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RETRACTED: Drug Resistance and Molecular Characteristics of Carbapenem-Resistant OXA-48-Producing *Klebsiella pneumoniae* Strains in Hainan, China

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Abstract: Background: The emergence and global spread of carbapenem-resistant hypervirulent *Klebsiella pneumoniae* (CR-hvKP) are of great concern to health services worldwide. These β-lactamases hydrolyze almost all β-lactams, are plasmid-encoded, and are easily transferable among bacterial species. They are mostly of the KPC types in CR-hvKp. OXA-48-producing hvKP strains have been rarely reported in the literature. Methods: OXA-48-producing hvKP strains were collected from clinical specimens at the First Affiliated Hospital of Hainan Medical University from January 2022 to March 2023. Hypervirulent strains were tested for virulence in a mouse lethality study and underwent whole genome sequencing to identify genomic features. Results: A total of 42 unique OXA-48-bearing *K. pneumoniae* strains were identified, including three CR-hvKP strains (KP2683-1, NCRE61, and KP2185), which were isolated from bacteremia, pulmonary abscess, and liver abscess separately. The three CR-hvKP strains belonged to two different clones of ST11 KL64 (KP2185 and NCRE61) and ST23 K1 (KP2683-1). The KP2683-1 strain had the highest virulence. Whole genome sequencing analysis indicated that NCRE61 and KP2185 acquired IncFIB-type plasmids with a set of virulence genes (*iroBCDN*, *iucABCD*, *iutA*, *rmpA*, and *rmpA2*), while KP2683-1 acquired an IncL-type bla_{OXA-48}-harboring plasmid. Consecutive cultures showed that the bla_{OXA-48}-harboring plasmids were highly stable in the three hvKP strains and could be transmitted to *Escherichia coli* J53 by conjugation. The drug susceptibility testing results show that Ceftazidime/avibactam is sensitive for OXA-48-producing hvKP. Conclusions: Our study highlighted the two evolutionary pathways of OXA-48-producing hvKP strains and confirmed their virulence through in vivo testing. Ceftazidime/avibactam may be a viable option for treating OXA-48-producing hvKP strains.

Keywords: *Klebsiella pneumoniae*; carbapenemase; hypervirulent strain; OXA-48

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1. Introduction

Klebsiella pneumoniae (KP) is a common cause of respiratory tract, intestinal tract, blood, and abdominal organ infections in the world [1]. Upon acquisition of a plasmid that harbors a carbapenemase gene, it becomes carbapenem-resistant *K. pneumoniae* (CRKP), a superbug that causes untreatable or hard-to-treat infections [1,2]. CRKP strains usually lead to nosocomial infections and make therapeutic options very limited, which are related to high mortality [2]. The main molecular mechanism in CRKP is the bearing of carbapenemase-encoding plasmids, and KPC are the most common types of carbapenemases [2]. Apart from antimicrobial resistance, another worrisome development is related to the evolution of hypervirulent KP (hvKP). hvKP strains, which is usually thought to be attributed to the carriage of a virulence plasmid that harbors two CPS regulator genes (rmpA and rmpA2)

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and a number of siderophore gene clusters [1,3]. The emergence of hvKP has also become a global concern because it poses a significant constraint on therapeutic strategies in clinics. The study [4–6] revealed that the virulence traits of these strains have been related to the carriage of a typical virulence plasmid including multiple virulence-encoding genes (e.g., *rmpA*, regulator of the mucoid phenotype A) or specific KP capsular polysaccharide types (K1, K2, etc.). Studies have shown that virulence and carbapenem-resistance evolved separately in two distinct KP clonal groups [5,6]. However, convergence of these two evolutionary paths has led to the emergence of KP strains that are hypervirulent and carbapenem-resistant at the same time; therefore, these strains are called carbapenem-resistant and hypervirulent KP (CR-hvKP) [6,7]. CR-hvKP is considered a real superbug that exhibits high transmissibility, hyper-resistance, and hypervirulence, posing a grave threat to human health. These CR-hvKP strains can be generated by at least three different evolution pathways. Firstly, CR-hvKP strains can arise from the transfer of a pLVPK-like virulence plasmid into a classic CRKP strain [5,6]. Secondly, the transfer of a hybrid carbapenemase encoding and virulence plasmid in a KP strain can result in the emergence of CR-hvKP strains [8–10]. Finally, CR-hvKP strains can acquire carbapenemase-encoding plasmids [6,7]. In existing research reports, most CR-hvKP strains harboring the $bla_{\text{KPC-2}}$ plasmid were identified in China [5–7]. However, CR-hvKP strains bearing $bla_{\text{OXA-48}}$ have never been documented in Hainan, China [11,12]. In this research, we aimed to determine the clinical, drug-resistant, microbiological, and genomic characteristics of OXA-48-bearing CR-hvKP strains in the First Affiliated Hospital of Hainan Medical University, China.

2. Materials and Methods

2.1. Data Collection and Strains Identification

We collected OXA-48-producing KP strains from clinical samples (sputum, urine, blood, bile, pleural fluid, etc.) in the microbiological laboratories at the First Affiliated Hospital of Hainan Medical University from January 2022 to March 2023. The experimental study was approved by the institutional ethics board of the First Affiliated Hospital of Hainan Medical University (HYLL-2023-033). All experimental protocols were performed in accordance with the relevant ethical guidelines and regulations. Only the first culture was included for patients who had more than one culture that tested positive for OXA-48-producing strains of CR-hvKP to avoid duplication. Bacterial identification was determined using matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (bioMérieux SA, Marcy l'Etoile, France).

2.2. Susceptibility Testing of Agents

All CRKP strains were cultured on Columbia blood agar (Oxoid, Hampshire, UK) containing 5% sheep blood (Luqiao, Beijing, China). Strain identity was confirmed by MALDI-TOF mass spectrometry (BrukerDaltonik GmbH, Bremen, Germany). Antimicrobial susceptibility was identified by the Vitek2 System, with the exception of tigecycline and colistin. The minimum inhibitory concentration (MIC) of tigecycline, ertapenem, and imipenem was determined by Etest, and colistin was determined by the broth microdilution method. The European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints were used for tigecycline and colistin susceptibility (http://www.eucast.org/clinical_breakpoints, accessed on 1 January 2020). All the tests were performed and interpreted as per Clinical and Laboratory Standard Institute (CLSI) 2020 guidelines [13].

2.3. Bacterial Conjugation

Plasmid conjugation was performed using the OXA-48-producing CR-hvKP strain as the donor strain (ertapenem-resistant) and *Escherichia coli* J53 (sodium azide-resistant) as the recipient strain. Standard bacterial strains used in the conjugation experiment were from urine and blood, respectively. The experiment was conducted using a previously described filter mating method [9]. About 1×10^8 colony-forming units (cfu) of both the

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recipient strain and the donor strain were mixed and dotted on a sterilized filter, which was then incubated on a luria Bertani (LB) agar plate for 18 h at 37 °C. Transconjugants were selected using LB agar plates supplemented with 100 mg/L sodium azide and 0.125 mg/L ertapenem. Polymerase chain reaction (PCR) for *rmpA*/*rmpA2* genes and $bla_{\text{OXA-48}}$ and the MICs for carbapenem were performed on the transconjugants.

2.4. Microbiological Characteristics

All strains were subjected to a multiplex PCR analysis for the rapid detection of common clonal types (ST65, 86, 23, 375, 11/258) [14]. A strain was considered genetically hypervirulent if it contained the *rmpA*/*rmpA2* and *iutA* genes as previously described [15]. Capsular genotyping, detection of the *rmpA*/*rmpA2* genes, and genes encoding for carbapenemases, $bla_{\text{OXA-48}}$, bla_{IMP}, bla_{VIM}, bla_{KPC}, and bla_{NDM} were detected by PCR as previously described [15,16].

2.5. Stability of bla_{OXA-48}-Encoding Plasmid in CR-hvKP Strains

Bacterial cultures were grown at 37 °C in a shaking incubator at 200 revolutions per minute (RPM) and daily passaged by a 1:1000 dilution in LB broth without antibiotics for 28 days. From the plated culture, 100 colonies were randomly chosen and replica-plated onto LB agar plates, one containing ertapenem and one without antibiotics. Colonies growing on the antibiotic-free LB agar plate but not on the ertapenem-containing plate were then tested for the presence of $bla_{\text{OXA-48}}$ using PCR.

2.6. Virulence Assessment

All animal care procedures and protocols were approved by the Institutional Animal Care and Use Committee of the First Affiliated Hospital of Hainan Medical University. Female C57BL/6 mice aged 6–8 weeks were given intraperitoneal injections of KP strains at various concentrations, according to previous studies [9,14–16]. Survival was determined by Kaplan-Meier analysis with a log-rank test using the Prism software package (GraphPad, Prism 9.0). The OXA-48-producing CR-hvKP strains and control strains were estimated in a mouse lethality study to calculate their 50% lethal dose (LD50).

2.7. Whole-Genome Sequencing (WGS) Analysis

The whole Genome Shotgun project has been deposited at GenBank under the bioproject accession PRJNA549322. Resistance genes were called by AMRFinderPlus v3.9.8 and Abricate v1.0.0 (https://github.com/tseemann/abricate (accessed on 30 November 2023)), with the combination of the NCBI Bacterial Antimicrobial Resistance Reference Gene Database and ResFinder v4.0 [17,18]. OXA-48-producing CR-hvKP strains were sequenced using the combination of Illumina HiSeq (Illumina, Inc., San Diego, CA, USA) short-read and Oxford Nanopore long-read sequencing and assembled using Unicycler v0.4.9 [19]. Sequence types (ST), capsular types, and virulence genes were characterized using Kleborate v2.3.0 [20] and Kaptive v2.0.4 [21]. Plasmid replicon analysis was conducted using plasmidFinder [22,23].

2.8. Statistical Analysis

All the statistical analysis was conducted using MedCalc online software (https://www.medcalc.org/calc/odds_ratio.php (accessed on 30 November 2023)) and GraphPad Prism Version 9 (San Diego, CA, USA: GraphPad Software; www.graphpad.com). Statistical significance for parametric and non-parametric variables between the two carbapenemase genes was tested by chi-square and Fisher's exact tests, and p-values < 0.05 were considered significant. Ninety-five percent confidence intervals (CIs) were calculated for various outcomes among the isolates producing the two genes to know the possible association between the presence of a gene and an outcome.

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3. Result

3.1. Clinical Features of Patients Infected with OXA-48-Producing CR-hvKP Strains

During this study period, a total of 42 patient episodes involving OXA-48-producing KP strains were identified. The most common capsular genotype was K64 (16/42), followed by KN2 (12/42), and K47 (5/42). The majority of these strains belonged to ST11 (n = 21). Three patients were infected with OXA-48-producing CR-hvKP strains (KP2683-1, KP2185, and NCRE61), as detected by the presence of *iutA*, *iroN*, and *rmpA*/*rmpA2*. Table 1 presents the clinical features and outcomes of these three patients.

Table 1. Clinical characteristics of patients infected with OXA-48-producing hvKP strains.

Case	NCRE61	KP2185	KP2683-1
Invasive procedures and devices at the time of culture	endotracheal intubation and a mechanical ventilator	tracheostomy urinary catheter, central venous catheter,	central venous catheter
Diagnosis Location at time of culture	respiratory medicine, ordinary ward	general surgery ordinary ward	bacteremia intensive care unit
28-day mortality or survivor	survivor	survivor	survivor
Prior antibiotic exposure within 1 month	imipenem	piperacillin	piperacillin, meropenem,
Definitive antimicrobial therapy	tigecycline and piperacillin	meropenem	meropenem and tigecycline
Co-morbidity	chronic obstructive pulmonary disease	diabetes cerebral infarction	hypertension diabetes
Length of stay after infection: days	64 days	55 days	73 days
Community- or hospital-acquired infection	hospital-acquired infection	hospital-acquired infection	hospital-acquired infection
Age, years	68	71	62
Gender	male	male	male

3.2. Bacterial Conjugation Experiment

A conjugation experiment was conducted to assess the transconjugation ability of the OXA-48-carrying plasmid. The results in Table 2 indicated that the carbapenem resistance phenotype of the three OXA-48-producing hvKP strains could be transferred to *E. coli* J53. The transconjugants all carried the $bla_{\text{OXA-48}}$ gene, but the virulent determinant *ituA* or *rmpA*/*rmpA2* were not detected in these transconjugants, suggesting the virulence plasmids were not co-transferred along with the OXA-48 plasmids.

Table 2. Conjugational efficiency of OXA-48-carrying plasmids and susceptibility of transconjugants.

Strain	Conjugational Efficiency	Minimal Inhibitory Concentration (μg/mL)		
		Ertapenem	Imipenem	Meropenem
NCRE61 transconjugant	8.2×10^{-4}	0.5	2	0.38
KP2683-1 transconjugant	5×10^{-5}	3	4	0.5
KP2185 transconjugant	3.5×10^{-4}	0.38	3	0.38
E. coli J53	-	0.003	0.19	0.016

3.3. Plasmid Stability

The three OXA-48-producing hvKP strains were passed daily for 28 days (~280 generations) to assess the stability of $bla_{\text{OXA-48}}$-bearing plasmids in CR-hvKP strains. At day 28, $bla_{\text{OXA-48}}$ was not detected in these mutants by PCR; however, two plasmid-cured mutants of KP2185 were identified, and other β-lactamases and virulence genes such as *rmpA* and *rmpA2* were retained. The MICs of imipenem and ertapenem decreased from 2 and 12 mg/L to 0.125 and 2 mg/L, respectively. Plasmid-cured KP2683-1 and NCRE-61 strains were not obtained in this experiment, suggesting that the $bla_{\text{OXA-48}}$-bearing plasmid is highly stable in CR-hvKP strains.

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3.4. Antibiotic Sensitivity of OXA-48-Producing hvKP Strains

Table 3 presents the detailed MICs for the three OXA-48-producing hvKP strains. These strains exhibited varying antimicrobial resistance profiles. NCRE61 and KP2185 were resistant to the above-mentioned aminoglycosides, fluoroquinolones, and cephalosporins. Specifically, KP2683-1 was susceptible to almost all antibiotics tested, including aminoglycosides (amikacin and gentamycin), fluoroquinolones (ciprofloxacin and levofloxacin), cephalosporins (cefepime, ceftriaxone, cefuroxime, cefazolin, and ceftazidime), colistin, and tigecycline, with the exception of imipenem and ertapenem. The three strains had similar MICs against imipenem (2–3 mg/L) and were all susceptible to ceftazidime/avibactam.

Table 3. Antimicrobial susceptibility test of the three OXA-48-producing hvKP strains.

	MIC (mg/L) [a]		
Antibiotics	**NCRE61**	**KP2185**	**KP2683-1**
Tigecycline	1.0	0.5	0.75
Imipenem	3	2	2
Ertapenem	4	12	8
Colistin	4	2	1
Ceftazidime/avibactam	1.0	1.0	0.25
Piperacillin/Tazobactam	≥128	≥128	≤128
Cefepime	≥64	≥64	≤1
Ceftriaxone	≥64	≥64	≤1
Cefuroxime	≥64	≥64	4
Cefazolin	≥64	≥64	16
Ceftazidime	16	≥64	≤1
Ciprofloxacin	≥4	≥4	≤0.25
Levofloxacin	≥8	≥8	≤0.12
Gentamicin	≥16	≥16	≤1
Amikacin	≥64	≥64	≤2

[a] The values were MIC-correlates determined by the Vitek2 System, except for ertapenem, imipenem, and tigecycline, determined by the E test, and colistin, determined by broth microdilution.

3.5. Microbiological Characteristics, In Vivo Virulence Assessment, and Genomic Analysis of OXA-48-Producing hvKP Strains

Table 4 presents the microbiological characteristics of the three OXA-48-producing hvKP strains. The two strains were of ST11 and had a KL64 capsule type; the ST23 strain KP2683-1 had a KL1 capsule type, which is a commonly observed background in hvKP strains. The KP-2683 strain (ST23) has one chromosome with a length of 5,284,538 bp and three plasmids belonging to the IncFIB (repB_KLEB_VIR)-IncHIB (pNDM-MAR), repA (pKOX), and IncLgroups, respectively. Ulteriorly, KP-2683 only harbors the chromosomal-based $bla_{\text{SHV-11}}$ and plasmid-borne $bla_{\text{OXA-48}}$ genes. The NCRE-61 strain has one chromosome with a length of 5,380,176 bp and seven plasmids ranging from 1307 to 205,089 bp, belonging to the IncFIB (repB_KLEB_VIR), IncC, IncFIB (pKPHS1), FII (pBK30683), IncL, colE1, and an unknown group, respectively. NCRE-61 carries genes that encode resistance to aminoglycosides, fluoroquinolones, macrolides, sulfonamides, trimethoprim, tetracyclines, and β-lactams ($bla_{\text{TEM-1}}$, $bla_{\text{DHA-1}}$, $bla_{\text{CTX-M-14}}$, and $bla_{\text{OXA-48}}$). The KP2185 strain contains one chromosome with a length of 5,287,957 bp and six plasmids ranging from 15,032 to 203,769 bp. The plasmids belong to the IncFIB (repB_KLEB_VIR), IncC, IncFIB (pKPHS1), FII (pBK30683), IncL, and colE1 groups, respectively. Like the NCRE-61 strain, KP2185 also contains multiple genes encoding antimicrobial resistance, including $bla_{\text{OXA-48}}$. A comprehensive list of antimicrobial resistance genes can be found in Table 5.

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Table 4. Microbiological characteristics of OXA-48-producing hvKP strains.

Strain No	NCRE61	KP2185	KP2683-1
Virulence factors associated with hypervirulent strains	*ΔrmpA*, *rmpD*, *rmpC*, *rmpA2*, *iroBCDN*, *iucABCD-iutA*, *ybtSXQPAUTE*, *irp1*, *irp2*	*ΔrmpA*, *rmpD*, *rmpC*, *rmpA2*, *iroBCDN*, *iucABCD-iutA*	*rmpADC*, *ΔrmpA2*, *iroBCDN*, *iucABCD-iutA*, *ybtSXQPAUTE*, *irp1*, *irp2*
β-lactamase resistance gene	OXA-48, DHA-1, CTX-M-14, TEM-1, SHV-11	OXA-48, DHA-1, CTX-M-14, TEM-1, SHV-11	OXA-48, SHV-11
LD50	2.4×10^6	5.1×10^6	2.1×10^3
Capsular type	K64	K64	K1
ST type	ST11	ST11	ST23
Date of isolation	22 March 2022	2 December 2022	28 January 2023
Specimen	Bronchoalveolar lavage fluid	Liver abscess	Blood

Table 5. Antimicrobial-resistant genes of OXA-48-producing hvKP.

Strain No	Antimicrobial Resistant Genes								
	β-Lactamases	Aminoglycoside	Fluoroquinolones	Chloramphenicol	Tetracycline	Macrolides	Rifampin	Sulfonamides	Trimethoprim
NCRE61	$bla_{\text{OXA-48}}$, $bla_{\text{SHV-11}}$, $bla_{\text{TEM-1}}$, $bla_{\text{DHA-1}}$, $bla_{\text{CTX-M-14}}$	*strB*, *strA*, *rmtB*, *aac3-IId*, *aadA16*, *aadA2*, *aph(3″)-Ib*, *aph(6)-Id*, *aac(6′)-Ib-cr5*	*qnrB4*	*floR2*	*tetA*, *tetG*	-	*arr3*	*sul1*, *sul2*	*dfrA27*, *dfrA12*
KP2683-1	$bla_{\text{OXA-48}}$, $bla_{\text{SHV-11}}$	-	-	-	-	-	-	-	-
KP2185	$bla_{\text{OXA-48}}$, $bla_{\text{SHV-11}}$, $bla_{\text{TEM-1}}$, $bla_{\text{DHA-1}}$, $bla_{\text{CTX-M-14}}$	*strB*, *strA*, *rmtB*, *aac3-IId*, *aadA2*, *aph(3″)-Ib*, *aph(6)-Id*	GyrA-83I; ParC-80I, *qnrB4*	*floR2*	*tetG*	*erm42*	-	*sul1*, *sul2*	*dfrA12*

NOTE: "-" negative.

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An in vivo virulence assessment was conducted using a septicemia mouse model through intraperitoneal injection, revealing higher virulence in these three strains compared to a carbapenem-resistant ST11 OXA-48-producing strain (NCRE35), with an LD50 of 1×10^7 cfu. However, the LD50 ($2.4–5 \times 10^6$) in the two ST11-K64 hvKP strains was significantly higher than that of the ST23-K1 strain (2.1×10^3), indicating that the ST23-K1 strain possesses a higher level of virulence compared to the ST11-K64 strains, despite having similar virulence genes. The presence of virulence genes encoding aerobactin (*iroBCDN*), salmochelin (*iucABCD* and *iutA*), and *rmpADC* and *rmpA2* was observed in all three OXA-48-producing hvKP strains. However, the yersiniabactin genes (*irp and ybt*) were only detected in KP-2683 and NCRE-61 and were missing in the ST11 strain KP-2185.

Figure 1 presents the plasmid structures of $bla_{\text{OXA-48}}$ harboring plasmids. Figure 2 presents the plasmid structure of virulence plasmids from NCRE-61, KP2185, NTUH-K2044, and KP2683. These virulence genes were found on the virulence plasmids of the three strains (pKP-2683-vir, pNCRE-61-vir, and pKP2185-vir). KP-2683 was unique in containing an additional copy of *rmpADC* on the chromosome in ICE*Kp1*. It should be noted that the *rmpA* gene on pKP-2185-vir and pNCRE-61-vir were truncated due to a frameshift, while the *rmpA2* gene on pKP2683-vir was also truncated. pKP2683-vir showed >99.9% nucleotide identity and ~98% query coverage against plasmid pK2044 from the K1 prototype strain NTUH-K2044. pNCRE-61-vir and pKP-2185-vir had 99.9% identity but ~92% query coverage against pK2044 (Figure 2). In addition, pNCRE-61-vir and pKP-2185-vir only contain a single replicon of IncFIB (repB_KLEB_VIR), while the second IncHIB (pNDM-MAR) found in p2683-Vir was missing. Overall, the three $bla_{\text{OXA-48}}$ harboring plasmids, pKP-2683-OXA, pNCRE-61-OXA, and pKP2185-OXA, are highly similar to the $bla_{\text{OXA-48}}$ prototype plasmid pOXA48a [22,24], with 94–96% query coverages and ~99.8% nucleotide identity (Figure 1). The main difference is that the $bla_{\text{OXA-48}}$ in pOXA48a is harbored by a Tn1999.1 element, while it was carried by a Tn1999.2 transposon in three plasmids sequenced in the current study.

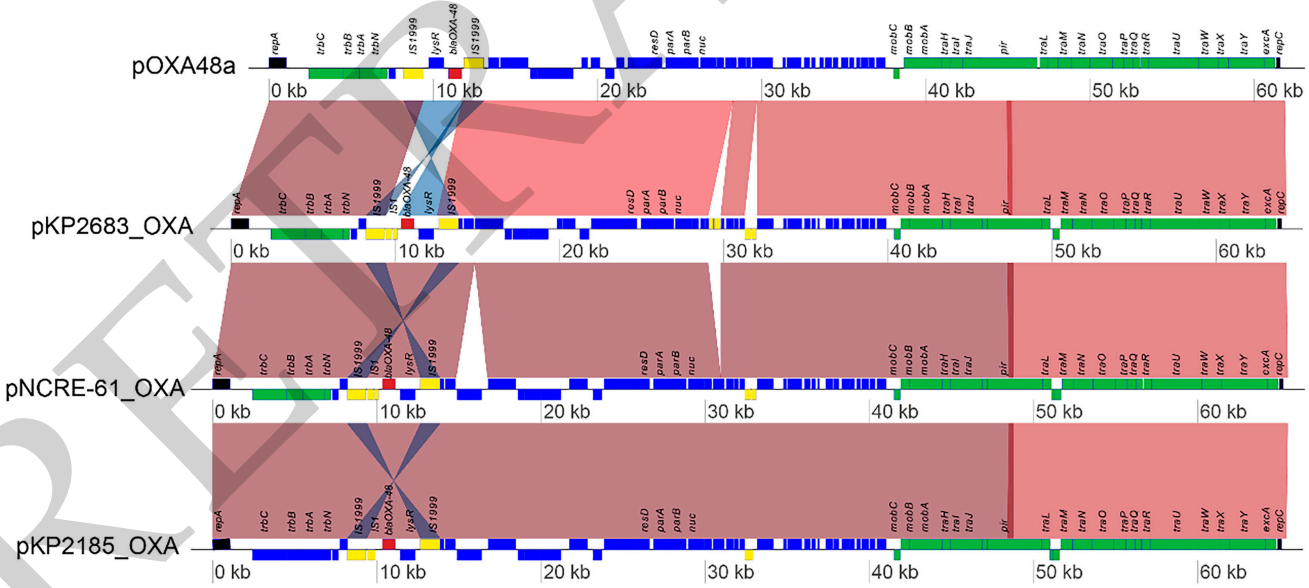

Figure 1. The plasmid structures of $bla_{\text{OXA-48}}$ harbor plasmids. The open reading frames were depicted as blocks. Black, yellow, red, green, and blue blocks represent replication genes, mobile elements, antimicrobial resistance genes, plasmid transfer genes, and plasmid backbone genes, respectively. Genes in the forward direction were located above the central line, while genes in the reverse direction were located below. The turkey red shading indicates regions of shared homology in the same direction among the different elements, while the blue shading signifies inverted shared homology.

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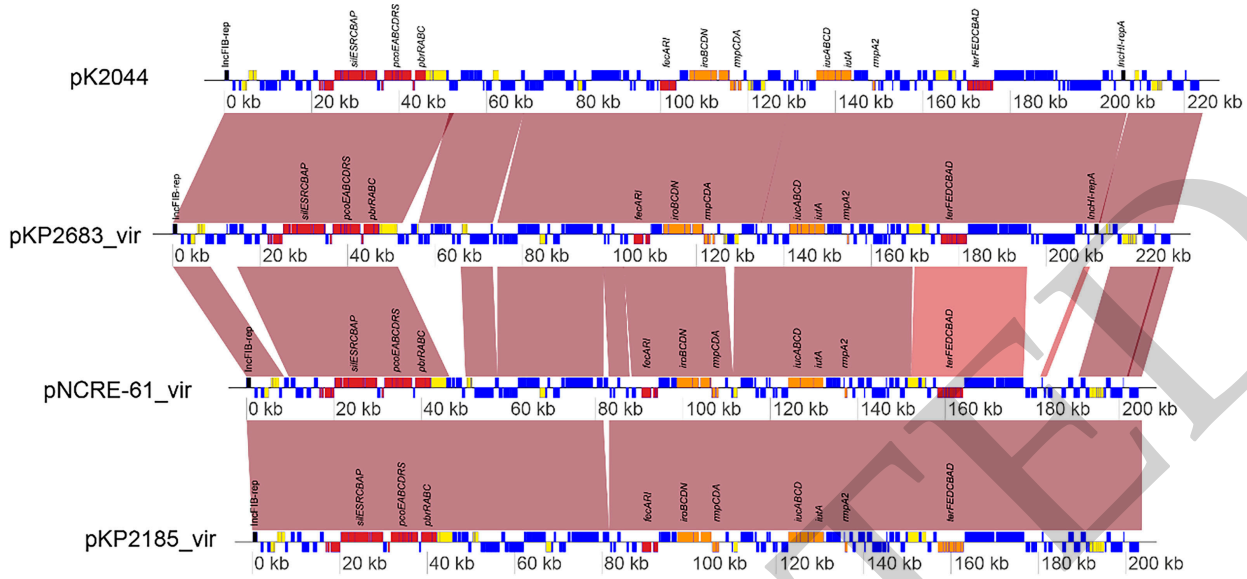

Figure 2. The plasmid structure of virulence plasmids from NCRE-61, KP2185, NTUH-K2044, and KP2683. The open reading frames were depicted as blocks. Black, yellow, red, orange, and blue blocks represent replication genes, mobile elements, heavy metal resistance genes, virulence genes, and plasmid backbone genes, respectively. The shading represents regions of shared homology among the different elements. Genes in the forward direction were located above the central line, while genes in the reverse direction were located below.

We conducted a core genome analysis and compared the two ST11 strains with an additional five non-hypervirulent ST11 KL64 strains collected from our hospital to increase resolution. The results showed that KP-2185 and NCRE-61 had 26 different core SNPs but differed from the other five ST11 strains by 78–81 core SNPs.

4. Discussion

K. pneumoniae, as a vital human pathogen, represents increasing multidrug-resistance, particularly to third-generation cephalosporins and carbapenems [11]. Taking into account the mortality and morbidity of patients, medical burden, global drug resistance trends, and other relevant standards, CRKP was undoubtedly considered a key priority pathogen in the priority antibiotic-resistant bacteria list by the World Health Organization (WHO) in 2017 [3,4]. Carbapenem resistance is primarily mediated by the production of carbapenemases (carbapenem hydrolyzing-lactamases), which have been found in KP isolates to fall into three categories: (1) metallo-lactamases or molecular class B lactamases (New Delhi metallo-lactamase/NDM-1, IMP, VIM) that hydrolyze all lactams except monobactams and are inhibited by ethylenediamine tetraacetic acid (EDTA) but not clavulanic acid; (2) serine-lactamases of molecular class A (NMC/IMI, SME, KPC, and GES) that hydrolyze even monobactams are inhibited by clavulanic acid and tazobactam but not by EDTA; (3) molecular class D serine β-lactamases (oxacillinases β-lactamases/OXA-48) that do not hydrolyze monobactams and are poorly inhibited by clavulanic acid and EDTA [4,5]. These properties are used for differentiation in the laboratory. Most of these carbapenemases are acquired either by mutation or by horizontal gene transfer.

The recent emergence of hvKP strains has drawn global attention as they can cause severe infections that are challenging to treat with current antibiotics. Most CR-hvKP strains reported to date involve KPC or NDM-producing strains [5,6]. OXA-48-producing hvKP strains have been reported in Europe, but the in vivo virulence of these strains has not been evaluated [12,22,24,25].

We discovered three CR-hvKP strains among 42 clinical OXA-48-producing KP strains. It should be noted that the rmpA2 gene in these plasmids is often structurally different from the WT rmpA2 gene by harboring various mutations or insertions/deletions; likewise,

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the rmpA gene in some plasmids also possessed mutations. Our findings revealed two evolutionary pathways for CR-hvKP. Firstly, the Prototypical ST23 hvKP strain KP2683-1 acquired an IncL/M type plasmid containing the OXA-48 gene; secondly, the ST11 CRKP strains KP2185 and NCRE61 acquired an IncFIB virulence plasmid carrying the genes *rmpA2*, *iut*, and *iro* gene clusters. In the above two evolutionary routes, the plasmids of the three CR-hvKP strains play a crucial role in the dissemination of resistance and virulence-associated genetic elements.

Our study first describes OXA-48-producing hvKP strains in the Hainan province of China and verifies their virulence through in vivo testing. The ST11 and capsular K64 strains (NCRE61 and KP2185) that acquired a virulence plasmid had an LD50 in the middle range compared to classic CRKP and KP2683-1, suggesting that the clonal backgrounds may have an impact on the virulence of CR-hvKP. The strain KP2683-1, with an ST23 and capsular K1 background, was resistant to carbapenems but retained susceptibility to most extended-generation cephalosporins, a profile consistent with the acquisition of the $bla_{\text{OXA-48}}$ gene on an Inc. L plasmid. KP2683-1 also showed the highest virulence among the strains tested. Our study also found that the OXA-48-harboring plasmids are transmissible through conjugation, indicating that their transmissible nature will likely further contribute to the disease burden of CR-hvKP infection.

A recent study suggested that a hvKP ST23 strain was found to have acquired the OXA-181-bearing plasmid, but this strain lost the plasmid in subsequent subcultures without the selective pressure of carbapenem [26]. It is widely accepted that antibiotic resistance often comes with a cost to the organism's fitness [27]. The hypothesis is that hvKP strains may suffer a negative impact on fitness if they carry certain carbapenemase-encoding plasmids, and that these genes may be relatively unstable in the absence of selective pressure.

The stability of the OXA-48-harboring IncL-type plasmid in CR-hvKP strains has not been documented in previous literature. Interestingly, we were the first to discover that these $bla_{\text{OXA-48}}$-bearing plasmids were highly stable in CR-hvKP strains in our study, even without the selective pressure of carbapenem, which may be linked to the specific CR-hvKP strain and plasmid structure, posing an additional threat from this superbug. Our findings reveal that ceftazidime/avibactam, a novel β-lactam/β-lactamase inhibitor, can be effective against CR-hvKP, even when the strain is carbapenem-resistant. This is supported by previous studies indicating the efficacy of ceftazidime/avibactam against KPC-2-producing CRKP [28]. Our results suggest that ceftazidime/avibactam may also be a viable option for treating OXA-48-producing hvKP. Our study emphasizes the need for new treatment options and highlights the difficulties in treating CR-hvKP strains.

Nevertheless, a few limitations exist in our study. First, to fully understand the virulence characteristics and strain backgrounds of these OXA-48-producing CR-hvKP strains, as well as their clinical impacts, a comprehensive molecular surveillance study on these strains is currently underway. This problem will be the focus of our future study. Second, this was a single-center study. We only collected strains from a tertiary hospital in China. Additionally, we did not perform the phenotypic disinfectant susceptibility testing of the CRKP strains in our study. Finally, despite only identifying three OXA-48-producing CR-hvKP strains, we were able to uncover two evolutionary routes in these strains.

5. Conclusions

In conclusion, we first reported three OXA-48-producing hvKP strains in Hainan Province, China, and demonstrated that $bla_{\text{OXA-48}}$-bearing plasmids are highly stable in two distinct clonal backgrounds. Our study highlighted the two evolutionary pathways of OXA-48-producing hvKP strains and confirmed their virulence through in vivo testing. The $bla_{\text{OXA-48}}$-encoding plasmid demonstrated high conjugation efficiency and stability in the CR-hvKP strains. Ceftazidime/avibactam may be a viable option for treating OXA-48-producing hvKP strains. Furthermore, our study emphasizes the need for further research on CR-hvKP strains worldwide to better comprehend the geographic variations

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and the relationship between virulence and resistance with the ongoing emergence of OXA-48-producing hvKP strains in Hainan Province, China.

Author Contributions: L.L., B.L. and M.Y. collected the epidemiological materials, clinical data, and samples. M.Y., B.L. and L.L. manipulated laboratory tests and statistical analysis of data. L.L. drafted the manuscript. X.Z. and Q.L. revised the final manuscript. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: This study was approved by the institutional ethics board of the First Affiliated Hospital of Hainan Medical University (HYLL-2023-033), and informed consent was waived by the First Affiliated Hospital of Hainan Medical University. This study is reported in accordance with ARRIVE guidelines.

Informed Consent Statement: All authors have been personally and actively involved in the substantive work leading to the report and will hold themselves jointly and individually responsible for its content. Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data will be made available to others on reasonable requests to the corresponding author. The availability of data and material also needs to be approved by the institutional ethics board of the First Affiliated Hospital of Hainan Medical University.

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