP114 as the reference genome. ST and KL types are annotated with specific designations. In the remaining annotation information, "+" indicates the presence of the gene, whereas "−" indicates its absence. The evolutionary tree is enhanced visually using iTOL.

(B) Sequence alignment of KPC-gene-related mobile genetic elements (MGEs) among various ST11 hypervirulent carbapenem-resistant *Klebsiella pneumoniae* (hv-CRKP) strains, using the Tn*6296* transposon as the reference sequence.

harbored by KP156 is readily conjugative and has the potential to spread.[42]

Conclusion

In this study, we identified a KPC-2-ST11-CRKP strain with a KL25 capsular serotype in Ningxia, characterized by extensive antibiotic resistance, and hypervirulence traits. Through comprehensive whole genome sequencing, we investigated its genetic makeup and potential transmission routes. This clone harbors numerous resistance genes, virulence factors, and mobile elements, which pose significant challenges for treatment and facilitate transmission. Therefore, it is imperative to enforce stringent surveillance and infection control measures in healthcare facilities to limit the spread of these strains.

Limitations of the study

Several limitations must be addressed in this study. Firstly, the correlation between KL25 capsular serotype and virulence trait requires verification through additional experiments. Secondly, our institution has not yet established a comprehensive monitoring and prevention system for ST11-hv-CRKP, primarily due to insufficient communication with the clinic and a high patient volume in the hospital. Thirdly, all mice used in the mouse infection model were female ICR mice; a balanced ratio of male to female mice (1:1) may yield more robust results. Lastly, additional screens of ST11 hv-CRKPs in the region are necessary to examine its distribution and transmission routes. This will enhance our understanding of the transmission mechanisms and help prevent the further spread of these clones in hospital settings.

RESOURCE AVAILABILITY

Lead contact

Further information or requests for reagents resources, and data should be addressed to the lead contact, Pengtao Wang (wangpengtao2021@163.com).

Materials availability

- This study did not generate new unique reagents.
- There are restrictions to the availability of the *Klebsiella* clinical isolate KP156 used in this study because it was stored in the hospital system that restricts distribution of the bacteria, but we can guide you to the proper contact.

Data and code availability

- WGS data of KP156, KP13, KP22, KP33, KP85, and KP114 have been deposited at NCBI Genbank database and are publicly available as of the date of publication. Accession numbers are listed in the key resources table.
- The sequence data of chromosomes and plasmids cited in this paper, including KSH203-Chromosome, NTUH-2044-Chromosome, CG43-Chromosome, pKP169-P2, pKPC-CR-hvKP-C789, pJNKPN52_kpc_fosA, pIM057_KPC, p16HN-263, pEBSI036-2-KPC, p3, pCRKP40-4, pD, pXJ-K2-3, pK2044, pLVPK, Tn*6296*, are extracted from other studies. Accession numbers and references are listed in the key resources table.
- Code of kSNP3 used in this study have been deposited at Figshare database and are publicly available as of the date of publication. DOI accession numbers are listed in the key resources table.
- Any other information reported in this paper will be shared by the lead contact upon request.

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AUTHOR CONTRIBUTIONS

Y.K., W.J., and P.W. designed the study. Y.K., C.X., and W.M. performed all experiments; Y.K., C.X., and Q.L. analyzed and interpreted the data. Y.K., W.J., and P.W. wrote and edited the article. All authors were involved in preparing the final manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and Viruses Strains		
K.pneumoniae KP156	This study	KP156
K.pneumoniae KP13	This study	KP13
K.pneumoniae KP22	This study	KP22
K.pneumoniae KP33	This study	KP33
K.pneumoniae KP85	This study	KP85
K.pneumoniae KP114	This study	KP114
K.pneumoniae NTUH-2044	Laboratory stock	NTUH-2044
K.pneumoniae ATCC-700603	Laboratory stock	ATCC-700603
Chemicals, peptides, and recombinant proteins		
LB Broth, powder	Solarbio	Cat: L8291
Deposited Data		
Sequence of KP156	This study	Genbank: CP154301, CP154303, CP154304, CP154305
Sequence of KP13	This study	Genbank: CP154632, CP154633, CP154634
Sequence of KP22	This study	Genbank: CP154293, CP154294, CP154295, CP154296
Sequence of KP33	This study	Genbank: CP154391, CP154392, CP154393, CP154394, CP154395
Sequence of KP85	This study	Genbank: CP154396, CP154397, CP154398, CP154399, CP154400
Sequence of KP114	This study	Genbank: CP152422, CP152423, CP152424
Sequence of KSH203-Chromosome	Fu et al.[23]	Genbank: CP034327
Sequence of NTUH-2044-Chromosome	Wu et al.[13]	Genbank: NC_012731
Sequence of CG43-Chromosome		Genbank: NC_022566
Sequence of pKP169-P2	Huang et al.[43]	Genbank: NZ_CP078123
Sequence of pKPC-CR-hvKP-C789	Zhang et al.[20]	Genbank: NZ_CP034417
Sequence of pJNKPN52_kpc_fosA	Hao et al.[44]	Genbank: NZ_MZ709016
Sequence of pIM057_KPC	Tang et al.[45]	Genbank: NZ_CP095423
Sequence of p16HN-263	Yang et al.[4]	Genbank: NZ_CP045264
Sequence of pEBSI036-2-KPC	Ahmed et al.[46]	Genbank: NZ_MT648513
Sequence of p3	Zhang et al.[5]	Genbank: CP054747
Sequence of pCRKP40-4	Hu et al.[31]	Genbank: CP107324
Sequence of pD	Hu et al.[30]	Genbank: CP069176
Sequence of pXJ-K2-3	Zhu et al.[32]	Genbank: CP032243
Sequence of pK2044	Wu et al.[13]	Genbank: NC_006625
Sequence of pLVPK	Chen et al.[47]	Genbank: AY378100
Sequence of Tn*6296*	Guo et al.[48]	Genbank: FJ628167
Experimental models: Organisms/strains		
ICR mice	Viewsolid	VSM10004
Software and Algorithms		
SPAdes software	Prjibelski et al.[49]	SPAdes
RAST software	Overbeek et al.[50]	RAST
Prokka software	Torsten Seemann.[51]	Prokka

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Kaptive tool	Wick et al.[52]	Kaptive
ResFinder tool	Florensa et al.[53]	ResFinder
PlasmidFinder tool	Carattoli et al.[54]	PlasmidFinder
VFDB dataset	Liu et al.[55]	VFDB
ISfinder tool	Siguier et al.[56]	ISfinder
VRprofile2 tool	Wang et al.[57]	VRprofile2
BRIG software	Alikhan et al.[58]	BRIG
Easyfig software	Sullivan et al.[59]	Easyfig
kSNP3 software	Gardner et al.[60]	kSNP3
Code for kSNP3 software	https://doi.org/10.6084/m9.figshare.27695658.v1	kSNP3
iTOL tool	Letunic et al.[61]	iTOL

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Bacterial strain

The hypervirulent KPC-2-producing multidrug-resistant *Klebsiella pneumoniae* KP156 was collected from the hospital information system of General Hospital of Ningxia Medical University. Initially, sputum was collected from a female patient, and then KP156 was obtained by sputum culture. Then we determined its mucoviscosity and identified KP156 as hv-CRKP by antimicrobial susceptibility testing and mouse intraperitoneal infection model. Then WGS was used to obtain sequence information of KP156, and subsequently we determined that KP156 was ST11 and KL25, and additional analysis including comparative genomics analysis were performed. Other *Klebsiella pneumoniae* isolates (KP13,KP22,KP33,KP85,KP114) in this study were collected and analyzed same to the procedure of KP156. *Klebsiella pneumoniae* NTUH-2044 and ATCC-700603 were obtained from laboratory stock. All *Klebsiella pneumoniae* isolates were cultured in Luria-Bertani (LB) broth at 37°C with shaking at 200 rpm.

Mice

The experimental animals used in this study were healthy female ICR mice, aged 6–8 weeks, purchased from Viewsolid. Animal experiment described in this study was approved by Medical Science Research Ethics Committee IRB of the General Hospital of Ningxia Medical University (Z2022/025, approved 10 November 2022).

METHOD DETAILS

Collection of clinical information and identification of bacterium

Case information on patients infected with *Klebsiella pneumoniae* (KP156) was collected from the hospital information system of General Hospital of Ningxia Medical University. The KP156 isolate was identified using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS).

Antimicrobial susceptibility testing

The minimum inhibitory concentration (MIC) values were performed by VITEK-2 compact system (bioMérieux, Marcy-l'Étoile, France), and the results were interpreted as recommended by the Clinical and Laboratory Standards Institute (CLSI, M100-S31), with *Escherichia coli* (ATCC25922) being the quality control bacterium.

Mucoviscosity assay

The mucoviscosity of the test strains was determined using a sedimentation assay.[62] Briefly, overnight cultures grown in LB media (37°C, 200rpm) were subcultured to an optical density (OD_{600}) of approximately 0.2 in fresh LB media and incubated at 37°C with shaking at 200 rpm for about 4h. Cultures were then normalized to an OD of 1.0 mL^{-1} and centrifuged for 5 min at 2500×g. The supernatant was gently removed without disturbing the pellet to measure the OD_{600}. The bacteria were cultured overnight on blood agar plates, then string test was performed by stretching the bacterial colonies on blood agar plates using an inoculation loop.[63] Results are presented as the mean and standard deviation (s.d.) of data from three independent experiments. One-way ANOVA test and post hoc test were conducted to analyze the statistical differences among the test strains. The analysis was performed using Prism 8 software.

Mouse intraperitoneal infection model

In accordance with Liu's study, intraperitoneal injections were administered to mice in order to assess the virulence characteristics of KP156.[64] Female ICR mice aged 6–8 weeks were sourced from Viewsolid. NTUH-2044 and 1×PBS were employed as the positive and negative controls for hypervirulence, respectively. Ten mice in each group were injected intraperitoneally with 5×10^7 CFU of NTUH-2044 or KP156, or an equivalent volume of 1×PBS. Subsequently, the survival of the mice was monitored for a period of 48 h. The methodology employed for mouse infection models for other strains, including KP13, KP22, KP33, KP85, and KP114, was consistent with that used for KP156. The analysis was performed using Prism 8 software. This study was approved by Medical Science Research Ethics Committee IRB of the General Hospital of Ningxia Medical University (Z2022/025, approved 10 November 2022).

Whole genome sequencing

Genomic DNA extracted from the KP156 strain and other strains (KP13, KP22, KP33, KP85, KP114) were subjected to whole genome sequencing utilizing a hybrid assembly system that incorporates DNBSEQ and PacBio technologies. DNBSEQ produces 350 bp paired-end sequences and filters out reads with a quality value of continuous $\leq$20 base numbers accounting for up to 40% of the total, as well as those containing N bases exceeding 0.1%. In contrast, the PacBio system constructs a 10-kb fragment library, with subreads N50 and N90 measuring 10,816 bp and 7,255 bp, respectively. Additionally, subreads shorter than 1,000 bp are excluded from the final dataset. The sequencing process involved short- and long-read whole-genome sequencing. DNA samples were disrupted using ultrasonic waves from a Covaris instrument to create short DNA fragments of appropriate length. Subsequently, the DNA sample was purified with the Agencourt AMPure XP-Medium kit, resulting in a sample concentration of approximately 300-400bp. The size and concentration of DNA library fragments were assessed using the Agilent 2100 Bioanalyzer with Agilent DNA 1000 Reagents. Sequencing was performed using combined probe anchoring polymerization (cPAS). Reads were filtered to remove low-quality data and adapter sequences to ensure more accurate and reliable results during sequencing on the PacBio platform. Prior to assembly, K-mer analysis was conducted to determine genome size, heterozygosity level, and duplication degree. *De novo* genome assembly was carried out with the SPAdes Genome Assembler (version 3.11.0).[49] Predicted genes were annotated using the RAST tool (version 2.0)[50] and Prokka (version 1.12.21).[51]

Bioinformatics analysis

Capsular type was determined using Kaptive (https://kaptive-web.erc.monash.edu/).[52] Antimicrobial resistance genes and plasmid replicons were identified through ResFinder and PlasmidFinder tools available at the Center for Genomic Epidemiology (mhttps://genomicepidemiology.org/services/).[53,54] Virulence genes were screened using the VFDB core database (http://www.mgc.ac.cn/VFs/main.htm).[55] Insertion sequences (IS), transposons, and integrons were analyzed with ISfinder (https://www-is.biotoul.fr/index.php)[56] and VRprofile2 (https://tool2-mml.sjtu.edu.cn/VRprofile/home.php).[57] Chromosomal and plasmid alignments for each strain were performed using BRIG 0.95 and Easyfig 2.2.3.[58,59] The evolutionary tree was constructed using the kSNP3 software,[60] while iTOL was utilized for the enhancement of visual presentation(https://itol.embl.de/).[61] The code for kSNP3 was provided in the key resources table.

QUANTIFICATION AND STATISTICAL ANALYSIS

All data were analyzed for normality distribution using the Shapiro-Wilk test. Statistical differences between multiple groups were performed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons tests, using GraphPad Prism 8.0 software (as shown in Figure 1A). The data are presented as the means ± SDs of 3 independent experiments. The significance levels were defined as: ns: not significant, $^{**}p < 0.01$, $^{***}p < 0.001$.