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Molecular and genetic features of a bla_{NDM-1} and bla_{SHV-12} coharboring hypermucoviscous *Klebsiella pneumoniae* of serotype K2 and ST65

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

Abstract

Purpose This study aimed to assess the resistance phenotype, virulence phenotype, and genetic characteristics of a bla_{NDM-1} and bla_{SHV-12} co-harboring ST65 K2 *Klebsiella pneumoniae* (KP114), which was isolated from General hospital of Ningxia Medical University.

Methods Antibiotic susceptibility test was determined by Vitek 2 Compact system. Multilocus Sequence typing (MLST), antimicrobial resistance and virulence genes were examined by PCR and Sanger sequencing. The virulence of KP114 was evaluated through string test, macrophage phagocytosis assay, serum resistance assay, and mouse infection model. Whole-genome sequencing was performed for further analysis of genetic information.

Results The presence of the bla_{NDM-1} and bla_{SHV-12} genes in KP114 confered resistance to multi-antibiotics. The hypervirulence of KP114 was demonstrated through various in vitro experiments and in vivo mouse infection model. KP114 was found to harbor two distinct plasmids: a drug-resistant plasmid (pKP114-NDM), classified as the IncX3 type, which contained various transfer elements including type IV coupling protein (T4CP) and type IV secretion system (T4SS), and a virulence plasmid (pKP114-vir) that exhibited a high sequence similarity with pLVPK. The results of the conjugation experiment showed that resistance and virulence traits were successfully transferred from KP114 to *Escherichia coli* EC600 and J53.

Conclusions We reported a Carbapenem-resistant hypervirulent *Klebsiella pneumoniae* (CR-hvKP) strain of ST65 K2 serotype carrying the bla_{NDM-1} and bla_{SHV-12}, which exhibited hypervirulence and drug resistance with potential for transmission. This finding allows improved clinical surveillance and control of this clone, thereby holding considerable value for clinical treatment.

Keywords *Klebsiella pneumoniae*, ST65, Serotype K2, bla_{NDM-1}, Drug resistance, Virulence plasmid

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Introduction

Klebsiella pneumoniae (KP) is a significant pathogen responsible for nosocomial infections in recent years, which have become the leading cause of morbidity and mortality [1–3]. This can be attributed to the rapid evolution of KP, resulting in the emergence of multi-drug resistant and highly virulent strains [4–6]. Among them, some gene-specific KP strains, such as serotype K1/K2, have evolved into hypervirulent *Klebsiella pneumoniae* (hvKP) [7–9]. hvKP exhibits susceptibility to the majority of antibiotics and was originally detected in Taiwan during the late 1980s among individuals suffering from severe liver abscesses [10]. It is believed to cause serious community-acquired infections in young and healthy individuals. The highly viscous phenotype is attributed to the upregulation of capsular expression caused by the regulation of *rmpA* and/or *rmpA2*, along with the elevated iron levels controlled by aerobactin, salmochelin, yersiniabactin, and enterobactin [11, 12]. The main capsular serotypes identified in reported hvKP strains include ST23, ST86, ST65, ST25, and ST375.

In the past decade, there has been a rise in multidrug resistance in KP, particularly in its resistance to carbapenems, which are the last resort antibiotics. Four major carbapenemase enzymes, namely KPC, OXA-48, NDM, and VIM, have been identified globally [13, 14]. KPC is the most commonly found carbapenemase in China. Additionally, NDM was initially discovered in India but has since spread across South Asia, becoming the second most significant factor contributing to carbapenem resistance in China [15]. bla_{NDM} gene, known for its wide range of antibacterial characteristics, is present in various conjugated plasmids and sequence types (STs), leading to the occurrence of prevalent nosocomial infections [16].

Carbapenem resistance and hypervirulence have traditionally been considered as two separate phenotypes. Carbapenem-resistant *Klebsiella pneumoniae* (CRKP) strains are generally less virulent, while hvKP strains are often susceptible to antibiotics [17]. However, in recent years, there has been an emergence of KP strains that exhibit both carbapenem resistance and high virulence, resulting in severe clinical outcomes [17, 18]. Studies have demonstrated hvKP strains have the ability to evolve into Carbapenem-resistant hypervirulent *Klebsiella pneumoniae* (CR-hvKP) strains by acquiring carbapenemase-encoding plasmids [19]. Furthermore, recent data suggests that CRKP strains can gain high virulence by acquiring pLVPK-like virulence plasmids [20]. The prevalence of CR-hvKP has been steadily increasing since 2010, and patients infected with CR-hvKP generally experienced a poorer prognosis [18]. In 2016, a fatal outbreak of CR-hvKP infection was reported in an intensive care unit in China, with 21 strains of ST11 KPC-2-producing CRKP isolated from five patients, all of whom died during hospitalization [21]. However, reports of CR-hvKP strains in Ningxia Hui Autonomous Region are still rare. In this study, we reported for the first time a clinically isolated ST65 K2 hvKP strain (KP114) that carries both $bla_{\mathrm{NDM-1}}$ and $bla_{\mathrm{SHV-12}}$. We conducted an analysis of the virulence, resistance traits, and genetic information of this strain, while investigating its molecular mechanism of transmission. The emergence of CR-hvKP in the region is a significant concern for healthcare networks, and urgent measures are needed to prevent its further dissemination within hospitals.

Methods and materials

CRKP isolated and antimicrobial susceptibility testing

The strain was isolated from the sputum of a 53-year-old male patient admitted to the Department of Vascular Surgery at the General Hospital of Ningxia Medical University. All protocols received ethical approval from the Ethics Committee of Ningxia Medical University to ensure that this study complied with national and international guidelines for human research. The VITEK2 Compact automatic microbial analyzer (BioMerieux, Paris, France) was used to for strain identification and antimicrobial susceptibility tests, according to guideline document M100-S26 established by Clinical and Laboratory Standards Institute.

Detection of virulence and antibiotic-resistance genes

The presence of virulence genes (*rmpA*, *rmpA2*, *iucA*, *iutA*, *fim*, *mrk*, *ybtS*, *iroB*, *mrkD*, *entB*, *peg344*) and antibiotic-resistance genes (bla_{KPC}, bla_{NDM},$bla_{\mathrm{OXA-48}}$ and bla_{VIM}) was screened and detected using specific primers in a PCR assay [22–24]. The resulting PCR products were then identified through agarose gel electrophoresis.

Multilocus sequence typing and capsular type identification

MLST was performed to detect seven housekeeping genes (*rpoB*, *gapA*, *mdh*, *pgi*, *phoE*, *infB*, *and tonB*) of the strain using PCR, following the protocol provided at http://bigsdb.pasteur.fr/klebsiella/primers_used.html. The sequencing of the products was carried out at Tsingke Biotechnology (Beijing, China). The obtained sequencing results were uploaded and compared to determine the allele number and sequence type (ST). The *wzi* alleles and K types were identified from the *K. pneumoniae* sequence typing database available at http://bigsdb.web.pasteur.fr.

String test

K. Pneumoniae strain KP114 was plated on blood agar and incubated overnight at 37 °C. A single colony was selected and streaked outward using an inoculation loop. The string test was considered positive if the length of the viscous string produced by the isolate exceeded 5 mm,

indicating a hypervirulent phenotype. The NTUH-K2044 strain was used as a positive control, and this experiment was conducted three times.

Serum resistance assay

The serum resistance assay was carried out as described previously with minor modifications [25]. All tested strains were grown overnight to mid-logarithmic phase at 37 °C, 200 rpm, shaking for 0, 1, 2 and 3 h, and plated on counting plates after gradient dilution. Following incubation, the samples underwent a 10-fold serial dilution process. Subsequently, they were placed on plates and incubated at 37 °C for 24 h to quantify bacterial colonies. The survival percent was determined by dividing the count of bacteria treated with normal serum by the count of bacteria treated with PBS.

Immune cell phagocytosis assay

The murine-derived alveolar macrophages, MH-S cells (CL-0597, Wuhan Pricella Biotechnology Co., China.), were used in the macrophage phagocytosis experiments. All the cells were cultured in RPMI-1640 medium (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA). Bacteria from overnight cultures were grown to the mid-logarithmic stage and subsequently added to the MH-S cells at a ratio of 10:1 for different incubation times (0 h, 0.5 h, 1 h, 2 h) at 37℃ in 5% CO_2. Following the infection, 0.5% Triton X-100 was added to the cell culture dish for 10 min to lyse the cells. Viable bacteria were quantified by plating serial dilutions, and the percentage of bacteria was calculated by dividing the number of colonies observed at different incubation times by the number of colonies counted at 0 h.

Mouse intraperitoneal infection model

This study was conducted in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. It was also approved by the Ethics Committee for the Use of Laboratory Animals of Ningxia Medical University (Z2019/023, approved on 10 November 2019). Female ICR mice, aged six or seven weeks, were obtained from Huachuang Sino Company and randomly divided into four groups. The bacteria were grown in LB broth until the logarithmic phase, and each mouse was injected with 5×10^7 CFU bacteria. The negative control group was treated with PBS, while the positive control group was treated with NTUH-K2044 isolate. The mortality rates were observed for 60 h. The survival analysis in the mouse model was performed by Kaplan-Meier estimate.

Whole genome sequencing

Genomic DNA was extracted from the KP114 strain and subjected to whole genome sequencing using two systems: the DNB system, which generates 350 bp paired-end sequences, and the PacBio System, which assembles a 10-kb fragment library. The sequencing process involved short- and long-read whole-genome sequencing. DNA samples were disrupted using ultrasonic waves generated by a Covaris instrument, resulting in the production of short DNA fragments of appropriate length. The disrupted DNA sample was then purified using the Agencourt AMPure XP-Medium kit, leading to a concentration of the sample around 300–400 bp. The size and concentration of the DNA library fragments were assessed using the Agilent 2100 Bioanalyzer with Agilent DNA 1000 Reagents. Finally, sequencing was carried out using combined probe anchoring polymerization (cPAS). The reads were filtered to remove low-quality data and adapter sequences to ensure more accurate and reliable results when sequencing on the PacBio platform. Prior to assembly, K-mer analysis was performed to estimate the genome size, degree of heterozygosity, and degree of duplication. De novo genome assembly was conducted using SPAdes Genome Assembler (version 3.11.0). The predicted genes were annotated using the RAST.

tool (version 2.0) and Prokka (version 1.12.21). Plasmid maps were generated using GenomeVx (http://wolfe.ucd.ie/GenomeVx/). Circular plasmid map comparisons were performed using the BLAST Ring Image Generator (version 0.95), while linear alignments of multiple genomic loci were conducted using EasyFigure (version 2.2.3). Plasmid incompatibility typing was identified using VRprofile 2.0 (https://tool2-mml.sjtu.edu.cn/VRprofile/).

Conjugation experiment

Conjugation experiments were conducted using *Escherichia coli* EC600 and J53 as the recipients and KP114 as the donor. The mixtures were incubated at 37 °C for 18 h. Subsequently, the mixtures were plated on MacConkey agar plates containing meropenem and rifampicin to screen for KP114:EC600 transconjugants, and on MacConkey agar with meropenem and sodium azide to identify KP114:J53 transconjugants, all incubated at 37 °C for 18 h. Further PCR amplification were carried out to determine the presence of resistance genes (bla_{NDM-1} and bla_{SHV-12}) and virulence genes (*rmpA2*, *iutA*, *iucA*, *iucB*, *iucC* and *iucD*) in the transconjugants. The sequence information of the primers was listed in Table 1. Antimicrobial susceptibility testing of the transconjugants was conducted using the VITEK2 compact automatic microbial analyzer, and the virulence phenotype of the transconjugants was identified with mouse intraperitoneal infection models, as described previously.

Statistical analysis

Statistical analysis was performed using using GraphPad Prism 8.0 software. Data were presented as medians

Table 1 Sequence information of the PCR primers used in this study

Genes	Forward (5′-3′)	Reverse(5′-3′)	Amplicon size (bp)
bla_{NDM-1}	GGTTTGGCGATCTGGTTTTC	CGGAATGGCTCATCACGATC	621
bla_{SHV-12}	TTATCTCCCTGTTAGCCACC	GATTTGCTGATTTCGCTCGG	816
rmpA	GAGTAGTTAATAAATCAATAGCA	CAGTAGGCATTGCAGCA	332
rmpA2	GTGCAATAAGGATGTTACATTA	GGATGCCCTCCTCCTG	430
iutA	GGGAAAGGCTTCTCTGCCAT	TTATTCGCCACCACGCTCTT	920
iucA	AATCAATGGCTATTCCCGCTG	CGCTTCACTTCTTTCACTGACAG	239
iucB	GATATTTATCGCCGCACCGC	AGCCAGCTTTGTACGTAGTGGG	594
iucC	AAACCTGGTTTACGCAACTGT	ACCCGTCTGCAAATCATGGAT	269
iucD	ACAAAAAGTTCTATCGCTTCC	CCTGATCCAGCTGATGCTC	714

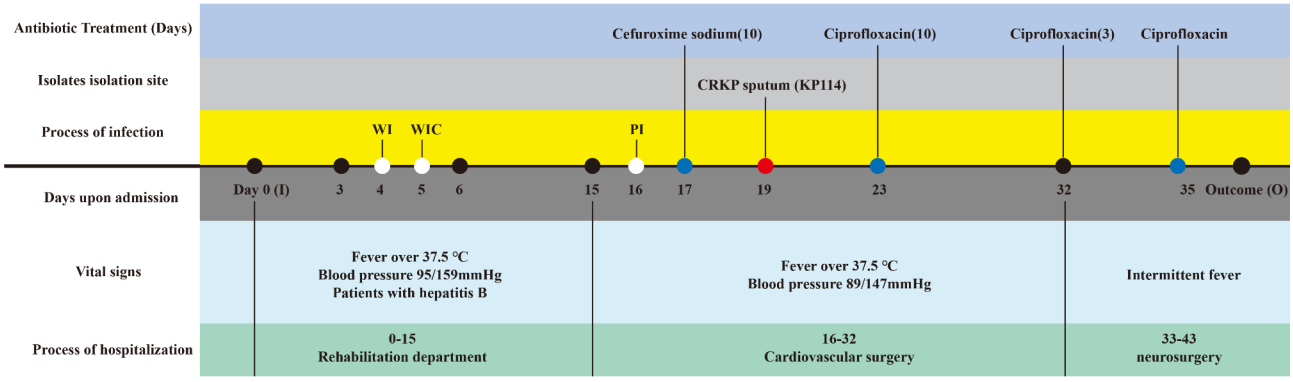

Fig. 1 The case history of the patient's admission, treatment, and isolate collection was summarized in a timeline. Abbreviations: I, admission; O, Discharge; WI, Wound infection; IC, Wound Infection control; PI, Pulmonary infection

or means ± standard deviation. Statistical differences between multiple groups were performed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons tests and $P < 0.05$ was considered statistically significant.

Results

Case description

A 53-year-old male patient was admitted to the department of vascular surgery at the General Hospital of Ningxia Medical University on June 29, 2021, due to a traumatic injury that occurred three months ago. The patient, who has a 20-year history of chronic hepatitis and liver cirrhosis, underwent a splenectomy surgery for liver cirrhosis 20 years ago and has no known allergies, with no previous occurrences of tuberculosis or other infectious diseases. He was initially diagnosed with common iliac artery entrapment on the right side and presented with symptoms such as fever, pulmonary artery embolism in the apical posterior segment of the right upper lobe of the lung, post-traumatic cerebral injury, lacunar cerebral infarction, severe pneumonia, and viral hepatitis B. The patient received a series of treatments including anticoagulation, blood pressure control, heart rate stabilization, and other necessary medications. On day 19, an analysis of the sputum culture revealed the presence of *Klebsiella pneumoniae* (KP114), *Pseudomonas aeruginosa*, *Streptococci* and coagulase-negative *Staphylococci*. The patient was prescribed an adjusted dosage of ciprofloxacin at 0.8 g per day, to be taken twice daily. After receiving active anti-infective symptomatic treatment and other related therapies, the patient's blood routine, calcitonin, blood sedimentation, CRP, and other indexes returned to normal levels. A follow-up chest CT scan revealed that the lesions in the bilateral lower lung infiltration site had resolved, and the patient's limb function, swallowing function, and daily life ability had improved compared to before. On day 43, the patient was discharged from the hospital. Timeline to summarize the case history of the patient admission, treatment, and isolate collection was described as Fig. 1.

Characteristics of carbapenem-resistant KP114

KP114 strain exhibited resistance to all tested β-lactam antibiotics, while remaining susceptible to amikacin, tobramycin, ciprofloxacin, levofloxacin, tetracycline, and colistin (Table 2). The results of the string test demonstrated that strain KP114 produced a viscous string with a diameter greater than 5 mm, indicating the hypermucoviscous phenotype of the strain. Multilocus sequence typing analysis (MLST) revealed that the KP114 strain belonged to sequence type 65 and had a capsular serotype of K2 based on capsular typing by wzi allele. Given the high levels of drug resistance and hypermucoviscous

Table 2 Antibiotic resistance characteristics of KP114 strain

Antimicrobial Agent	MIC (μg/mL)	*R/S*
Piperacillin-Tazobactam	≥ 128	R
Amoxicillin-clavulanate	≥ 32	R
Ticarcillin-clavulanate	≥ 128	R
Cefoperazone-Sulbactam	≥ 64	R
Ceftazidime	≥ 64	R
Cefepime	≥ 32	R
Aztreonam	≥ 64	R
Imipenem	≥ 16	R
Meropenem	≥ 16	R
Amikacin	≤ 2	S
Tobramycin	≤ 1	S
Ciprofloxacin	≤ 0.25	S
Levofloxacin	≤ 0.12	S
Tetracycline	≤ 0.5	S
Colistin	≤ 0.5	S

Abbreviations: MIC, Minimum inhibitory concentration; R, resistant; S, susceptible

Table 3 Features of virulence and resistance of KP114 strain

Isolate	KP114
Isolation origin	sputum
Hypermucoviscosity	+
Capsule serotype	K2
Sequence type	ST65
HM phenotype regulator genes	
rmpA	+
rmpA2	+
Siderophore systems	
Enterobactin (*entABCEF*)	–
Aerobactin (*iucABCDcluster*)	+
Aerobactin receptor (*iutA*)	+
Yersiniabactin (*ybt and irp complex*)	+
Salmochelin (*iroBCD*)	–
Salmochelin receptor (*iroN*)	–
Fimbrial genes	
Type 3 fimbrial genes (*mrk cluster*)	+
Type 1 fimbrial genes (*fim cluster*)	+
Genotoxin	
Colibactin (*clbA* to *clbS* cluster)	+
Ferric uptake	
kfuABC cluster	–
Antibiotic-resistant genes	
CR-genes	bla_{NDM-1}
ESBLs	bla_{SHV-12}
Aminoglycoside resistance genes	–

phenotype, the presence of antimicrobial resistance genes and virulence factors was investigated by PCR. The strain coharbored the bla_{NDM-1} carbapenemase gene, as well as bla_{SHV-12} β-lactamase gene. Furthermore, the CRKP strain also exhibited the presence of more hypervirulent markers (*rmpA*, *rmpA2*, *iroB*, *iucA*, *iutA*, *fim*, *mrk*, *ybt* genes), suggesting that it belonged to CR-hvKP (Table 3).

Virulence potential of KP114 as measured by in vivo and in vitro models

To further verify the virulence potential of KP114, we conducted both in vivo mouse infection experiments and in vitro experiments on serum resistance and macrophage phagocytosis. In our study, we utilized a clinical CRKP strain KP26 as a negative control, which showed a negative string test and did not possess any of the virulence genes that were tested. In comparison to a 90% survival rate in the mouse model infected with KP26, the mouse model infected with KP114, as well as the previously reported hvKP strain NTUH-K2044 (ST23, K1 serotype), exhibited a 0% survival rate after 24 h (Fig. 2A). The results indicated that the ST65 serotype K2 strain KP114 exhibited similar levels of virulence as the well-documented ST23 serotype K1 hvKP strain in the mouse infection model. In the serum resistance assay, both KP114 and NTUH-K2044 showed higher levels of serum resistance compared to KP26. However, KP114 displayed a lower serum resistance capacity than NTUH-K2044 at 2 and 3 h after incubation with serum (Fig. 2B). The results of the in vitro experiments on KP114 phagocytosis by MH-S cells indicated a significantly higher survival rate in the KP114 group compared to the KP26 control group at 0.5 h, 1 h and 2 h. These findings were in line with the positive control NTUH-K2044 (Fig. 2C).

KP26 was used as a negative control which showed a negative string test and did not possess any of the virulence genes that were tested. Additionally, NTUH-K2044 was employed as a positive control due to its known hypervirulence. (ns, non-significant; * $P<0.05$; ** $P<0.01$; *** $P<0.001$ in one-way ANOVA with Tukey's multiple comparison).

Genetic characteristics of KP114 identified by whole-genome sequencing

Although molecular typing, detection of antibiotic and virulence-related genes, in vitro and in vivo models of virulence all indicated that KP114 is a hypervirulent CRKP strain, its genetic information remains unclear. Whole-genome sequencing allowed us to obtain the detailed genetic information of chromosomes and plasmids in the KP114. Our analysis showed that the KP114 genome was a single chromosome of 5,486,094 bp and two plasmids: pKP114-vir (169506 bp) and pKP114-NDM (55234 bp). Sequence alignments using BLAST showed that KP114 exhibited 99% identity with the hypervirulent strain NTUH-K2044 (GenBank: NC_012731), as well as 99% identity with the hypervirulent strain KPCG43 (GenBank: NC_022566) (Fig. 3A). The final draft genome revealed 41.22% G + C, with a total of 3405 annotated protein-coding sequences, 74 tRNA sequences and 18 rRNA sequences (Table 4). The functional annotation of the KP114 genome based on the COG database revealed

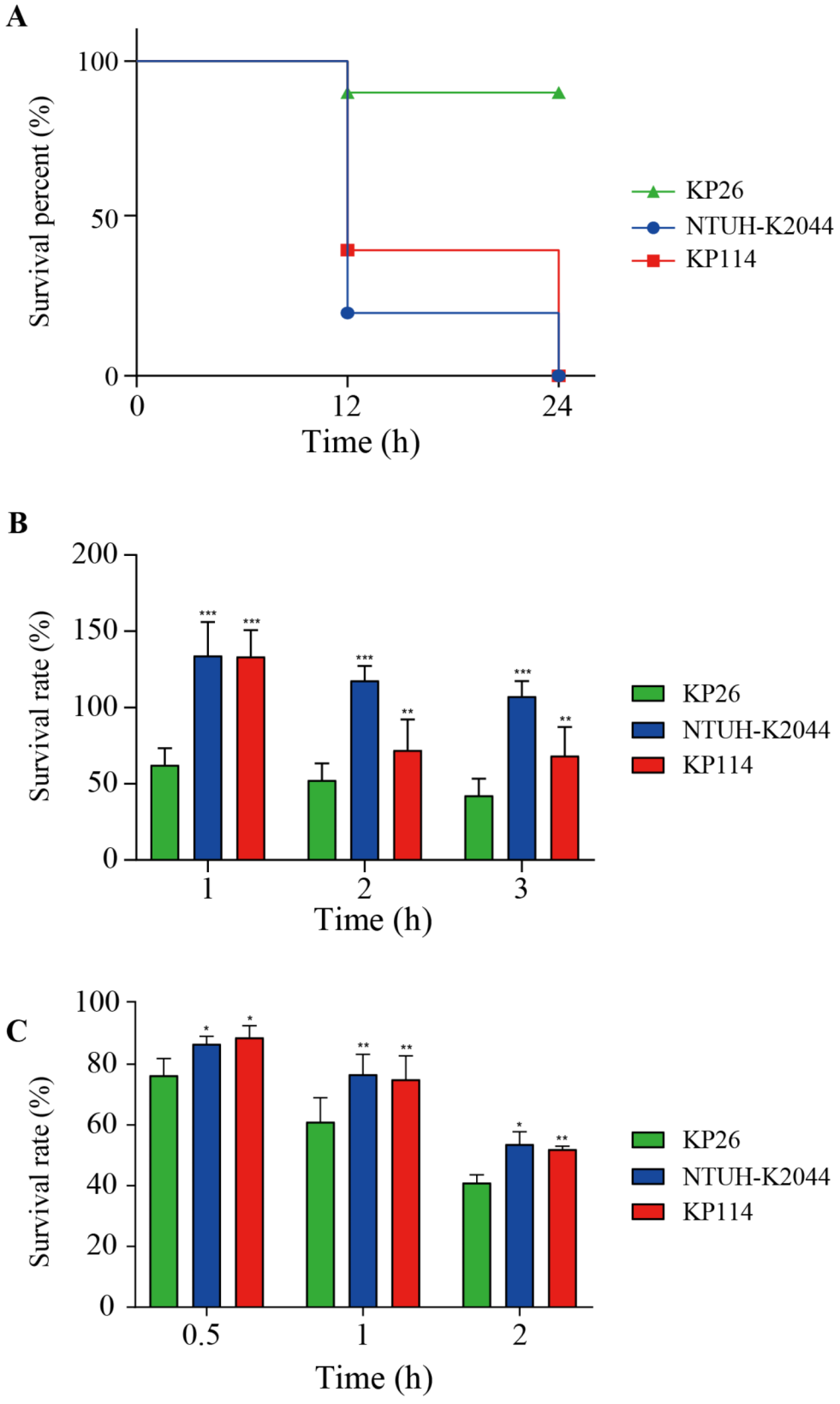

Fig. 2 The virulence characteristics of KP114 were assessed through various assays. (**A**) The mouse infection model was employed to evaluate the in vivo virulence of KP114. (**B**) The serum resistance assay was conducted to examine the in vitro virulence of KP114. (**C**) The MH-S cell phagocytosis assay was performed to investigate the anti-phagocytosis of KP114

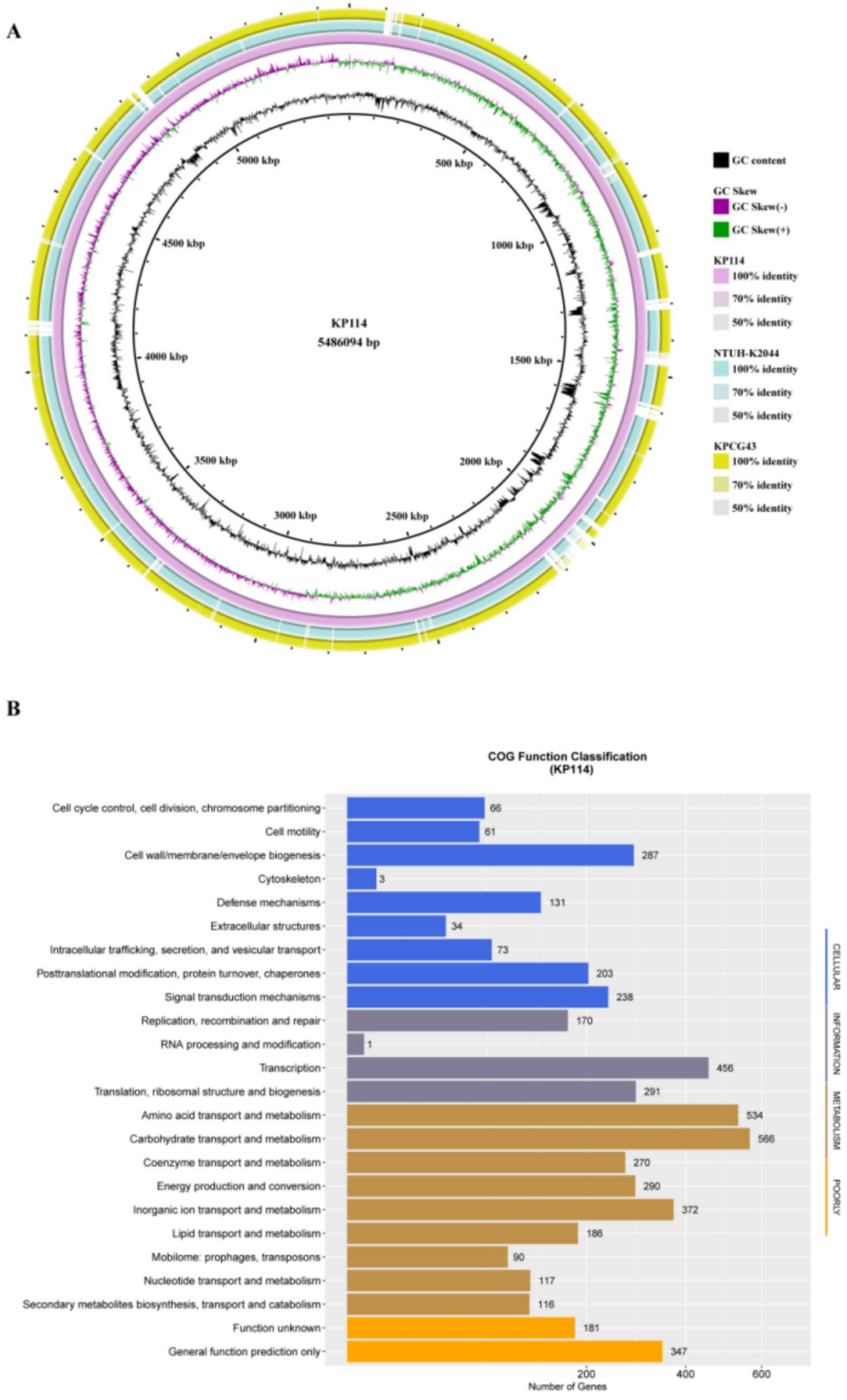

Fig. 3 Genomic analysis of the KP114 strain chromosome was conducted. (**A**) Comparative genomic analysis was performed on the KP114 strain and other sequenced hypervirulence KP strains, including NTUH-K2044 and KPCG43. (**B**) The COG functional categories for KP114 were determined using the NCBI COG database and its corresponding function descriptions (http://www.ncbi.nlm.nih.gov/COG/)

Table 4 General genomic features of KP114 strain

Elements and characteristics	Value
Genome size (bp)	5,710,834
N50 (bp)	11,404
DNA coding region (%)	87.04
DNA GC content (%)	58.06
Contig number	3
Total genes	5,474
sRNA genes	42
tRNA genes	89
Plasmids	2
Protein-coding genes	5,464
Genes assigned to COGs	4,438

that the majority of genes are related to carbohydrate transport and metabolism (12.7%), amino acid transport and metabolism (12%), transcription (10.2%), and cell wall biogenesis (6.4%) (Fig. 3B).

Comparative genomic analysis of pKP114-vir

The completely assembled virulence plasmid with a full length of ~160 kbp from KP114 strain aligned well to the virulence plasmids pLVPK and pvir-CR-HvKP4 using the Basic Local Alignment Search Tool (BLAST; https://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequence alignment showed it shares 99.76% identity, 75% coverage with pLVPK and 99.74%identity, 90% coverage with pvir-CR-HvKP4 (Fig. 4A). A couple of virulence determinants, including *rmpA2* encoding mucoid regulators, *iucA*, *B*,*C*, *D* encoding a cluster of aerobactin production, were located in a approximately 16 kbp region of pKP114-vir plasmid which belongs to IncHI1B/repB virulence plasmid (Fig. 4A). Moreover, we also found that pKP114-vir carries mobilizable elements (https://tool2-mml.sjtu.edu.cn/VRprofile/), including an origin of transfer (oriT) region and several transposase families, such as those from the IS3, IS5, IS1, Tn3, IS630, and ISKox1 families, but lacks a type IV secretion system (T4SS), which indicates a limited capacity to genes transfer by conjugation (Fig. 4B).

Sequence analysis of pKP114-NDM like plasmids harboring bla_{NDM} with IncX3 replicon

In the KP114 strain, bla_{NDM-1} was located on another plasmid pKP114-NDM which belonged to IncX3 plasmid that is the most prevalent plasmid harboring the bla_{NDM} gene. Plasmid pKP114-NDM was observed to harbor relaxase, type IV coupling protein (T4CP), and type IV secretion system (T4SS), indicating a potential horizontal transmission of virulence genes between different plasmids or isolates. The length of plasmid pKP114-NDM was determined to be 55,234 bp, with a total of 14 ORFs (open reading frames) present. This particular plasmid can be divided into two distinct parts: a 41,210 bp backbone and a 14,024 bp region responsible for multiple drug resistance (MDR). Within the backbone, there are several important genes that play essential roles in plasmid replication (*repB*), conjugal transfer (*TrbC*, *TrbE*), and plasmid stability (*parA*). On the other hand, the MDR region includes the Tn3 transposon, which encodes several proteins, including the β-lactamases bla_{NDM-1} and bla_{SHV-12}, as well as a transposase (encoded by the tnpA gene) and a resolvase (encoded by the *tnpR* gene). Additionally, it contains Tn125-like transposon that encodes the bla_{NDM-1}, ble_{MBL}, *trpF*, *dsbC*, *groES*, *groL*, and *insE* genes, along with IS26, IS30 and IS5 sequences. The genome sequence of pKP114-NDM showed a high level of similarity (>90%) with plasmids from different bacterial species such as *K. pneumoniae*, *E. coli*, *E. cloacae* and *Citrobacter freundii.* Specifically, the plasmids pNDM-HN380 (GenBank: JX104760) pNDM-HF727 (GenBank: KF976405), pECS5S-NDM4 (GenBank: KX470734), pKP04-NDM (GenBank: KU314941), pl12298-NDM (GenBank: KP987216), pincX-SHV (GenBank: JN247852), and pSCE516-2 (GenBank: KX023261) exhibited sequence similarities with pKP114-NDM. Additionally, plasmids pNDM-HN380, pNDM-HF727 and pSCE516-2 also showed a high level of similarity (>90%) to pKP114-NDM (Fig. 5).

Transmission of resistance and virulence characteristics in KP114 evaluated through a conjugation assay

To assess the transferability of the KP114 resistance and virulence characteristics, we conducted conjugation experiments using the KP114 strain as the donor and the *E. coli strain* J53 and EC600 as the recipients. Two transconjugants, KP114:J53 (TC1) and KP114:EC600 (TC2), were identified and chosen for subsequent analysis. Drug susceptibility tests revealed that these transconjugants exhibited resistance to Ticarcillin-clavulanate, Ceftazidime, Aztreonam, Imipenem, and Meropenem. However, they were found to be sensitive to Amikacin, Tobramycin, Ciprofloxacin, and Levofloxacin. These transconjugants have similar resistance characteristics to KP114, but with a lower level of resistance compared to KP114 (Table 5). The presence of resistance genes bla_{NDM-1} and bla_{SHV-12} may contribute to the carbapenem resistance observed in transconjugants (Table 6). The in vivo virulence of transconjugants was analyzed using a mouse infection assay. The survival rates of mice infected with KP114, J53, TC1, EC600, and TC2 strains were 0%, 90%, 20%, 90%, and 30%, respectively, at 60 h after bacterial infection (Fig. 6). The findings indicate that transconjugants, similar to KP114, exhibited increased virulence in comparison to the recipient bacteria. This is further supported by PCR detection which revealed the presence of specific virulence genes (*iutA* and *iucA*) in the transconjugates (Table 6). Therefore, it is evident that resistance

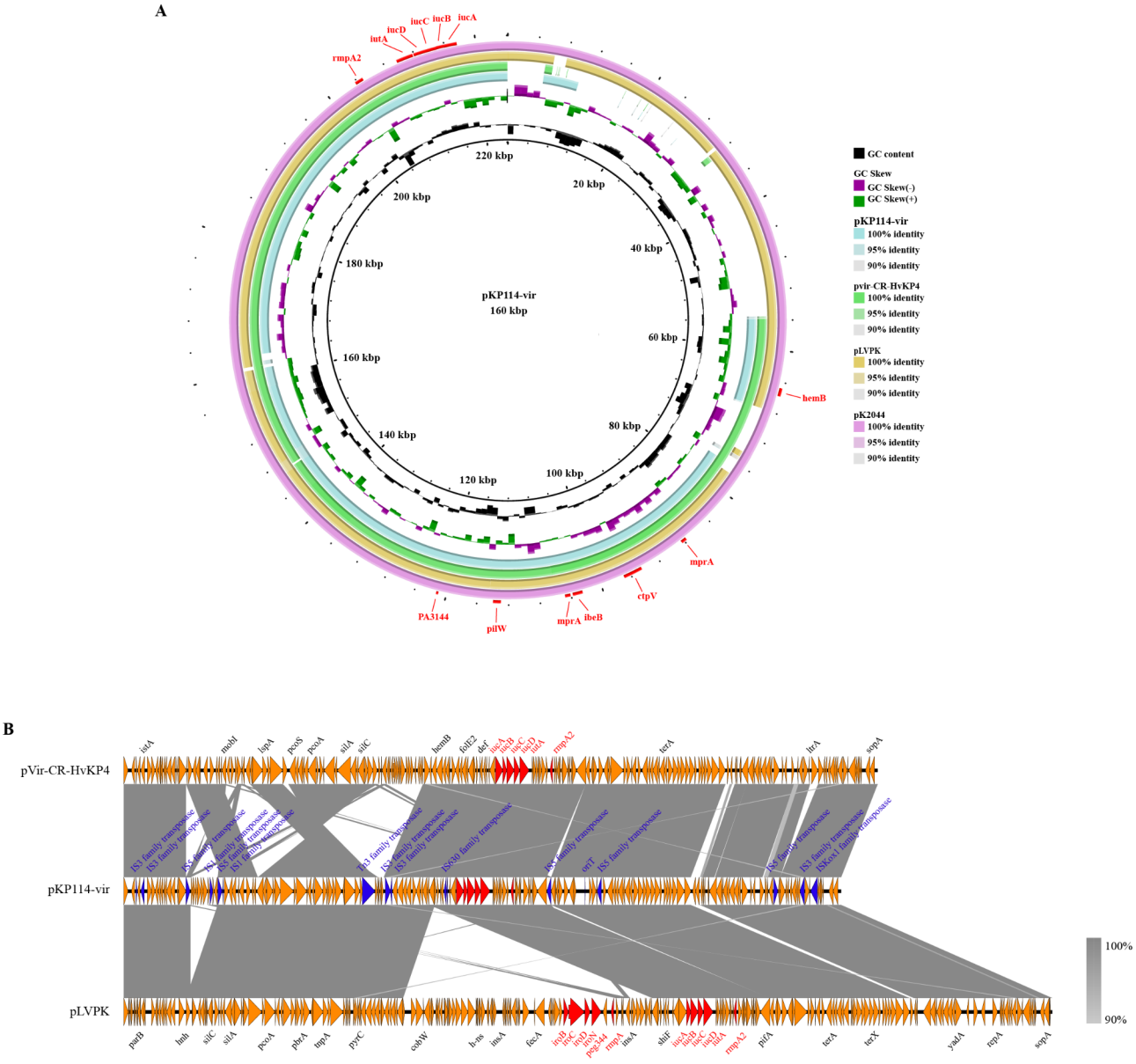

Fig. 4 Comparative genomic analysis of the virulence plasmid from the KP114 strain was performed. (**A**) Alignment of pKP114-vir (GenBank: CP152424.1) against three known virulence plasmids: pLVPK (GenBank: AY378100.1), pK2044 (GenBank: CP026012.1) and pvir-CR-HvKP4 (GenBank: NZ_MF437313.1). The circular map was generated with the BLAST Ring Image Generator. (**B**) Linear genome alignment analysis among pKP114-vir, pLVPK and pvir-CR-HvKP4 were generated based on EasyFigure software. Colored arrows indicate ORFs and the shaded region reflects sequence similarity. The virulence associated genes are indicated in red and mobilizable elements are indicated in blue

and virulence traits in KP114 can be transferred to *E. coli* strains such as J53 and EC600.

Discussion

Infections caused by hypervirulent carbapenem-resistant *Klebsiella pneumoniae* (CR-hvKP) is difficult to treat and associated with high mortality and morbidity rates due to their drug resistance, high virulence, and efficient transmission [17, 26, 27]. In our study, we isolated a capsular serotype K2 and ST65 CR-hvKP strain (KP114) from a patient with common iliac artery dissection, which carried virulence genes (*rmpA/A2*, *ituA*, *iucABCD*, *wcaJ*, *film*, *mrk*, and *ybt*) and antibiotic resistance genes (bla_{NDM-1} and bla_{SHV-12}), indicating that this strain has the characteristics of high virulence and carbapenem resistance. The high virulence potential of KP114 was further verified through the observation of hypermucoviscosity phenotype, high serum resistance, antiphagocytosis, and a high lethality rate in a mouse model. Whole genome sequencing revealed the presence of virulence plasmid and resistance plasmid, both of which contain multiple mobile genetic elements, suggesting the

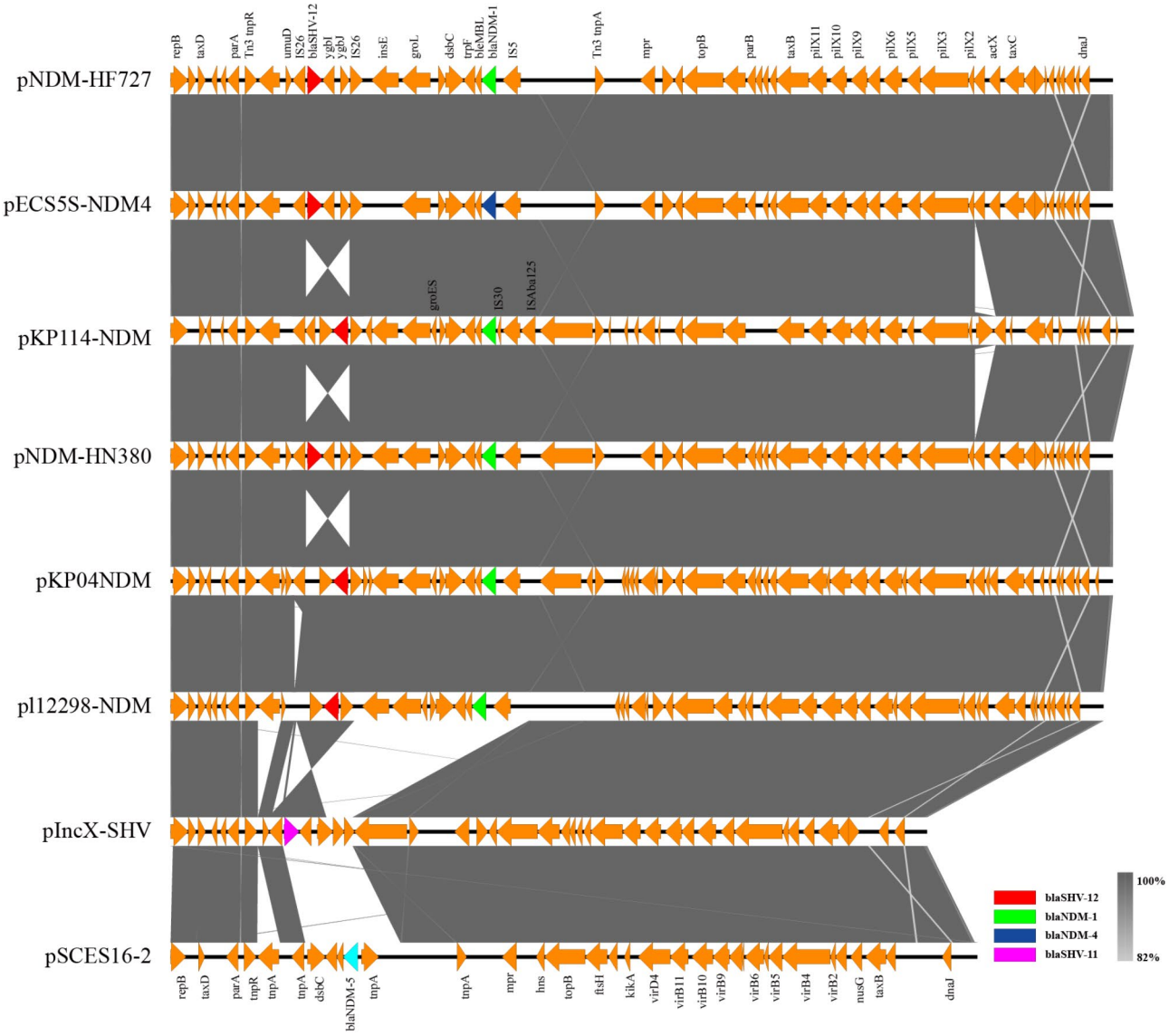

Fig. 5 The genetic structure features of pKP114-NDM-like plasmids were analyzed. The bla_{NDM-1}, bla_{NDM-4}, bla_{SHV-2} and bla_{SHV-12} genes are indicated in red, purple, baby blue and yellow, respectively

occurance of horizontal gene transfer between bacteria of the same or different species. Therefore, more attention should be focused on the evolution and transmission of CR-hvKP strain, especially in the hospital settings.

Although KPC is the most common carbapenemase in China, the presence of bla_{NDM} in CR-hvKP has also been reported [18, 25, 28, 29]. Of the bla_{NDM} variants discovered to date, bla_{NDM-1} is the most widespread and exhibits significant resistance to clinically relevant β-lactam antibiotics [16]. The presence of bla_{NDM-1} renders KP114 resistant to all tested β-lactam antibiotics but sensitive to aminoglycosides, quinolones, and tetracyclines. *Klebsiella pneumoniae* was not detected in sputum culture samples from the patient after ciprofloxacin treatment. The patient had no prior history of pulmonary infection and was probably infected from the hospital environment, suggesting the possibility of nosocomial transmission and infection by *Klebsiella pneumoniae* [30]. Despite the patient's improved condition, these colonizing bacteria can still cause infection when the patient's immune system is weakened, potentially worsening the disease. This has been reported in several previous studies [31–33]. Furthermore, conjugation experiments revealed the high transferability of the bla_{NDM-1} gene from KP114 to EC600 and J53, suggesting the potential horizontal transmission of bla_{NDM-1} between different bacteria species [34, 35]. It is important to note that there is a potential risk of bla_{NDM-1} transmission to *streptococci* and coagulase-negative *staphylococci*, both of which are also

Table 5 Antibiotic resistance characteristics of transconjugants

Antimicrobial Agent	MIC (μg/mL)				
	KP114	TC1	TC2	J53	EC600
Piperacillin-Tazobactam	≥ 128(R)	≥ 64(R)	≥ 64(R)	≤ 4(S)	≤ 4(S)
Ticarcillin-clavulanate	≥ 128(R)	≥ 32(R)	≥ 32(R)	≤ 8(S)	≤ 8(S)
Cefoperazone-Sulbactam	≥ 64(R)	≥ 32(I)	≥ 32(I)	≤ 8(S)	≤ 8(S)
Ceftazidime	≥ 64(R)	≥ 64(R)	≥ 32(R)	≤ 1(S)	≤ 1(S)
Cefepime	≥ 32(R)	≥ 64(R)	≥ 64(R)	≤ 1(S)	≤ 1(S)
Aztreonam	≥ 64(R)	≥ 32(R)	≥ 64(R)	≤ 1(S)	≤ 1(S)
Imipenem	≥ 16(R)	≥ 16(R)	≥ 16(R)	≤ 1(S)	≤ 1(S)
Meropenem	≥ 16(R)	8(R)	8(R)	≤ 0.25(S)	≤ 0.25(S)
Amikacin	≤ 2(S)	≤ 2(S)	≤ 2(S)	≤ 2(S)	≤ 2(S)
Tobramycin	≤ 1(S)	≤ 1(S)	≤ 1(S)	≤ 1(S)	≤ 1(S)
Ciprofloxacin	≤ 0.25(S)	≤ 0.25(S)	≤ 0.25(S)	≤ 0.25(S)	≤ 0.25(S)
Levofloxacin	≤ 0.12(S)	≤ 0.5(S)	≤ 0.5(S)	≤ 0.12(S)	≤ 0.12(S)
Tetracycline	≤ 0.5(S)	≤ 0.5(S)	≤ 0.5(S)	≤ 0.5(S)	≤ 0.5(S)
Minocycline	≤ 1(S)	≤ 1(S)	≤ 1(S)	≤ 1(S)	≤ 1(S)
Colistin	≤ 0.5(S)	≤ 0.5(S)	≤ 0.5(S)	≤ 0.5(S)	≤ 0.5(S)

Abbreviations: MIC, Minimum inhibitory concentration; TC, transconjugant; R, resistant; S, susceptible

Table 6 Features of virulence genes and resistance genes of TCs

Genes	Isolates				
	KP114	TC1	TC2	J53	EC600
bla_{NDM-1}	+	+	+	–	–
bla_{SHV-12}	+	+	+	–	–
rmpA2	+	+	–	–	–
iutA	+	+	+	–	–
iucA	+	+	+	–	–
iucB	+	–	+	–	–
iucC	+	+	–	–	–
iucD	+	–	–	–	–

present in the patient. Further experiments will be conducted to verify this hypothesis.

The presence of bla_{NDM} on plasmids has been documented in various replicon types, with the majority falling under limited replicon types such as IncX3, IncFII, or IncC [16, 36]. According to a study conducted by Wenjing Wu et al. [16], it was found that out of the 355 plasmids available in GenBank carrying the bla_{NDM} gene, approximately one-third (117) were found to carry IncX3 replicants. Our study also confirmed that pKP114-NDM belonged to IncX3 plasmid through whole genome sequencing analysis. These results indicated that the IncX3 plasmid is an important plasmid for the global distribution of bla_{NDM-1}. BLAST analysis revealed a significant sequence similarity between pKP114-NDM and

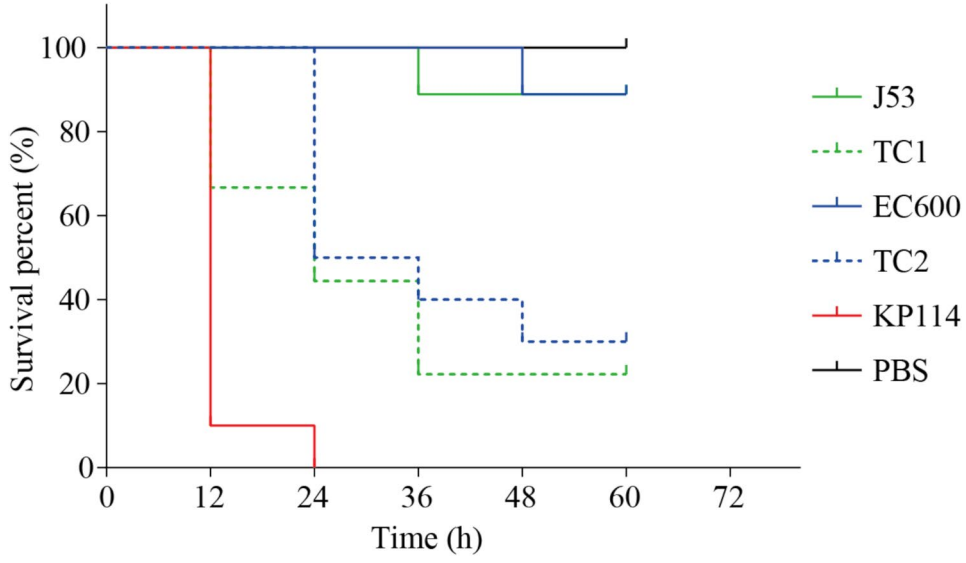

Fig. 6 The virulence of the transconjugants was assessed using a mouse infection model. The positive control group consisted of KP114, while the negative control group consisted of EC600 and J53. Each group comprised 10 mice, and each mouse was injected with 5×10^7 CFU bacteria

the previously reported plasmid pNDM-HN380 (GenBank entry JX104760) in the backbone and genetic load region, suggesting that they share a common ancestor [37]. The IncX3 plasmid possesses advantageous characteristics such as adaptability and stability under selective antibiotic pressure, which minimizes the risk of plasmid loss during vertical transmission [38, 39]. Our study illustrated that the type IV secretion system of IncX3 plasmid promotes efficient bla_{NDM-1} transfer. Furthermore, previous studies have shown that this specialized secretion system can also facilitate the biofilm formation and other auxiliary functions in the host strain [40, 41]. Collectively, these experimental findings highlight the crucial role of IncX3 plasmid as a significant vector for the dissemination and carriage of the bla_{NDM-1} resistance gene by bacteria.

In addition to the drug-resistant plasmid pKP114-NDM, KP114 also contains a virulence plasmid pKP114-vir. This plasmid has the IncHI1B-type replicons, specifically known as pLVPK-related replicons, which are associated with high virulence. Previous studies have demonstrated that other IncHI1B plasmids display high virulence phenotypes due to the presence of various virulence factors [42–44]. Whole-genome sequence analysis showed that pKP114-vir may have arisen due to extensive deletion of large fragments of the pLVPK sequence but retained important virulence and survival factors, such as *rmpA2*, *iucA*, *iucB*, *iucC*, *and iucD*. Like pvir-CR-hvKP4, pKP114-vir carries the *rmpA2* gene without the *rmpA* gene but still exhibits high virulence [44, 45]. In addition, several virulence-related genes on chromosome KP114 (such as *rmpA*, *wcaJ*, *film*, *mrk*, and *ybt*) also play a crucial role in regulating the virulence, growth, and replication of strains during host colonization and infection by encoding various siderophores, efflux pumps, capsule polysaccharide synthesis proteins, and adhesins [12, 18]. It is commonly assumed the virulence plasmid pLVPK is non-conjugated and therefore cannot be automatically transferred to other strains [12, 18, 46]. pLVPK-like plasmids are classified as mobile due to the inclusion of a mobility (MOB) component, which consists of an origin of transfer (oriT) and relies on conjugative helper plasmids and other transfer elements to facilitate the transfer of these virulence plasmids. This mechanism could potentially elucidate the transmission of virulence traits from KP114 to *E. coli* strains. Our study observed the absence of certain virulence genes in the transconjugants. This absence may be attributed to horizontal gene transfer, which can result in the emergence of new genetic variants, including gene amplification, mutation, and deletion, all of which may contribute to adaptive evolution. Further experimental verification is necessary to ascertain whether other variants exist.

Multilocus sequence typing (MLST) and capsular serotyping are extensively employed as powerful methods for genotyping and characterizing strains of bacteria. Among the hvKP strains, the most common serotypes are K1 and K2 [7, 47]. Serotype K1 is associated with sequence type ST23, while serotype K2 exhibits a greater diversity of genotypes, including ST65, ST86, and ST380, which have been proven to be highly virulent [48]. These results are consistent with the high virulence of the ST65 K2 CR-hvKP strain isolated from Ningxia Hospital in our study. Recently, sporadic instances of ST36, ST1797, and ST25 in CR-hvKP have been reported [49–51]. In addition to K1 and K2, other serotypes such as K64, K62, and K35 were also identified in hvKP isolates [7, 52]. This suggests that hvKP has developed a wider range of variants to better adapt to different environmental stress, thereby enhancing bacterial survival.

However, there are several limitations in this study. Firstly, we did not conduct a follow-up on the patient after his discharge, thus lacking information on the final outcomes of patients infected with KP114. Secondly, at present, we have only identified one CR-hvKP case and further analysis of clinical data is needed to screen CR-hvKP infected populations and investigate its epidemiology and molecular infection mechanisms. Finally, it was not sufficient to conduct experiments with only 2 transconjugants in this study. In the future, we need to study more transconjugants to validate their resistance and virulence phenotypes.

Conclusion

In conclusion, we isolated a CR-hvKP strain of ST65 K2 serotype from Ningxia, China, that carries the bla_{NDM-1} and bla_{SHV-12} genes, demonstrating hypervirulence and drug resistance, along with potential for transmission. This finding underscores the urgent need to enhance monitoring and control measures to prevent the further spread and infections caused by this strain.

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Author contributions

The conception of the study was carried out by P.W. and W.J. The design of the work was developed by P.W., Y.K., and Q.L. The execution of the experiments was conducted by Y.K., Q.L., W.M., C.X., and Z.Q. Data analysis was performed by Y.K., Q.L., W.M., and C.X. The interpretation of the data was undertaken by P.W., W.J., and Y.K. The initial draft of the manuscript was written by P.W., Y.K., and Q.L., with subsequent revisions made by P.W., Y.K., Q.L., and Z.Q. All authors have read and approved the final manuscript.

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Data availability
The genomic DNA sequence data supporting the findings of this study have been deposited in the National Library for Biotechnology Information (https://www.ncbi.nlm.nih.gov/) under the primary accession numbers CP152422, CP152423, and CP152424. Additional data is provided within the manuscript file.

Declarations

Ethics approval and consent to participate
This study was approved by the Medical Science Research Ethics Committee IRB of the General Hospital of Ningxia Medical University (Z2022/025, approved on 10 November 2022) and conducted in accordance with ARRIVE guidelines. The patient who agreed to participate signed an informed consent form to participate in the study. This study follows the Declaration of Helsinki.

Competing interests
The authors declare no competing interests.

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