



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 610041, China; gongyq@hbmzu.edu.cn (Y.G.); feng_yu@scu.edu.cn (Y.F.)

² Department of Infection Control, Minda Hospital, Hubei Minzu University, Enshi 445000, China

³ Center for Pathogen Research, West China Hospital, Sichuan University, Chengdu 610041, China

⁴ Division of Infectious Diseases, State Key Laboratory of Biotherapy, Chengdu 610041, China

* Correspondence: lvxj@scu.edu.cn

Abstract: This study describes KPC-204, a novel variant of *Klebsiella pneumoniae* carbapenemase, characterized by a Lys-Asp-Asp (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* DH5 α , 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. Docking simulations revealed a weaker interaction between KPC-204 and avibactam compared to KPC-2. 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.



Citation: Gong, Y.; Feng, Y.; Lv, X. Identification of a Novel KPC Variant, KPC-204, Conferring Resistance to Both Carbapenems and Ceftazidime–Avibactam in an ST11 *Klebsiella pneumoniae* Strain.

Microorganisms **2024**, *12*, 1193.

<https://doi.org/10.3390/microorganisms12061193>

Academic Editor: Teresa Nogueira

Received: 22 May 2024

Revised: 8 June 2024

Accepted: 11 June 2024

Published: 13 June 2024



Copyright: © 2024 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

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 the 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 the mutation in KPC-encoding genes [4,5]. Previously reported mutations like KPC-31/KPC-33(D179Y) [6,7], T243A (unassigned) [8], KPC-128 (D179Y/T243M) [8], KPC-134 (D178A with an insert sequence of aspartic acid–aspartic acid–asparagine–arginine–alanine–proline–asparagine–lysine) [9], KPC-93 (T237S and H274Y) [10], KPC-74 (G239_V240 deletion) [11], and KPC-71 (S182 insertion) [12], these genetic alterations are associated with increased CZA minimum inhibitory concentration (MIC) and decreased meropenem (MEM) MIC in comparison to wild-type isolates. The 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 [13]. We identified a novel KPC variant, KPC-204, in an ST11 *K. pneumoniae* isolate from China. This variant contains a KDD insertion at Ambler position 269 within loop 267–275 [14], representing a mutational hotspot distinct from KPC-2. KPC-204 significantly decreases susceptibility to CZA, yet remains effective against carbapenems.

2. Materials and Methods

2.1. The Strains and In Vitro Susceptibility

The *K. pneumoniae* strain, designated 130125, was isolated from the 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). The MICs of antimicrobial agents were determined using the microdilution method of the Clinical and Laboratory Standards Institute (CLSI) [15]. Avibactam was added at a fixed concentration of 4 mg/L, tazobactam at 4 mg/L, relebactam at 4 mg/L, and vaborbactam at 8 mg/L.

2.2. Whole Genome Sequencing and Analysis

The genomic DNA of strain 130125 was prepared using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) and sequenced using both HiSeq X10 (Illumina; San Diego, CA, USA) and MinION (Nanopore; Oxford, UK) platforms. Both short (Illumina) and long (Nanopore) reads were utilized to generate a de novo hybrid assembly using Unicycler v0.5.0 [16] under conservative mode and polished using Pilon v1.24 [17]. FastANI v1.33 [18] was used to calculate the 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 [19]. The sequence type (ST) was determined by querying the PubMLST database [20] using MLST v2.23.0 (<https://github.com/tseemann/mlst>; accessed on 28 April 2024), while capsule (KL) and outer membrane porins were typed using Kleborate v2.3.2 [21]. Antimicrobial resistance genes and plasmid replicons were identified from the genome sequences using the ABRicate v1.0.0 (<https://github.com/tseemann/abricate>; accessed on 28 April 2024) to query the ResFinder database (<http://genepi.food.dtu.dk/resfinder>; accessed on 28 April 2024) and PlasmidFinder [22] database (accessed on 28 April 2024), respectively. Plasmid comparison was performed using BRIG v0.95 [23] in the default settings. Insertion sequences were identified using ISFinder (<https://isfinder.biotoul.fr/>; accessed on 28 April 2024) [24].

2.3. qRT-PCR

The expression of the *bla*_{KPC} gene was assessed using qRT-PCR. RNA was extracted from late-exponential-phase cultures using the TRIzol reagent (Invitrogen China Limited, Beijing, China). Genomic DNA was removed, and cDNA was synthesized using the PrimeScript RT Reagent Kit with gDNA Eraser (TaKaRa Biotechnology (Dalian), Dalian, China). qRT-PCR was conducted on a LightCycler 96 (Roche, Basel, Switzerland) using SYBR Premix Ex Taq II (Tli RNaseH plus kit) (TaKaRa Biotechnology (Dalian), Dalian, China) according to the manufacturer's instructions. The assays utilized primers for *bla*_{KPC} and the housekeeping gene *rpoB* listed in Table S1. The expression of *bla*_{KPC} was normalized to the housekeeping gene *rpoB*. The relative expression was calibrated against isolate 015093. Relative transcript levels were calculated using the $2^{-\Delta\Delta CT}$ formula based on the mean values. For each strain, three independent cultures were used to extract RNA as three biological replicates, and for each RNA sample, the whole process of qRT-PCR was repeated in triplicate as technical replicates.

2.4. 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 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* DH5 α (Tsingke, Beijing, China), as described before [25]. 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* DH5 α .

2.5. Kinetic Assay and Determination of IC₅₀ 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 [26]. Extinction coefficients and wavelengths were adopted from earlier studies [11]. 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 IC₅₀ values for the 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 μ M in PBS and incubated for 10 min, after which 100 μ M of nitrocefin was added. Absorbance at 482 nm was recorded after 30 min and analyzed with GraphPad Prism 9.0 (GraphPad Software, San Diego, CA, USA). This procedure was replicated in three independent experiments.

2.6. Structure Prediction

The crystal structure of the KPC-2 protein was retrieved from the PDB database (PDB ID: 2OV5, <https://doi.org/10.2210/pdb2OV5/pdb>, accessed on 28 April 2024) [27]. AlphaFold v2.2.3 [28] was employed to generate a structural model for the KPC-204 mutant. All software utilized, including AlphaFold v2.2.3 for CC312 modeling and the openMM Amber force field for protein relaxation, ran on a local server equipped with a powerful dual-channel E5-2697 Intel 24-core CPU, Nvidia A100 (40 GB) GPU, 96 GB of memory, for efficient computation. Notably, AlphaFold's "monomer" model searched all genetic databases used in CASP14 to create the most accurate model, with the final selection based on the highest plddt confidence score. This combined approach ensured the availability of reliable protein structures for further analysis.

2.7. Comparative Secondary and Stereoscopic Structures

Amino acid sequence alignments and secondary structure predictions were conducted using ESPript 3 [29], with the crystal structure of KPC-2 (PDB ID: 2OV5) as a reference for secondary structure depiction. Stereoscopic structures of KPC-204 and KPC-2 were aligned using PyMOL v2.5.2 software.

2.8. Molecular Docking

AutoDock4.2.6 [30] software was used for the molecular docking simulations. Three-dimensional protein structures were retrieved from the Protein Data Bank (PDB) database and AlphaFold modeling. Ligand structures were built using Chem3D v20.1 software. The protein and ligand structures were then prepared for docking calculations by converting

them into the pdbqt file format according to the AutoDock protocol. All docking parameters were maintained at their default settings, except for the maximum number of energy evaluations (eval) and the number of genetic algorithm (GA) runs. The docking simulations were executed using the prepareCovalent.py Python script, and the resulting interactions were analyzed using PyMOL software. A site-specific approach ensured ligands explored the relevant binding pocket of the KPC enzymes. The grid box, with a spacing of 0.375 Å and dimensions of 60 × 60 × 60 points, was centered at coordinates (59.28, −23.36, −4.18) Å.

2.9. 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 [31]. Transconjugants were selected on LB agar with 4 mg/L of ceftazidime and 150 mg/L of 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.

3. Results

3.1. Antimicrobial Susceptibility

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

Table 1. MIC (mg/L) of antimicrobial agents for isolate 130125, 015093 and *E. coli* DH5α 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
DH5α::pEKPC-2	>512	>512	>512	>512	512	128	0.5	16	0.125	8	0.03	8
DH5α::pEKPC-204	>512	256	>512	512	256	128	64	16	0.125	16	0.125	8
DH5α::pET28a	1	1	2	0.06	0.125	0.25	0.25	0.25	0.06	≤0.015	≤0.015	≤0.015
<i>E. coli</i> 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, and vaborbactam at 8 mg/L.

3.2. 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 harbored the *bla*_{KPC-204} gene, encoding KPC-204, 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. 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 was classified as ST11, a prevalent type of CRKP in China, and exhibited the KL64 capsule type. The genetic assessment of *ompK35* and *ompK36* genes identified characteristic mutations in ST11 *K. pneumoniae* strains included a truncation in *ompK35* and an insertion of GD amino acids at positions 134–135 in *ompK36* [32].

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	-	<i>bla</i> _{SHV-158}	<i>aadA2</i> , <i>fosA6</i>
pKPC204_130125	CP148997	154,728	IncR, IncFII	<i>bla</i> _{KPC-204} , <i>bla</i> _{TEM-1} , <i>bla</i> _{CTX-M-65}	<i>rmtB1</i>
p1_130125	CP148998	10,060	ColRNAI		
p2_130125	CP148999	5596	-		

3.3. 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} [33] on the chromosome. Additionally, the novel carbapenemase gene *bla*_{KPC-204}, alongside *bla*_{CTX-M-65} and *bla*_{TEM-1}, is located on a 154-kb IncFII/IncR plasmid, designated as pKPC204_130125 (Table 2). 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 1). The *bla*_{KPC-204} gene is located within a composite transposon, flanked downstream by *ISKpn27* and upstream by *ISKpn6*. Moreover, *bla*_{KPC-204}, in conjunction with *bla*_{CTX-M-65}, is part of a 10 kb integrative composite transposon, bounded by IS26 sequences (Figure 1). This arrangement underscores the genetic mobility potential, facilitating the dissemination of antibiotic resistance.

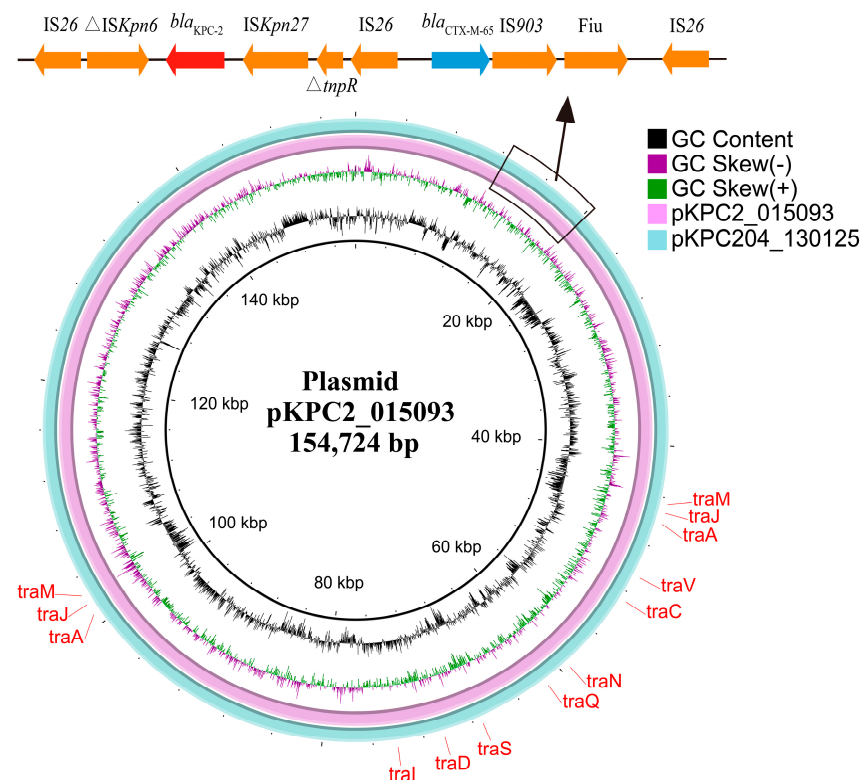


Figure 1. Alignment of pKPC204_130125 with pKPC2_015093. pKPC2_015093 is used as a reference. The alignment, conducted as a pairwise BLASTn comparison using the BLAST Ring Image Generator (BRIG) [23], between plasmid pKPC204_130125 and pKPC2_015093 (GenBank accession no. CP036301) demonstrated a 100% coverage and 99.97% identity. The *bla*_{KPC-204} gene is located within a composite transposon, flanked downstream by *ISKpn27* and upstream by *ISKpn6*. Moreover, *bla*_{KPC-204}, in conjunction with *bla*_{CTX-M-65}, is part of a 10 kb integrative composite transposon, bounded by IS26 sequences. The locations of *tra* genes, pivotal for conjugation [34], are indicated.

3.4. Relative bla_{KPC} Gene's Expression Levels

Quantitative Real-Time PCR (qRT-PCR) was employed to assess the relative bla_{KPC} gene's expression levels using *K. pneumoniae* 015093 [35] (an ST11 isolate that produces bla_{KPC-2} and exhibits susceptibility to CZA, sourced from our institution, with MIC values presented in Table 1) as a reference. The expression of bla_{KPC} in isolate 130125 showed a (1.104 ± 0.135) -fold increase compared to isolate 015093, a difference that was statistically insignificant ($p > 0.05$) (Figure 2).

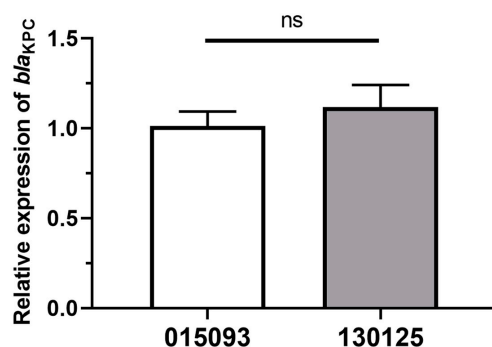


Figure 2. The relative expression level of bla_{KPC} compared to strain 015093. The relative expression of bla_{KPC} mRNA in isolate 130125 was 1.104 ± 0.135 times that of isolate 015093 ($p > 0.05$); ns, $p > 0.05$ (Student's *t*-test).

3.5. Identification of $bla_{KPC-204}$ 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* DH5 α , pEKPC-204 conferred resistance to a broad spectrum of β -lactams, notably to CZA (MIC 64/4 mg/L), as well as 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 DH5 α ::pEKPC-204 was 128 times higher than that for DH5 α ::pEKPC-2 (Table 1). When tested with a fixed ceftazidime concentration of 2 mg/L, the MIC for avibactam against DH5 α ::pEKPC-204 was 16 mg/L, four times the MIC for DH5 α ::pEKPC-2 (4 mg/L), highlighting the significant elevation in avibactam resistance attributable to the KPC-204 variant.

3.6. 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.

β -Lactam	KPC-2			KPC-204		
	K_m (μ M)	k_{cat} (s^{-1})	k_{cat}/K_m (μ M $^{-1}$ · s^{-1})	K_m (μ M)	k_{cat} (s^{-1})	k_{cat}/K_m (μ M $^{-1}$ · 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. IC₅₀ of β-lactamases inhibitors against KPC-2 and KPC-204 ^a.

Inhibitor	IC ₅₀ (μM)	
	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.

3.7. Comparative Secondary and Stereoscopic Structures of KPC-204 and Related Variants

The secondary structures of KPC-204 and other KPC variants with insertions at Ambler position 269 were aligned using data from the NCBI database (April 2024) (Figure 3a). This alignment focused on the Omega loop (residues 164–179) and loop 267–275 regions, both crucial for enzyme activity and antibiotic resistance. Notably, KPC-204 and KPC-29 share an identical KDD insertion at position 269 despite their distinct evolutionary origins (KPC-2 and KPC-3, respectively), indicating potential convergent evolution. KPC-67 contains a KDDKDD insertion at the same position. KPC-29 and KPC-67 were previously reported to exhibit resistance to both CZA and MEM [36], warranting further studies on their enzyme kinetics and structure.

Stereoscopic structural analysis between KPC-204 and KPC-2 reveals a high degree of similarity in the Omega loop (residues 164–179). However, significant divergence is observed in loop 267–275 (Figure 3b). In KPC-204, the insertion of three amino acids (DDK) at position 269 extends the loop 267–275 range.

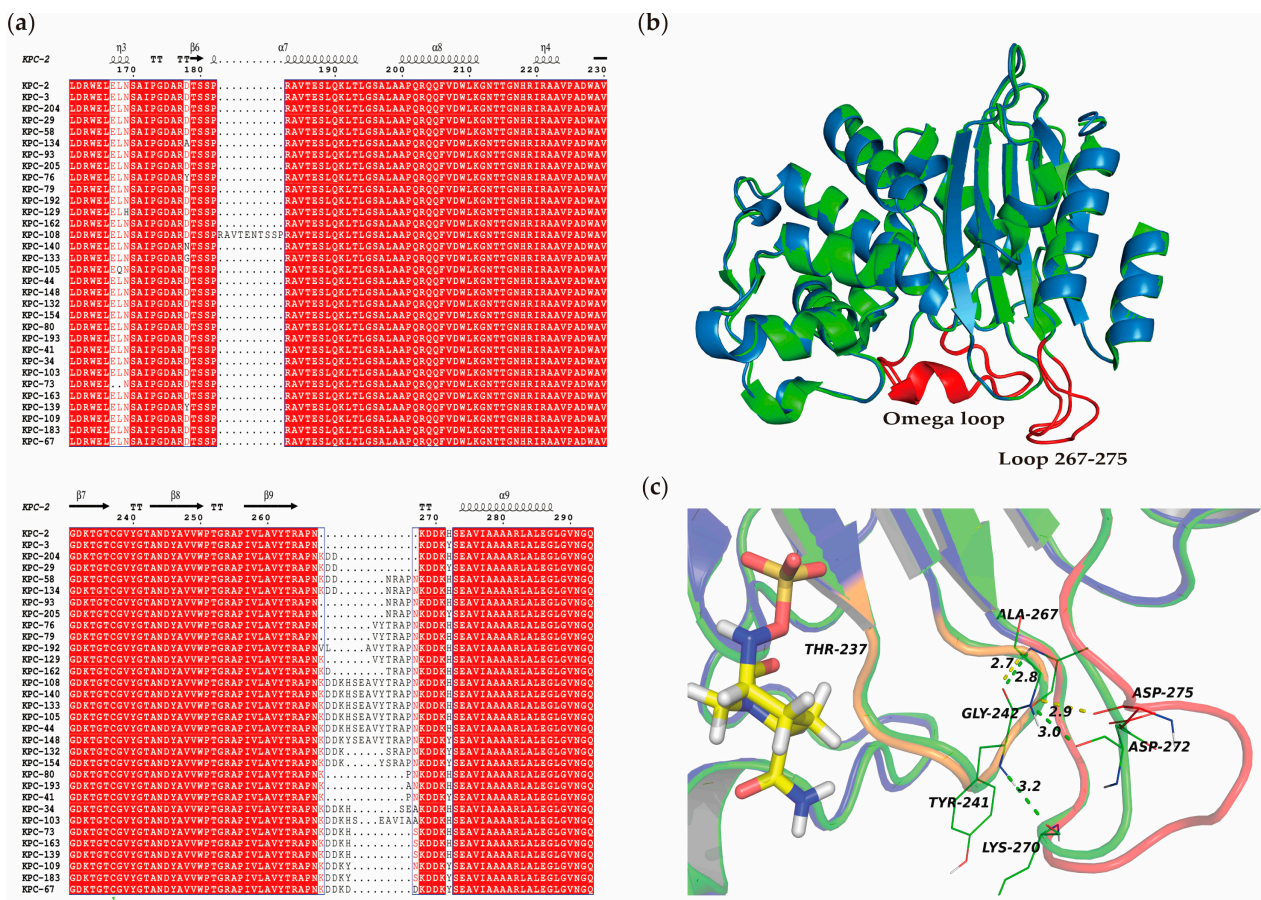


Figure 3. The secondary structure of KPC-204 compared with closely related KPC variants and the stereoscopic structure alignment of KPC-204 with KPC-2. (a) The alignment of secondary structures.

Closely related KPC variants are those with insertions at Ambler position 269, available in the NCBI database (April 2024). The image shows the Omega loop (residues 164–179) and loop 267–275 regions. Secondary structure elements, α helices, β sheets, and 3_{10} -helices (represented by η), are indicated. β -strands are rendered as arrows, and strict β -turns are shown as TT letters. (b) Stereoscopic structure alignment. KPC-204 and KPC-2 are colored in blue and green, respectively. The Omega loop and loop 267–275 of both KPCs are highlighted in red. (c) The comparison of key hydrogen bonds in loop regions 237–243 and 267–275 between KPC-2 and KPC-204. In KPC-2, Tyr241 and Lys270 form a hydrogen bond with a bond length of 3.2 Å, Gly242 and Asp272 form a hydrogen bond with a bond length of 3.0 Å, and Tyr241 and Ala267 form a hydrogen bond with a bond length of 2.8 Å. In KPC-204, the insertion of three amino acids (DDK) at position 269 extends the loop 267–275 range, resulting in a hydrogen bond between Gly242 and Asp275 with a bond length of 2.9 Å and a hydrogen bond between Tyr241 and Ala267 with a bond length of 2.7 Å. The hydrogen bond between Tyr241 and Lys270 is no longer present. KPC-204 and KPC-2 are colored in blue and green, respectively. The loop 237–243 and loop 267–275 of KPC-204 are highlighted in brown and pink, respectively. Hydrogen bonds are depicted as green dashed lines with bond lengths indicated in Å in KPC-2, and yellow dashed lines in KPC-204.

A comparison of key hydrogen bonds in loop regions 237–243 and 267–275 between KPC-2 and KPC-204 shows that in KPC-2, Tyr241 and Lys270 form a hydrogen bond with a bond length of 3.2 Å, Gly242 and Asp272 form a hydrogen bond with a bond length of 3.0 Å, and Tyr241 and Ala267 form a hydrogen bond with a bond length of 2.8 Å. In KPC-204, Gly242 and Asp275 form a hydrogen bond with a bond length of 2.9 Å, and Tyr241 and Ala267 form a hydrogen bond with a bond length of 2.7 Å. The hydrogen bond between Tyr241 and Lys270 is no longer present (Figure 3c).

3.8. Molecular Docking of KPC-204 and KPC-2 with Ceftazidime, Avibactam and Meropenem

We then performed molecular docking simulations to visualize the interactions between KPC-204 and KPC-2 enzymes with ceftazidime, avibactam and meropenem (Figure 4). Interestingly, both KPC-204 and KPC-2 display similar binding scores for ceftazidime (−11.26 kcal/mol and −11.62 kcal/mol, respectively), with slight variations in the specific hydrogen bonding residues (Asn170 vs. Leu169). In contrast, avibactam lacks the hydrogen bond with the side chain of Thr235 in KPC-204, which is present in KPC-2. Moreover, the binding free energy is significantly weaker in KPC-204 (−5.09 kcal/mol) compared to KPC-2 (−8.92 kcal/mol), indicating a reduced affinity for the protein. This diminished interaction may contribute to the resistance observed in KPC-204. KPC-204's KDD insertion at position 269 likely weakens its meropenem resistance compared to KPC-2. This is suggested by both the weaker binding affinity observed in docking analysis (−6.33 kcal/mol vs. −8.72 kcal/mol) and the absence of a key hydrogen bond with Glu166, which is present in KPC-2.

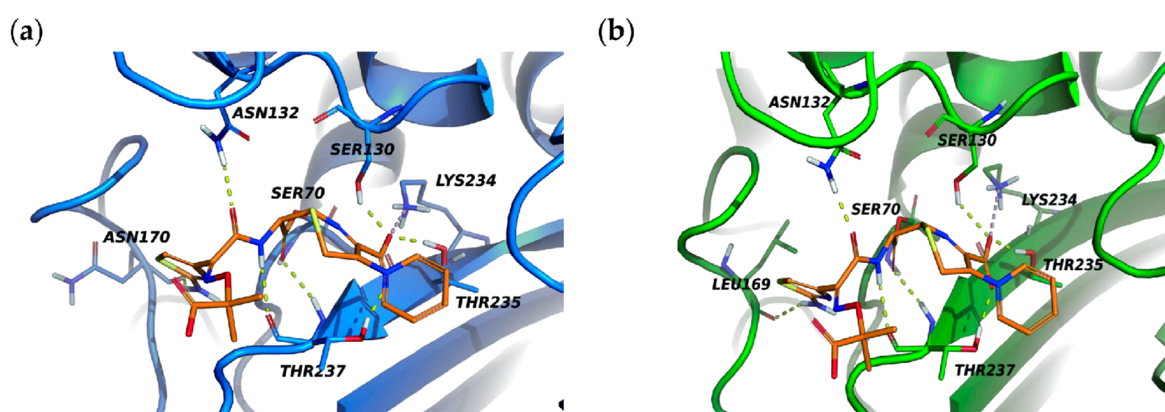


Figure 4. Cont.

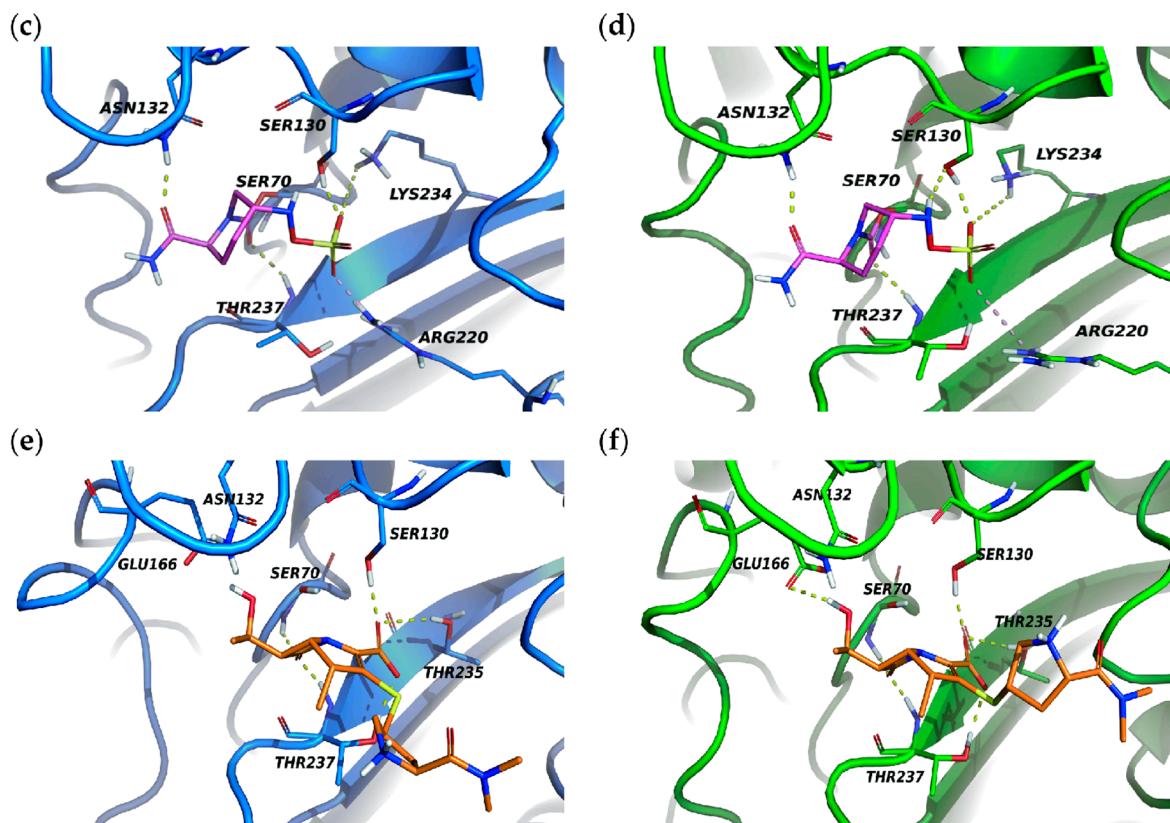


Figure 4. Molecular docking analysis of KPC-204 and KPC-2 interactions with ceftazidime and avibactam. KPC-204 and KPC-2 are colored in blue and green, respectively. Amino acids forming polar bonds with the substrate are shown. (a) KPC-204 with ceftazidime: docking score = -11.62 kcal/mol; H-bonds with Asn170, Asn132, Thr237, Thr235, Ser130; and salt bridge with Lys234. (b) KPC-2 with ceftazidime: docking score = -11.26 kcal/mol; H-bonds with Leu169, Asn132, Thr237, Thr235, Ser130; and salt bridge with Lys234. (c) KPC-204 with avibactam: docking score = -5.09 kcal/mol; H-bonds with Asn132, Thr237, Ser130, Lys234; and salt bridge with Arg220. (d) KPC-2 with avibactam: docking score = -8.92 kcal/mol; H-bonds with Asn132, Thr237, Thr235, Ser130, Lys234; and salt bridge with Arg220. (e) KPC-204 with meropenem: docking score = -6.33 kcal/mol and H-bonds with Ser130 Thr235 Thr237. (f) KPC-2 with meropenem: docking score = -8.72 kcal/mol and H-bonds with Glu166 Ser130 Thr235 Thr237.

3.9. *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 that 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).

4. Discussion

Carbapenem antibiotics are the last-resort drugs for treating infections caused by multidrug-resistant Gram-negative bacteria, and CZA is crucial for treating CRKP infections. KPC-2 and KPC-3 are the most prevalent KPC enzymes [4]. KPC-2-producing ST11-type CRKP strains have emerged as a prevalent clonal lineage in China, posing significant clinical challenges [37,38]. With the global utilization of CZA, resistance to CZA has increased, primarily due to novel mutations in the genes encoding the KPC enzyme [4]. Reports of CZA-resistant KPC variants are common. 194 KPC variants were been identi-

fied in the NCBI Reference Sequences (RefSeq) database (<https://www.ncbi.nlm.nih.gov/pathogens/refgene/#KPC>; accessed on 28 April 2024).

Simultaneous resistance to CZA and MEM is rare. Muresu et al. reported [39] a *K. pneumoniae* strain harboring *bla*_{KPC-31} and *bla*_{OXA-181} that was resistant to both CZA and MEM, but the cloning of *bla*_{KPC-31} [40] and *bla*_{OXA-181} [41] did not show MEM resistance. Arcari et al. reported [42] a *K. pneumoniae* strain carrying *bla*_{KPC-154} that showed MEM resistance, but resistance was not observed when the *bla*_{KPC-154} gene was cloned. This suggests the involvement of *bla*_{KPC} gene over-expression and porin modifications, reflecting the complexity of resistance mechanisms.

We identified an ST11-type *K. pneumoniae* strain simultaneously resistant to CZA and MEM, with no prior exposure to CZA. Compared to the control strain 015093, there were no significant differences in *bla*_{KPC} gene expression levels in strain 130125. Our study identified KPC-204, featuring a KDD insertion at position 269 within loop 267–275, which demonstrated resistance to both CZA and MEM after cloning. Notably, instances of CZA resistance in *K. pneumoniae* have been reported even in the absence of prior CZA exposure [36,39,43]. Given the high genomic similarity between this variant and the ST11 KPC2 strain 015093, it suggests a single case arising from a spontaneous mutation.

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). The alignment of these KPC variants, including the Omega loop (residues 164–179) and loop 266–275, is shown in Figure 3. Among these, KPC-29, -93, -76, -44, -154, -41, and -67 are associated with resistance to CZA [10,36,42,44–46]. Additionally, variants KPC-29 and -154 have also been reported to exhibit resistance to both CZA and MEM [36].

Enzyme kinetic analyses revealed that KPC-204 maintains similar catalytic activity towards ceftazidime and meropenem compared to KPC-2. However, KPC-204 displays a significantly reduced affinity for avibactam, reflected in a 16-fold increase in the IC50 value. Docking simulations support these findings, revealing a weaker interaction between KPC-204 and avibactam compared to KPC-2. The DDK insertion at position 269 of the KPC-204 is situated in the loop 267–275 region, which is peripheral and does not directly interact with the binding pocket or the substrate. KPC-204 may alter the conformation of the binding pocket and substrate affinity by modifying loop 267–275, subsequently affecting loop 237–243.

The KPC-type carbapenemase gene frequently resides on self-conjugative plasmids, facilitating its spread across bacterial populations [4,47–49]. Specifically, *bla*_{KPC-204} is harbored on an IncFII/IncR plasmid, known for its capability to transfer horizontally 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 *ISKpn27* and upstream by *ISKpn6*. This arrangement is similar to that found in pKP048 from *K. pneumoniae* isolates in China, yet it diverges from Tn4401 [50,51]. Additionally, *bla*_{KPC-204}, together with *bla*_{CTX-M-65}, forms part of a 10 kb integrative composite transposon, enclosed by IS26 sequences, indicating the 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.

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 documented a rare 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. Docking simulations support these

findings, revealing a weaker interaction between KPC-204 and avibactam compared to KPC-2. 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 the 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: <https://www.mdpi.com/article/10.3390/microorganisms12061193/s1>, 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). References [4,10,20,26,28–30,52–54] are cited in the Supplementary Materials.

Author Contributions: Writing—original draft: Y.G.; writing—review and editing: Y.G. and 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.

Funding: This research was funded by the Elderly Health Project of Sichuan Provincial Health Commission, grant number GBKT20021.

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 number for pKPC2_015093 is CP036301.

Acknowledgments: We gratefully acknowledge Zhiyong Zong for his guidance and assistance in reading the literature.

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

References

1. Ma, J.; Song, X.; Li, M.; Yu, Z.; Cheng, W.; Yu, Z.; Zhang, W.; Zhang, Y.; Shen, A.; Sun, H.; et al. Global Spread of Carbapenem-Resistant *Enterobacteriaceae*: Epidemiological Features, Resistance Mechanisms, Detection and Therapy. *Microbiol. Res.* **2023**, *266*, 127249. [[CrossRef](#)] [[PubMed](#)]
2. Kalil, A.C.; Klompas, M. Ceftazidime-Avibactam versus Meropenem for the Treatment of Nosocomial Pneumonia. *Lancet Infect. Dis.* **2018**, *18*, 229–231. [[CrossRef](#)]
3. Bush, K.; Bradford, P.A. Interplay between β -Lactamases and New β -Lactamase Inhibitors. *Nat. Rev. Microbiol.* **2019**, *17*, 295–306. [[CrossRef](#)] [[PubMed](#)]
4. Hobson, C.A.; Pierrat, G.; Tenaillon, O.; Bonacorsi, S.; Bercot, B.; Jaouen, E.; Jacquier, H.; Birgy, A. *Klebsiella pneumoniae* Carbapenemase Variants Resistant to Ceftazidime-Avibactam: An Evolutionary Overview. *Antimicrob. Agents Chemother.* **2022**, *66*, e0044722. [[CrossRef](#)] [[PubMed](#)]
5. Ding, L.; Shen, S.; Chen, J.; Tian, Z.; Shi, Q.; Han, R.; Guo, Y.; Hu, F. *Klebsiella pneumoniae* Carbapenemase Variants: The New Threat to Global Public Health. *Clin. Microbiol. Rev.* **2023**, *36*, e0000823. [[CrossRef](#)] [[PubMed](#)]
6. Nicola, F.; Cejas, D.; González-Espinosa, F.; Relloso, S.; Herrera, F.; Bonvehí, P.; Smayevsky, J.; Figueroa-Espinosa, R.; Gutkind, G.; Radice, M. Outbreak of *Klebsiella pneumoniae* ST11 Resistant To Ceftazidime-Avibactam Producing KPC-31 and the Novel Variant KPC-115 during COVID-19 Pandemic in Argentina. *Microbiol. Spectr.* **2022**, *10*, e03733-22. [[CrossRef](#)]
7. Shi, Q.; Yin, D.; Han, R.; Guo, Y.; Zheng, Y.; Wu, S.; Yang, Y.; Li, S.; Zhang, R.; Hu, F. Emergence and Recovery of Ceftazidime-Avibactam Resistance in *bla*_{KPC-33}-Harboring *Klebsiella pneumoniae* Sequence Type 11 Isolates in China. *Clin. Infect. Dis.* **2020**, *71*, S436–S439. [[CrossRef](#)]
8. Shields, R.K.; Nguyen, M.H.; Press, E.G.; Chen, L.; Kreiswirth, B.N.; Clancy, C.J. In Vitro Selection of Meropenem Resistance among Ceftazidime-Avibactam-Resistant, Meropenem-Susceptible *Klebsiella pneumoniae* Isolates with Variant KPC-3 Carbapenemases. *Antimicrob. Agents Chemother.* **2017**, *61*, e00079-17. [[CrossRef](#)]
9. Huang, X.; Shen, S.; Chang, F.; Liu, X.; Yue, J.; Xie, N.; Yin, L.; Hu, F.; Xiao, D. Emergence of KPC-134, a KPC-2 Variant Associated with Ceftazidime-Avibactam Resistance in a ST11 *Klebsiella pneumoniae* Clinical Strain. *Microbiol. Spectr.* **2023**, *11*, e0072523. [[CrossRef](#)]
10. Wu, Y.; Yang, X.; Liu, C.; Zhang, Y.; Cheung, Y.C.; Wai Chi Chan, E.; Chen, S.; Zhang, R. Identification of a KPC Variant Conferring Resistance to Ceftazidime-Avibactam from ST11 Carbapenem-Resistant *Klebsiella pneumoniae* Strains. *Microbiol. Spectr.* **2022**, *10*, e0265521. [[CrossRef](#)]
11. Li, X.; Quan, J.; Ke, H.; Wu, W.; Feng, Y.; Yu, Y.; Jiang, Y. Emergence of a KPC Variant Conferring Resistance to Ceftazidime-Avibactam in a Widespread ST11 Carbapenem-Resistant *Klebsiella pneumoniae* Clone in China. *Front. Microbiol.* **2021**, *12*, 724272. [[CrossRef](#)] [[PubMed](#)]

12. Li, X.; Ke, H.; Wu, W.; Tu, Y.; Zhou, H.; Yu, Y. Molecular Mechanisms Driving the In Vivo Development of KPC-71-Mediated Resistance to Ceftazidime-Avibactam during Treatment of Carbapenem-Resistant *Klebsiella pneumoniae* Infections. *mSphere* **2021**, *6*, e0085921. [CrossRef]
13. Cano, A.; Guzman-Puche, J.; Garcia-Gutierrez, M.; Caston, J.J.; Gracia-Ahufinger, I.; Perez-Nadales, E.; Recio, M.; Natera, A.M.; Marfil-Perez, E.; Martinez-Martinez, L.; et al. Use of Carbapenems in the Combined Treatment of Emerging Ceftazidime/Avibactam-Resistant and Carbapenem-Susceptible KPC-Producing *Klebsiella pneumoniae* Infections: Report of a Case and Review of the Literature. *J. Glob. Antimicrob. Resist.* **2020**, *22*, 9–12. [CrossRef] [PubMed]
14. Hobson, C.A.; Bonacorsi, S.; Jacquier, H.; Choudhury, A.; Magnan, M.; Cointe, A.; Bercot, B.; Tenaillon, O.; Birgy, A. KPC Beta-Lactamases Are Permissive to Insertions and Deletions Conferring Substrate Spectrum Modifications and Resistance to Ceftazidime-Avibactam. *Antimicrob. Agents Chemother.* **2020**, *64*, e01175-20. [CrossRef] [PubMed]
15. CLSI M100-S32; Performance Standards for Antimicrobial Susceptibility Testing 32nd Informational Supplement. CLSI: Wayne, PA, USA, 2022.
16. Wick, R.R.; Judd, L.M.; Gorrie, C.L.; Holt, K.E. Unicycler: Resolving Bacterial Genome Assemblies from Short and Long Sequencing Reads. *PLoS Comput. Biol.* **2017**, *13*, e1005595. [CrossRef]
17. Walker, B.J.; Abeel, T.; Shea, T.; Priest, M.; Abouelliel, A.; Sakthikumar, S.; Cuomo, C.A.; Zeng, Q.; Wortman, J.; Young, S.K.; et al. Pilon: An Integrated Tool for Comprehensive Microbial Variant Detection and Genome Assembly Improvement. *PLoS ONE* **2014**, *9*, e112963. [CrossRef] [PubMed]
18. Jain, C.; Rodriguez-R, L.M.; Phillippy, A.M.; Konstantinidis, K.T.; Aluru, S. High Throughput ANI Analysis of 90K Prokaryotic Genomes Reveals Clear Species Boundaries. *Nat. Commun.* **2018**, *9*, 5114. [CrossRef]
19. Richter, M.; Rosselló-Móra, R. Shifting the Genomic Gold Standard for the Prokaryotic Species Definition. *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 19126–19131. [CrossRef] [PubMed]
20. Jolley, K.A.; Bray, J.E.; Maiden, M.C.J. Open-Access Bacterial Population Genomics: BIGSdb Software, the PubMLST.Org Website and Their Applications. *Wellcome Open Res.* **2018**, *3*, 124. [CrossRef] [PubMed]
21. Wyres, K.L.; Wick, R.R.; Gorrie, C.; Jenney, A.; Follador, R.; Thomson, N.R.; Holt, K.E. Identification of *Klebsiella* Capsule Synthesis Loci from Whole Genome Data. *Microb. Genom.* **2016**, *2*, e000102. [CrossRef]
22. Carattoli, A.; Zankari, E.; García-Fernández, A.; Voldby Larsen, M.; Lund, O.; Villa, L.; Møller Aarestrup, F.; Hasman, H. In Silico Detection and Typing of Plasmids Using PlasmidFinder and Plasmid Multilocus Sequence Typing. *Antimicrob. Agents Chemother.* **2014**, *58*, 3895–3903. [CrossRef] [PubMed]
23. Alikhan, N.-F.; Petty, N.K.; Ben Zakour, N.L.; Beatson, S.A. BLAST Ring Image Generator (BRIG): Simple Prokaryote Genome Comparisons. *BMC Genom.* **2011**, *12*, 402. [CrossRef] [PubMed]
24. Siguier, P.; Perochon, J.; Lestrade, L.; Mahillon, J.; Chandler, M. ISfinder: The Reference Centre for Bacterial Insertion Sequences. *Nucleic Acids Res.* **2006**, *34*, D32–D36. [CrossRef] [PubMed]
25. Sambrook, J.; Russell, D. *Molecular Cloning: A Laboratory Manual*, 3rd ed.; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, USA, 2001.
26. Liu, S.; Jing, L.; Yu, Z.-J.; Wu, C.; Zheng, Y.; Zhang, E.; Chen, Q.; Yu, Y.; Guo, L.; Wu, Y.; et al. ((S)-3-Mercapto-2-Methylpropanamido) Acetic Acid Derivatives as Metallo- β -Lactamase Inhibitors: Synthesis, Kinetic and Crystallographic Studies. *Eur. J. Med. Chem.* **2018**, *145*, 649–660. [CrossRef] [PubMed]
27. Burley, S.K.; Bhikadiya, C.; Bi, C.; Bittrich, S.; Chao, H.; Chen, L.; Craig, P.A.; Crichlow, G.V.; Dalenberg, K.; Duarte, J.M.; et al. RCSB Protein Data Bank (RCSB.Org): Delivery of Experimentally-Determined PDB Structures alongside One Million Computed Structure Models of Proteins from Artificial Intelligence/Machine Learning. *Nucleic Acids Res.* **2023**, *51*, D488–D508. [CrossRef] [PubMed]
28. Highly Accurate Protein Structure Prediction with AlphaFold | Nature. Available online: <https://www.nature.com/articles/s41586-021-03819-2> (accessed on 11 May 2023).
29. Robert, X.; Gouet, P. Deciphering Key Features in Protein Structures with the New ENDscript Server. *Nucleic Acids Res.* **2014**, *42*, W320–W324. [CrossRef] [PubMed]
30. Morris, G.M.; Huey, R.; Lindstrom, W.; Sanner, M.F.; Belew, R.K.;Goodsell, D.S.; Olson, A.J. AutoDock4 and AutoDockTools4: Automated Docking with Selective Receptor Flexibility. *J. Comput. Chem.* **2009**, *30*, 2785–2791. [CrossRef]
31. Coque, T.M.; Oliver, A.; Pérez-Díaz, J.C.; Baquero, F.; Cantón, R. Genes Encoding TEM-4, SHV-2, and CTX-M-10 Extended-Spectrum Beta-Lactamases Are Carried by Multiple *Klebsiella pneumoniae* Clones in a Single Hospital (Madrid, 1989 to 2000). *Antimicrob. Agents Chemother.* **2002**, *46*, 500–510. [CrossRef] [PubMed]
32. Shen, Z.; Ding, B.; Ye, M.; Wang, P.; Bi, Y.; Wu, S.; Xu, X.; Guo, Q.; Wang, M. High Ceftazidime Hydrolysis Activity and Porin OmpK35 Deficiency Contribute to the Decreased Susceptibility to Ceftazidime/Avibactam in KPC-Producing *Klebsiella pneumoniae*. *J. Antimicrob. Chemother.* **2017**, *72*, 1930–1936. [CrossRef]
33. Tian, X.; Wang, Q.; Perlaza-Jiménez, L.; Zheng, X.; Zhao, Y.; Dhanasekaran, V.; Fang, R.; Li, J.; Wang, C.; Liu, H.; et al. First Description of Antimicrobial Resistance in Carbapenem-Susceptible *Klebsiella pneumoniae* after Imipenem Treatment, Driven by Outer Membrane Remodeling. *BMC Microbiol.* **2020**, *20*, 218. [CrossRef]
34. Virolle, C.; Goldlust, K.; Djermoun, S.; Bigot, S.; Lesterlin, C. Plasmid Transfer by Conjugation in Gram-Negative Bacteria: From the Cellular to the Community Level. *Genes* **2020**, *11*, 1239. [CrossRef]

35. Liu, Y.; Zhang, X.; Cai, L.; Zong, Z. Enhanced Survival of ST-11 Carbapenem-Resistant *Klebsiella pneumoniae* in the Intensive Care Unit. *Infect. Control Hosp. Epidemiol.* **2020**, *41*, 740–742. [[CrossRef](#)] [[PubMed](#)]
36. Carattoli, A.; Arcari, G.; Bibbolino, G.; Sacco, F.; Tomolillo, D.; Di Lella, F.M.; Trancassini, M.; Faino, L.; Venditti, M.; Antonelli, G.; et al. Evolutionary Trajectories toward Ceftazidime-Avibactam Resistance in *Klebsiella pneumoniae* Clinical Isolates. *Antimicrob. Agents Chemother.* **2021**, *65*, e0057421. [[CrossRef](#)]
37. Zhang, Y.; Wang, Q.; Yin, Y.; Chen, H.; Jin, L.; Gu, B.; Xie, L.; Yang, C.; Ma, X.; Li, H.; et al. Epidemiology of Carbapenem-Resistant *Enterobacteriaceae* Infections: Report from the China CRE Network. *Antimicrob. Agents Chemother.* **2018**, *62*, e01882-17. [[CrossRef](#)]
38. Zhang, R.; Liu, L.; Zhou, H.; Chan, E.W.; Li, J.; Fang, Y.; Li, Y.; Liao, K.; Chen, S. Nationwide Surveillance of Clinical Carbapenem-Resistant *Enterobacteriaceae* (CRE) Strains in China. *EBioMedicine* **2017**, *19*, 98–106. [[CrossRef](#)] [[PubMed](#)]
39. Muresu, N.; Del Rio, A.; Fox, V.; Scutari, R.; Alteri, C.; Are, B.M.; Terragni, P.; Sechi, I.; Sotgiu, G.; Piana, A. Genomic Characterization of KPC-31 and OXA-181 *Klebsiella pneumoniae* Resistant to New Generation of β -Lactam/ β -Lactamase Inhibitor Combinations. *Antibiotics* **2022**, *12*, 10. [[CrossRef](#)]
40. Antonelli, A.; Giani, T.; Di Pilato, V.; Riccobono, E.; Perriello, G.; Mencacci, A.; Rossolini, G.M. KPC-31 Expressed in a Ceftazidime/Avibactam-Resistant *Klebsiella pneumoniae* Is Associated with Relevant Detection Issues. *J. Antimicrob. Chemother.* **2019**, *74*, 2464–2466. [[CrossRef](#)] [[PubMed](#)]
41. Potron, A.; Poirel, L.; Nordmann, P. Origin of OXA-181, an Emerging Carbapenem-Hydrolyzing Oxacillinase, as a Chromosomal Gene in *Shewanella xiamenensis*. *Antimicrob. Agents Chemother.* **2011**, *55*, 4405–4407. [[CrossRef](#)]
42. Arcari, G.; Cecilia, F.; Oliva, A.; Polani, R.; Raponi, G.; Sacco, F.; De Francesco, A.; Pugliese, F.; Carattoli, A. Genotypic Evolution of *Klebsiella pneumoniae* Sequence Type 512 during Ceftazidime/Avibactam, Meropenem/Vaborbactam, and Cefiderocol Treatment, Italy. *Emerg. Infect. Dis.* **2023**, *29*, 2266–2274. [[CrossRef](#)]
43. Cui, X.; Shan, B.; Zhang, X.; Qu, F.; Jia, W.; Huang, B.; Yu, H.; Tang, Y.W.; Chen, L.; Du, H. Reduced Ceftazidime-Avibactam Susceptibility in KPC-Producing *Klebsiella pneumoniae* from Patients without Ceftazidime-Avibactam Use History—A Multicenter Study in China. *Front. Microbiol.* **2020**, *11*, 1365. [[CrossRef](#)]
44. Räsänen, K.; Koivula, I.; Ilmavirta, H.; Puranen, S.; Kallonen, T.; Lyytikäinen, O.; Jalava, J. Emergence of Ceftazidime-Avibactam-Resistant *Klebsiella pneumoniae* during Treatment, Finland, December 2018. *Eurosurveillance* **2019**, *24*, 1900256. [[CrossRef](#)]
45. Shi, Q.; Han, R.; Guo, Y.; Yang, Y.; Wu, S.; Ding, L.; Zhang, R.; Yin, D.; Hu, F. Multiple Novel Ceftazidime-Avibactam-Resistant Variants of *bla*_{KPC-2}-Positive *Klebsiella pneumoniae* in Two Patients. *Microbiol. Spectr.* **2022**, *10*, e0171421. [[CrossRef](#)]
46. Mueller, L.; Masseron, A.; Prod'Hom, G.; Galperine, T.; Greub, G.; Poirel, L.; Nordmann, P. Phenotypic, Biochemical, and Genetic Analysis of KPC-41, a KPC-3 Variant Conferring Resistance to Ceftazidime-Avibactam and Exhibiting Reduced Carbapenemase Activity. *Antimicrob. Agents Chemother.* **2019**, *63*, e01111-19. [[CrossRef](#)]
47. Liu, L.; Feng, Y.; Wei, L.; Xiao, Y.; Zong, Z. KPC-2-Producing Carbapenem-Resistant *Klebsiella pneumoniae* of the Uncommon ST29 Type Carrying OXA-926, a Novel Narrow-Spectrum OXA β -Lactamase. *Front. Microbiol.* **2021**, *12*, 701513. [[CrossRef](#)]
48. Qin, J.; Feng, Y.; Lü, X.; Zong, Z. KPC-12 with a L169M Substitution in the Ω Loop Has Reduced Carbapenemase Activity. *Eur. J. Clin. Microbiol. Infect. Dis.* **2021**, *40*, 1761–1766. [[CrossRef](#)]
49. Wang, L.; Shen, W.; Zhang, R.; Cai, J. Identification of a Novel Ceftazidime-Avibactam-Resistant KPC-2 Variant, KPC-123, in *Citrobacter Koseri* Following Ceftazidime-Avibactam Treatment. *Front. Microbiol.* **2022**, *13*, 930777. [[CrossRef](#)]
50. Jiang, Y.; Yu, D.; Wei, Z.; Shen, P.; Zhou, Z.; Yu, Y. Complete Nucleotide Sequence of *Klebsiella pneumoniae* Multidrug Resistance Plasmid pKP048, Carrying *bla*_{KPC-2}, *bla*_{DHA-1}, *qnrB4*, and *armA*. *Antimicrob. Agents Chemother.* **2010**, *54*, 3967–3969. [[CrossRef](#)]
51. Cuzon, G.; Naas, T.; Nordmann, P. Functional Characterization of Tn4401, a Tn3-Based Transposon Involved in *bla*_{KPC} Gene Mobilization. *Antimicrob. Agents Chemother.* **2011**, *55*, 5370–5373. [[CrossRef](#)]
52. Ding, L.; Shi, Q.; Han, R.; Yin, D.; Wu, S.; Yang, Y.; Guo, Y.; Zhu, D.; Hu, F. Comparison of Four Carbapenemase Detection Methods for *bla*_{KPC-2} Variants. *Microbiol. Spectr.* **2021**, *9*, e0095421. [[CrossRef](#)] [[PubMed](#)]
53. Scandorieiro, S.; de Camargo, L.C.; Lancheros, C.A.C.; Yamada-Ogatta, S.F.; Nakamura, C.V.; de Oliveira, A.G.; Andrade, C.G.T.J.; Duran, N.; Nakazato, G.; Kobayashi, R.K.T. Synergistic and Additive Effect of Oregano Essential Oil and Biological Silver Nanoparticles against Multidrug-Resistant Bacterial Strains. *Front. Microbiol.* **2016**, *7*, 760. [[CrossRef](#)] [[PubMed](#)]
54. Chiu, S.-K.; Ma, L.; Chan, M.-C.; Lin, Y.-T.; Fung, C.-P.; Wu, T.-L.; Chuang, Y.-C.; Lu, P.-L.; Wang, J.-T.; Lin, J.-C.; et al. Carbapenem Nonsusceptible *Klebsiella pneumoniae* in Taiwan: Dissemination and Increasing Resistance of Carbapenemase Producers during 2012–2015. *Sci. Rep.* **2018**, *8*, 8468. [[CrossRef](#)]

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.