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Yanqiao Gong , Yu Feng , Xiaoju Lv *

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Article

Identification of a Novel KPC Variant, KPC-204, Conferring Resistance to Both Carbapenems and Ceftazidime-Avibactam in an ST11 *Klebsiella pneumoniae* Strain

Yanqiao Gong [1,2], Yu Feng [1,3] and Xiaoju Lv [1,4,*]

[1] Center of Infectious Diseases, West China Hospital, Sichuan University, Chengdu, China; gongyq@hbmzu.edu.cn (Y.G.); feng_yu@scu.edu.cn (Y.F.)
[2] Department of Infection Control, Minda Hospital, Hubei Minzu University, Hubei, China
[3] Center for Pathogen Research, West China Hospital, Sichuan University, Chengdu, China
[4] Division of Infectious Diseases, State Key Laboratory of Biotherapy, Chengdu, China
* Correspondence: lvxj@scu.edu.cn

Abstract: This study describes KPC-204, a novel variant of *Klebsiella Pneumoniae* carbapenemase, characterized by a KDD amino acid insertion at Ambler position 269 deviates from KPC-2. This variant was identified in an ST11-type clinical isolate of carbapenem-resistant *Klebsiella Pneumoniae* from China. Notably, KPC-204 exhibits resistance to both ceftazidime-avibactam and carbapenems. Genetic analysis revealed that $bla_{KPC-204}$ was located on a highly mobile IncFII/IncR plasmid within a complex genetic structure that facilitates its spread. Functional analysis, achieved through cloning into *E. coli* DH5α, validates KPC-204's contribution to increased resistance to ceftazidime-avibactam. The kinetic parameters showed that KPC-204 exhibited similar affinity to KPC-2 toward ceftazidime and reduced sensitivity to avibactam. Mating experiments demonstrated the resistance's transmissibility. This investigation underscores the evolving diversity of KPC variants affecting ceftazidime-avibactam resistance, highlighting the necessity for continuous monitoring.

Keywords: KPC-204; ceftazidime-avibactam; *Klebsiella pneumoniae*

1. Introduction

Carbapenem-resistant *Klebsiella Pneumoniae* (CRKP) with the *Klebsiella Pneumoniae* carbapenemase (KPC) gene is a significant concern worldwide[1]. KPC enzymes degrade β-lactam antibiotics, including carbapenems, and are resistant to conventional β-lactamase inhibitors. Ceftazidime-avibactam (CZA) has been crucial for its effectiveness and safety in treating the infections caused by carbapenem-resistant *Enterobacteriaceae* (CRE)[2,3]. Nonetheless, the rise of CZA resistance among KPC-producing CRKP strains has become alarming, necessitating vigilant surveillance. The mechanisms of resistance to CZA in KPC-producing strains could be due to coproduction of less sensitive β-lactamases, to changes in membrane permeability including loss or mutations in porins, or to efflux pumps[4]. Nevertheless, the most frequent mechanism remains as mutations in KPC-encoding genes[4,5]. Previously reported mutations like KPC-33(D179Y),T243A (unassigned)[6], KPC-128 (D179Y/T243M)[6], KPC-134 (D178A with an insert sequence of aspartic acid-aspartic acid-asparagine-arginine-alanine-proline-asparagine-lysine)[7], KPC-93 (T237S and H274Y)[8], KPC-74 (G239_V240 deletion)[9], and KPC-71 (S182 insertion)[10], these genetic alterations are associated with increased CZA minimum inhibitory concentration(MICs) and decreased meropenem (MEM) MICs in comparison to wild-type isolates. Extension of resistance to CZA is associated with a trade-off in the lose resistance to carbapenem[4]. This balance is clinically beneficial, allowing for the combined use of CZA and carbapenems in treatment[11]. We identified a

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novel KPC variant, KPC-204, in an ST11 *K. Pneumoniae* isolate from China, which features a KDD insertion at Ambler position 269 within loop 267–275, a mutation hotspot divergent from KPC-2[12]. KPC-204 significantly decreases susceptibility to CZA, yet remains effective against carbapenems.

2. Results

2.1. Antimicrobial Susceptibility

The *K. Pneumoniae* isolate 130125 was resistant to piperacillin, piperacillin-tazobactam, cefoxitin, cefepime, ceftazidime, aztreonam, imipenem, meropenem, ertapenem, but was susceptible to imepenem-relebactam, meropenem-vaborbactam (Table 1). Moreover, the isolate was resistant to CZA, with a minimum inhibitory concentration (MIC) of 256 mg/L.

Table 1. MIC (mg/L) of antimicrobial agents for isolate 130125, 015093 and *E. coli* DH5*α* expressing KPC-204 or KPC-2.

Strains	MICs (mg/L)[a]											
	PIP	TZP	FOX	FEP	ATM	CAZ	CZA	IPM	IMR	MEM	MEV	ETP
130125	>512	256	>512	>512	512	>512	256	64	0.25	64	0.5	64
015093	>512	>512	>512	>512	512	>512	0.5	128	0.25	256	0.06	64
DH5*α*::pEKPC-2	>512	>512	>512	>512	512	128	0.5	16	0.125	8	0.03	8
DH5*α*::pEKPC-204	>512	256	>512	512	256	128	64	16	0.125	16	0.125	8
DH5*α*::pET28a	1	1	2	0.06	0.125	0.25	0.25	0.25	0.06	≤0.015	≤0.015	≤0.015
E. coli J53	1	1	1	0.06	0.125	0.25	0.125	0.125	0.06	≤0.015	≤0.015	≤0.015
J53::pKPC2_015093	>512	>512	>512	512	512	512	0.5	32	0.25	32	0.03	32
J53::KPC204_130125	>512	256	>512	512	512	512	64	32	0.25	64	0.25	32

[a]Abbreviations: PIP, Piperacillin; TZP, Piperacillin-tazobactam; FOX, Cefoxitin; FEP, Cefepime; CAZ, Ceftazidime; CZA, Ceftazidime-avibactam; ATM, Aztreonam; IPM, Imipenem; IMR, Imipenem-relebactam; MEM, Meropenem; MEV, Meropenem-vaborbactam; ETP, Ertapenem; Avibactam was added at a fixed concentration of 4 mg/L, tazobactam at 4 mg/L, relebactam at 4 mg/L, vaborbactam at 8 mg/L.

2.2. Genomic Analysis of Clinical K. pneumoniae Isolate 130125

The complete genome sequence of strain 130125 was obtained by *de novo* hybrid assembly of both short (Illumina) and long (Nanopore) reads and had a 5.4-Mb circular chromosome and three plasmids (Table 2). Strain 130125 was identified as *K. Pneumoniae*, exhibiting 99.67% average nucleotide identity (ANI) with reference strain HS11286 (GCF_000240185.1), exceeding the classification threshold of ≥ 96% ANI for bacterial species. Strain 130125 is classified as ST11, a prevalent type of CRKP in China, and exhibits the KL64 capsule type. The genetic assessment of *ompK35* and *ompK36* genes identified characteristic mutations in ST11 *K. Pneumoniae* strains: a truncation in *ompK35* and an insertion of GD amino acids at positions 134-135 in *ompK36*[13].

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Table 2. The complete genome and antimicrobial resistant genes of isolate 130125.

	Accession no.	Size,bp	Replicon type	Resistance genes	
				β-Lactam	Other
130125_chr	CP148996	5,462,753	-	*bla*SHV-158	*aadA2*, *fosA6*
pKPC204_130125	CP148997	154,728	IncR, IncFII	*bla*KPC-204, *bla*TEM-1, *bla*CTX-M-65	*rmtB1*
p1_130125	CP148998	10,060	ColRNAI		
p2_130125	CP148999	5,596	-		

*2.3. Genetic Context of bla*KPC-204*-Carrying Plasmid*

Strain 130125 harbors genes for four β-lactamases including narrow-spectrum β-lactamases gene *bla*SHV-187[14] on chromosome, a novel carbapenemase gene *bla*KPC-204 alongside *bla*CTX-M-65 and *bla*TEM-1, was located on a 154-kb IncFII/IncR plasmid, designated as pKPC204_130125 (Table 2). KPC-204 is a novel variant with a three-amino-acid insertion (Lys-Asp-Asp) between amino acids 268 and 269 within loop 267–275, diverging from KPC-2 (Figure 1). An alignment of pKPC204_130125 with pKPC2_015093(GenBank accession no. CP036301), revealed a 100% coverage and 99.97% identity, indicating significant genetic similarity (Figure 2). The *bla*KPC-204 gene is located within a composite transposon, flanked downstream by IS*Kpn27* and upstream by IS*Kpn6*. Moreover, *bla*KPC-204, in conjunction with *bla*CTX-M-65, is part of a 10-kb integrative composite transposon, bounded by IS*26* sequences (Figure 2). This arrangement underscores the genetic mobility potential, facilitating the dissemination of antibiotic resistance.

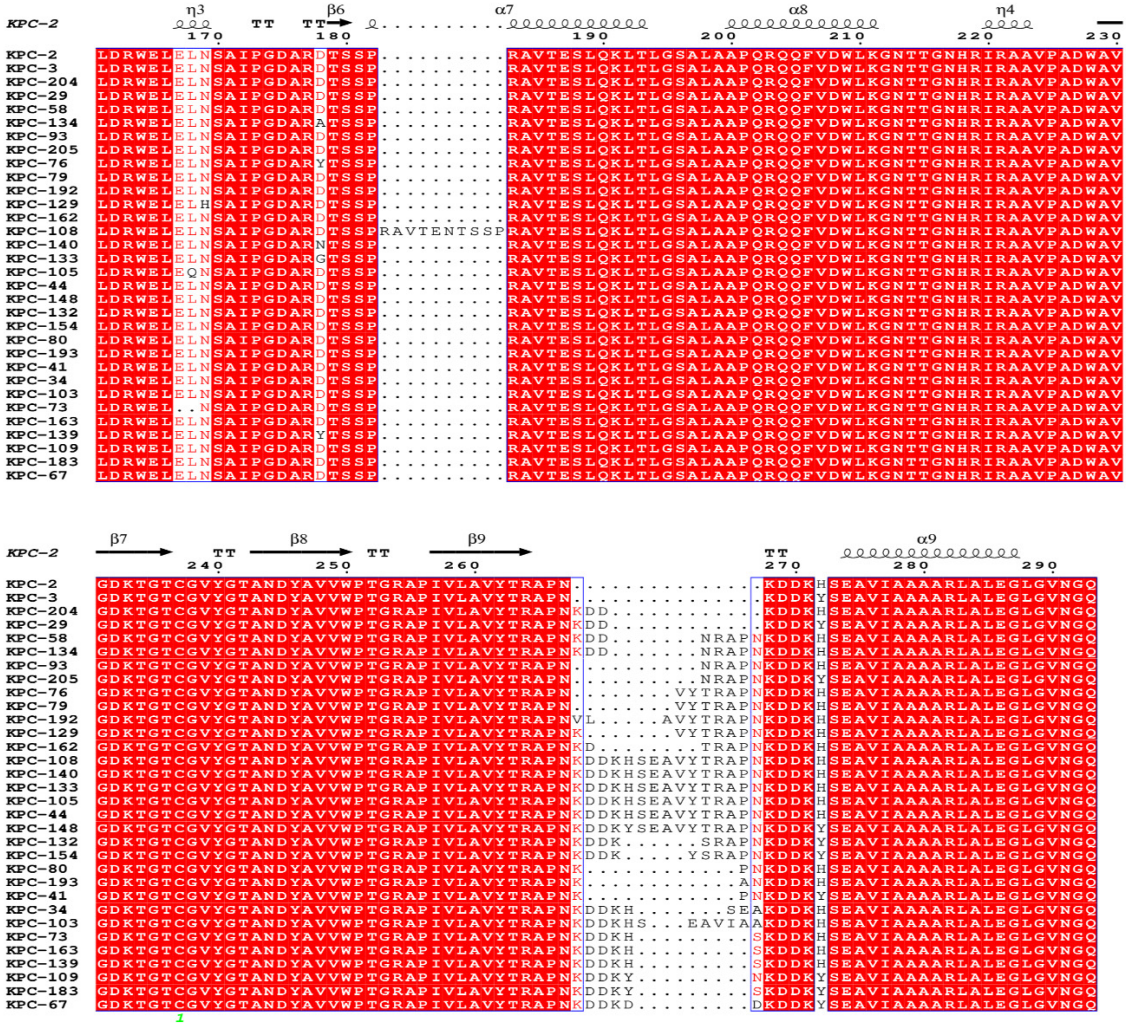

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Figure 1. Alignment of KPC variants with insertions at Ambler position 269, available in the NCBI database (April 2024), including the Omega loop (residues 164-179) and loop 266-275. The alignment of amino acid sequences and the prediction of secondary structures were performed using ESPript 3[15]. Secondary structure elements, α helixes, β sheets, and 310-helixes (representing by η), are indicated. β-strands are rendered as arrows, and strict β-turns are shown as TT letters.

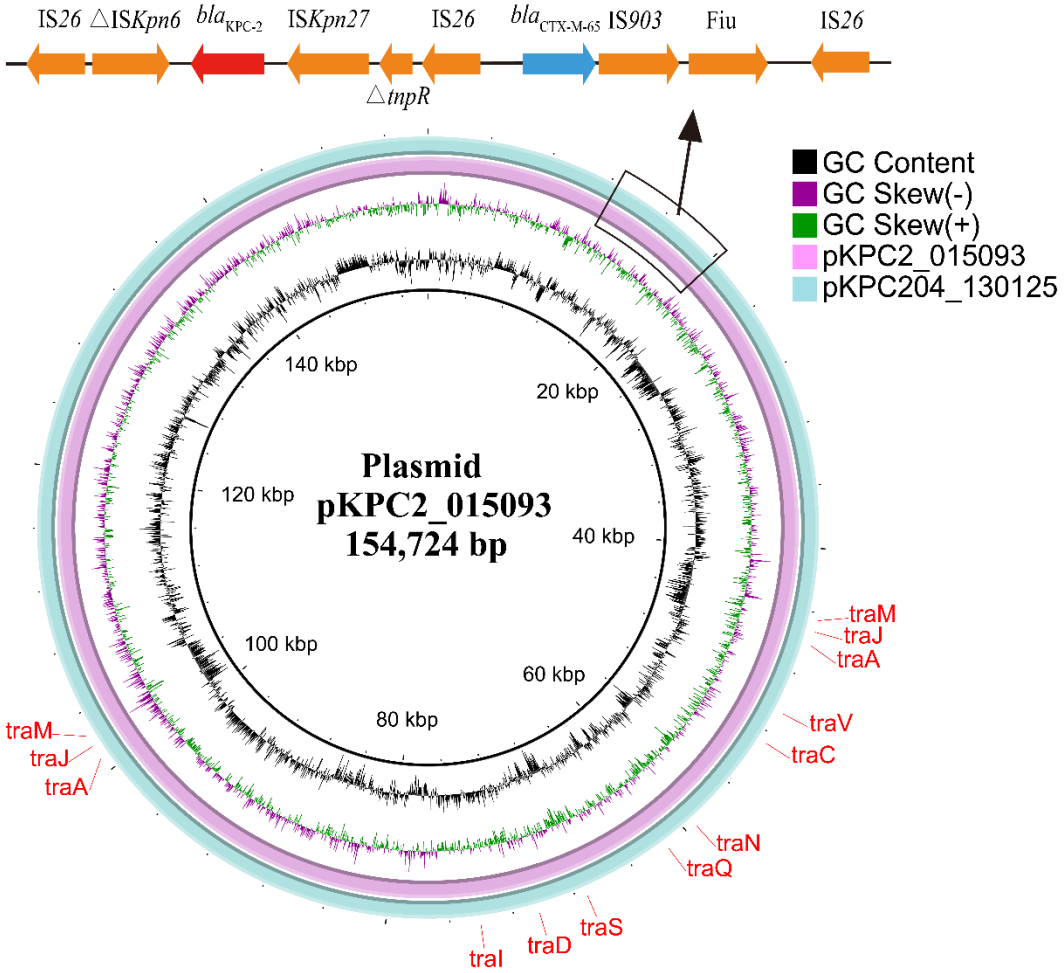

Figure 2. Alignment of pKPC204_130125 with pKPC2_015093. pKPC2_015093 is used as a reference. The alignment, conducted as a pairwise BLASTn comparison using the BLAST Ring Image Generator (BRIG)[16], between plasmid pKPC204_130125 and pKPC2_015093 (GenBank accession no. CP036301) demonstrated a 100% coverage and 99.97% identity. The *bla*KPC-204 gene is located within a composite transposon, flanked downstream by IS*Kpn27* and upstream by IS*Kpn6*. Moreover, *bla*KPC-204, in conjunction with *bla*CTX-M-65, is part of a 10-kb integrative composite transposon, bounded by IS*26* sequences. The locations of *tra* genes, pivotal for conjugation[17], are indicated.

2.4. Identification of blaKPC-204 Involved in CZA Resistance

*bla*KPC-204 and *bla*KPC-2 were successfully cloned into pET28a, generating pEKPC-204 and pEKPC-2. In *E. coli* DH5α, pEKPC-204 conferred resistance to a broad spectrum of β-lactams, notably to CZA (MIC 64/4 mg/L), and to imipenem, meropenem, and ertapenem (MICs 16 mg/L, 16 mg/L, and 8 mg/L, respectively), while remaining susceptible to imipenem-relebactam and meropenem-vaborbactam (MICs 0.125/4 mg/L and 0.125/8 mg/L, respectively). Remarkably, the CZA MIC for DH5α::pEKPC-204 was 128 times higher than that for DH5α::pEKPC-2(Table 1). When tested with a fixed ceftazidime concentration of 2 mg/L, the MIC for avibactam against DH5α::pEKPC-204 was 16 mg/L, four times the MIC for DH5α::pEKPC-2 (4 mg/L), highlighting the significant elevation in avibactam resistance attributable to the KPC-204 variant.

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2.5. Enzyme Kinetic Parameters and IC_{50} Values

The enzymatic kinetics analyses revealed that KPC-204 exhibits comparable catalytic efficiencies with substrates such as Nitrocefin, ceftazidime, and meropenem to those of KPC-2, as shown in Table 3. The hydrolytic profile of KPC-204 was consistent with the MIC observations presented above (Table 1). Moreover, the IC_{50} value, defined as the concentration required to achieve 50% inhibition, of avibactam was approximately 16 times greater for KPC-204 compared to KPC-2. This suggests that the insertion at position 269 (ins_269_KDD) in KPC-204 correlates with a reduced affinity and diminished sensitivity to avibactam. In contrast, the inhibitory effects of tazobactam and clavulanic acid on KPC-204 were markedly stronger than on KPC-2, displaying approximately 21-fold and 7-fold lower IC_{50} values, respectively (Table 4).

Table 3. Kinetic parameters of purified β-lactamases KPC-2 and KPC-204[a].

	KPC-2			KPC-204		
β-Lactam	**K_m (μM)**	**k_{cat}(s^{-1})**	**k_{cat}/K_m ($\mu M^{-1}\cdot s^{-1}$)**	**K_m (μM)**	**k_{cat}(s^{-1})**	**k_{cat}/K_m ($\mu M^{-1}\cdot s^{-1}$)**
Nitrocefin	22.124	97.589	4.411	31.178	116.419	3.734
Ceftazidime	870.413	5.226	0.006	975.154	7.801	0.008
Meropenem	15.283	5.194	0.34	14.157	8.325	0.588

[a]Data are the means of three independent experiments. Standard deviations were within 15% of the mean value.

Table 4. IC_{50} of β-lactamases inhibitors against KPC-2 and KPC-204[a].

	IC_{50} (μM)	
Inhibitor	**KPC-2**	**KPC-204**
Avibactam	0.045	0.569
Tazobactam	1.782	0.083
Clavulanic acid	0.887	0.124

[a]Data are the means of three independent experiments. Standard deviations were within 15% of the mean value.

2.6. $bla_{KPC-204}$ Was Located in a Self-Transmissible Plasmid

The transfer frequencies of plasmids pKPC2_015093 and pKPC204_130125 to *E. coli* J53 AziR were 2.4×10^{-5} and 8.6×10^{-4}, respectively, based on the ratio of transconjugants to recipients, highlighting pKPC204_130125 is readily self-transmissible. MICs for meropenem in *E. coli* J53 AziR carrying the respective plasmids were 32 mg/L and 64 mg/L. MICs for CZA were 0.5 mg/L for J53 with pKPC2_015093 and 64 mg/L for J53 with pKPC204_130125 (Table 1).

3. Discussion

KPC-2-producing ST11-type carbapenem-resistant *Klebsiella Pneumoniae* (CRKP) strains have emerged as a prevalent clonal lineage in China, posing significant clinical challenges[18,19]. With the global utilization of CZA, resistance to CZA has increased, primarily due to novel mutations in the genes encoding the KPC enzyme[4]. By April 2024, 194 KPC variants have been identified in the NCBI Reference Sequences (RefSeq) database (https://www.ncbi.nlm.nih.gov/pathogens/refgene/#KPC). Notably, instances of CZA resistance in *K. Pneumoniae* have been reported even in the absence of prior CZA exposure[20,21]. In this study, we describe a novel KPC variant which features a KDD insertion at Ambler position 269 within loop 267–275. This variant was identified in an ST11-type clinical isolate of CRKP from China, with no prior exposure to CZA.

The amino acid loop 267–275 in the KPC enzyme is a key mutation hotspot. Notably, mutations involving insertions at Ambler position 269 have been identified in several KPC variants, including KPC-204, -29, -58, -134, -93, -205, -76, -79, -192, -129, -162, -108, -140, -133, -105, -44, -148, -132, -154, -80, -193, -41, -34, -103, -73, -163, -139, -109, -183, and -67 (Table S2). Alignment of these KPC variants, including the Omega loop (residues 164-179) and loop 266-275, is shown in Figure 1. Among these,

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KPC-29, -93, -76, -44, -154, -41, and -67 are associated with resistance to CZA[8,21–25]. Additionally, variants KPC-29, -44, -154, and -67 have also been reported to exhibit resistance to both CZA and MEM. The kinetic parameters showed that KPC-204 exhibited similar affinity to KPC-2 toward ceftazidime and reduced sensitivity to avibactam. Unlike KPC variants in the Omega loop, where point mutations predominate, mutations in the loop 267–275 primarily involve amino acid insertions, resulting in duplications of amino acids[4]. Moreover, while extensions of resistance to CZA in KPC variants within the Omega loop are typically associated with a trade-off in carbapenem susceptibility, this trade-off is absent in some KPC variants with mutants of loop 267–275. Specifically, the resistance phenotype to both CZA and carbapenems is exclusively associated with mutations in loop 267–275.

The KPC-type carbapenemase gene frequently resides on self-conjugative plasmids, facilitating its spread across bacterial populations[4,26–28]. Specifically, KPC-204 is harbored on an IncFII/IncR plasmid, known for its capability for horizontal transfer via conjugation, thus highlighting the need for rigorous monitoring. The genetic context of *bla*$_{KPC-204}$ and plasmid pKPC2_015093 shows a high degree of similarity, positioned within a composite transposon, flanked downstream by IS*Kpn27* and upstream by IS*Kpn6*. This arrangement is similar to that found in pKP048 from *Klebsiella Pneumoniae* isolates in China, yet it diverges from Tn*4401*[29,30]. Additionally, *bla*$_{KPC-204}$, together with *bla*$_{CTX-M-65}$, forms part of a 10-kb integrative composite transposon, enclosed by IS*26* sequences, indicating potential for mobility and spread that warrants heightened attention. On a positive note, recent studies have demonstrated the efficacy of novel inhibitor combinations, such as imipenem-relebactam and meropenem-vaborbactam, in addressing these resistant strains.

We acknowledge the limitations of this study, specifically the absence of analyses in protein structure, molecular docking, molecular simulation, and other aspects of structural and computational chemistry, areas beyond our specialization. Nonetheless, our research provides preliminary insights into the antimicrobial resistance profile of *bla*$_{KPC-204}$.

4. Materials and Methods

4.1. The Strains and In Vitro Susceptibility

The *K. Pneumoniae* strain, designated 130125, was isolated from respiratory tract secretion of a patient in 2017 in the Intensive Care Unit (ICU) at West China Hospital. The patient received treatment with Penicillin and Meropenem for a duration of 12 days, followed by a 3-day course of Cefoperazone-Sulbactam before the sample was taken. Preliminary species identification was performed by Vitek II (bioMérieux, Marcy-l'Étoile, France). MICs of antimicrobial agents were determined using the microdilution method of the Clinical and Laboratory Standards Institute (CLSI) [31]. Avibactam was added at a fixed concentration of 4 mg/L, tazobactam at 4 mg/L, relebactam at 4 mg/L, vaborbactam at 8 mg/L.

4.2. Whole Genome Sequencing and Analysis

Genomic DNA of strain 130125 was prepared using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) and was subjected to whole genome sequencing using both HiSeq X10 (Illumina; San Diego, CA, United States) and MinION (Nanopore; Oxford, United Kingdom) platforms. Both short (Illumina) and long (Nanopore) reads were utilized to generate a *de novo* hybrid assembly using Unicycler v0.5.0[32] under conservative mode and polished using Pilon v1.24[33]. FastANI v1.33[34] was used to calculate pairwise average nucleotide identity (ANI) between 130125 and the type strain of *K. Pneumoniae* (GCF_000240185) for precise species identification, with a cut-off of 96% applied to define a bacterial species[35]. Sequence type (ST) was determined by querying the PubMLST database[36] using MLST v2.23.0 (https://github.com/tseemann/mlst), while capsule (KL) and outer membrane porins were typed using Kleborate v2.3.2[37]. Antimicrobial resistance genes and plasmid replicons were identified from the genome sequences using the ABRicate v1.0.0 (https://github.com/tseemann/abricate) to query the ResFinder database (https://cge.cbs.dtu.dk/services/ResFinder/) and PlasmidFinder[38] database, respectively. Plasmid

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comparison was performed using BRIG[16] in the default settings. Insertion sequences were identified using ISFinder[39].

4.3. Cloning Experiment

To evaluate the KPC-204 variant's role in CZA resistance in *K. Pneumoniae* 130125, the *bla*$_{KPC-204}$ and *bla*$_{KPC-2}$ genes, along with their promoter regions, were amplified from strains 130125 and 015093 (an ST11 isolate that produces *bla*$_{KPC-2}$ and exhibits susceptibility to CZA, from our institution) using primers KPC_NdeI_F and KPC_EcoRI_R listed in Table S1. The amplified products and pET-28a vector were digested with NdeI and EcoRI enzymes, ligated with T4 ligase, and transformed into *E. coli* DH5α(Tsingke, Beijing, China) as described before[40]. Transformants were selected on Luria-Bertani agar plates containing 50 mg/L kanamycin, confirmed through PCR employing primers KPC-F and KPC-R listed in Table S1, followed by Sanger sequencing validation. A control utilizing the empty vector pET-28a was similarly established in *E. coli* DH5α.

4.4. Kinetic Assay and Determination of IC_{50} Values

The *bla*$_{KPC}$ gene sequence (residues 25-293) was cloned into the pET-28a vector using In-Fusion HD Cloning Kits (Takara Bio, Kusatsu, Japan). The *bla*$_{KPC}$ gene sequence (residues 25-293) and the pET-28a vector were amplified with the primers listed in Table S1. The resultant recombinants were then transformed into *E. coli* Transetta (DE3) competent cells (Novagen, Sacramento, CA, USA). Protein purification was achieved via Ni-NTA affinity chromatography, as described previously[41]. Extinction coefficients and wavelengths were adopted from earlier studies[9]. The enzyme kinetics assay was performed in triplicate using a SPECTROstar Nano microplate reader (BMG Labtech, Ortenberg, Germany) for 15 min in each round. Kinetic parameters (K_m and k_{cat}) were determined using non-linear regression analysis with GraphPad Prism 9.0 (GraphPad Software, San Diego, CA, USA).

The IC_{50} values for inhibition of KPC-2 and KPC-204 proteins by avibactam, tazobactam, and clavulanic acid were determined using nitrocefin as the substrate. The enzymes were mixed with these inhibitors at concentrations ranging from 0 to 30 μM in PBS and incubated for 10 minutes, after which 100 μM nitrocefin was added. Absorbance at 482 nm was recorded after 30 minutes and analyzed with GraphPad Prism 9.0 (GraphPad Software, San Diego, CA, USA). This procedure was replicated in three independent experiments.

4.5. Mating Experiments

Mating experiments were performed in broth and on filters, using *E. coli* J53 AziR (an azide-resistant variant of J53) as the recipient, at 25°C and 37°C, as described previously[42]. Transconjugants were selected on LB agar with 4 mg/L ceftazidime and 150 mg/L sodium azide. The *bla*$_{KPC-204}$ gene and plasmid replicons in transconjugants were verified via PCR using primers listed in Table S1, followed by confirmation through Sanger sequencing.

5. Conclusions

In conclusion, our study is notable for several reasons: Firstly, we reported an ST11-type clinical CRKP isolate that produces KPC-204, a novel plasmid-borne KPC variant that confers CZA resistance. Secondly, we document a unique antimicrobial resistance profile, demonstrating resistance to both CZA and meropenem. Thirdly, we investigated the enzymatic changes induced by the KDD insertion at position 269, which diminishes the inhibitory efficacy of avibactam, leading to resistance. Lastly, we examined the genetic context of KPC-204, located on a highly transmissible IncFII/IncR plasmid within a composite transposon, presenting a potential for mobility and spread that warrants significant attention. These findings emphasize the need for vigilant monitoring and development of novel therapeutic strategies to manage such resistant bacterial strains effectively.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org, Table S1: Primers used in this study. Table S2: List of KPC alleles with insertions at Ambler position 269, available in the NCBI database (April 2024).

Author Contributions: writing—original draft: Y.G.; writing—review and editing: Y.G., X.L.; conceptualization: Y.G. and X.L.; methodology: Y.G.; software: Y.G. and Y.F.; data analysis: Y.G. and Y.F.; project administration: X.L.; funding acquisition: X.L. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: This work was approved by the West China Hospital Ethics Committee (2020-739).

Data Availability Statement: The sequence of KPC-204 has been deposited in the NCBI database under GenBank accession number OR979533. The accession numbers for strain 130125 range from CP148996 to CP148999. The accession numbers for pKPC2_015093 is CP036301.

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Conflicts of Interest: The authors declare no conflicts of interest.

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