

# Phenotypic and Genetic Analysis of KPC-49, a KPC-2 Variant Conferring Resistance to Ceftazidime–Avibactam and Maintaining Resistance to Imipenem and Meropenem

Meng Yu, Qingzheng Wei, Weiqing Song, Jiangshui Yuan

Clinical Laboratory, University of Health and Rehabilitation Sciences (Qingdao Municipal Hospital), Qingdao, Shandong, People's Republic of China

Correspondence: Jiangshui Yuan; Weiqing Song, Clinical Laboratory, University of Health and Rehabilitation Sciences (Qingdao Municipal Hospital), Qingdao, Shandong, People's Republic of China, Tel +86-0532-6602-7876, Email yjs19790104@163.com; songweiqing68@163.com

**Purpose:** *Klebsiella pneumoniae*, a gram-negative bacterium, poses a severe hazard to public health, with many bacterial hosts having developed resistance to most antibiotics in clinical use. The goal of this study was to look into the development of resistance to both ceftazidime–avibactam and carbapenems, including imipenem and meropenem, in a *K. pneumoniae* strain expressing a novel *K. pneumoniae* carbapenemase-2 (KPC-2) variant, referred to as KPC-49.

**Methods:** After 1 day of incubation of K1 on agar containing ceftazidime–avibactam (MIC = 16/4 mg/L), a second KPC-producing *K. pneumoniae* strain (K2) was recovered. Antimicrobial susceptibility assays, cloning assays, and whole genome sequencing were performed to analyse and evaluate antibiotic resistance phenotypes and genotypes.

**Results:** *K. pneumoniae* strain (K1), that produced KPC-2, was susceptible to ceftazidime–avibactam but resistant to carbapenems. The K2 isolate harboured a novel *bla*<sub>KPC-49</sub> variant, which differs from *bla*<sub>KPC-2</sub> by a single nucleotide (C487A), and results in an arginine-serine substitution at amino acid position 163 (R163S). The mutant K2 strain was resistant to both ceftazidime–avibactam and carbapenems. We demonstrated the ability of KPC-49 to hydrolyse carbapenems, which may be attributed to high KPC-49 expression or presence of an efflux pump and/or absence of membrane pore proteins in K2. Furthermore, *bla*<sub>KPC-like</sub> was carried on an IncFII (pHN7A8)/IncR-type plasmid within a *TnAs1*-orf-orf-orf-orf-orf-ISKpn6-*bla*<sub>KPC</sub>-ISKpn27 structure. The *bla*<sub>KPC-like</sub> gene was flanked by various insertion sequences and transposon elements, including the Tn3 family transposon, such as *TnAs1*, *TnAs3*, IS26, and IS481-ISKpn27.

**Conclusion:** New KPC variants are emerging owing to sustained exposure to antimicrobials and modifications in their amino acid sequences. We demonstrated the drug resistance mechanisms of the new mutant strains through experimental whole genome sequencing combined with bioinformatics analysis. Enhanced understanding of laboratory and clinical features of infections due to *K. pneumoniae* of the new KPC subtype is key to early and accurate anti-infective therapy.

**Keywords:** KPC, antibiotic resistance, whole genome sequencing, ceftazidime–avibactam, susceptibility testing

## Introduction

The worldwide dissemination of carbapenem-resistant *Enterobacteriaceae* (CRE), particularly carbapenem-resistant *K. pneumoniae* (CRKP), poses a significant risk to public health. CRKP can cause various infections, such as urinary tract infections, bloodstream infections, and pneumonia, leading to high morbidity and mortality.<sup>1</sup> Prevention and control of *K. pneumoniae* infection are becoming more challenging due to the development of antibiotic resistance. In addition to their resistance against most  $\beta$ -lactam antibiotics, including carbapenems, additional non- $\beta$ -lactam antibiotic resistance genes, such as those for aminoglycosides and quinolones, are also present in the CRKP.<sup>2</sup> The synthesis of carbapenemases encoded by the *bla*<sub>KPC</sub> gene is the leading cause of CRKP.<sup>3</sup> In particular, *K. pneumoniae* carbapenemase 2 (KPC-2) and *K. pneumoniae* carbapenemase 3 (KPC-3) have been frequently associated with the development of CRKP.

Therefore, the development of newer, more effective antibiotics that can overcome this resistance is of high clinical importance in managing CRKP infections.

Inhibiting class A, class C, and some class D  $\beta$ -lactamases is the function of the novel enzyme inhibitor avibactam. Following the demonstration that ceftazidime–avibactam therapy significantly increased antibiotic activity against CRE, the Food and Drug Administration (FDA) and European Medicines Agency (EMA) authorized it for clinical use in 2015.<sup>4,5</sup> The combined use of avibactam and ceftazidime (ceftazidime–avibactam) has provided an effective treatment for *K. pneumoniae* infections. However, new variants resistant to ceftazidime–avibactam are being found in clinical settings, with their emergence being attributed to mutations in the *bla*<sub>KPC</sub> gene in CRE.<sup>6–9</sup> Mutations in KPC, such as mutations in Asp179Tyr, Val240Gly, Ala240Val, Ala177Glu, Thr243Met, Pro169Leu, Asn179Asp, Tyr179Asp, Gln169Leu, and Gly130Ser, are the most important mechanism leading to ceftazidime–avibactam resistance, a widely reported phenomenon.<sup>7,10–13</sup> In addition, combination of several resistance mechanisms, such as membrane pore protein mutations, increases KPC expression. Moreover, an increased efflux pump activity can also produce ceftazidime–avibactam resistance.<sup>14</sup> These findings motivated us to conduct selection experiments using ceftazidime–avibactam to discover further mutations that may be accountable for the increasing rates of ceftazidime–avibactam-resistant variants.

## Materials and Methods

### Bacterial Isolates and Single-Step Mutant Selection

The parental strain of *K. pneumoniae* (K1) harboured *bla*<sub>KPC-2</sub>. The bacterial strains were identified by matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF MS). The selection procedure was performed as previously described.<sup>15</sup> A 10<sup>9</sup> CFU/mL bacterial sample from an overnight broth culture was distributed on Mueller–Hinton agar (MHA) containing ceftazidime–avibactam (fixed at 4 mg/L). The concentrations of ceftazidime–avibactam were 2 to 16 times greater than the minimum inhibitory concentration (MIC) as demonstrated previously by the CLSI broth microdilution method. After continuous observation for several days, the strains growing on all plates were recorded, saved, and passed for 10 consecutive times. Three consecutive ceftazidime–avibactam concentrations were measured on the obtained strains. Mutants were found to have MIC values four times greater than those of the original strain.

### Antimicrobial Susceptibility Tests and Identification of Carbapenem Resistance Genes

The MICs of meropenem, imipenem, ceftazidime–avibactam, ceftazidime, cefpodoxime, amikacin, polymyxin B, tigecycline, sulfamethoxazole, ciprofloxacin, and aztreonam were determined using the broth microdilution method. Antimicrobial breakpoints were determined according to the standards recommended by the European Committee for Antimicrobial Susceptibility Testing (EUCAST) for polymyxin B,<sup>16</sup> the FDA for tigecycline,<sup>17</sup> and the American Clinical and Laboratory Standards Institute (CLSI) for other antimicrobial agents.<sup>18</sup> Carbapenemase was initially identified via phenotyping, immunochromatography, and the carbapenemase inhibition enhancement assay using phenylboronic acid (PBA) and EDTA (PBA-EDTA assay).<sup>19</sup> PCR and whole genome sequencing (WGS) were performed to confirm the isolation of carbapenemase. *Escherichia coli* strain ATCC 25922 and *K. pneumoniae* strain ATCC 700603 served as controls for MIC testing.

### Cloning Experiments

The *bla*<sub>kpc</sub> gene was amplified using the Q5<sup>®</sup> High-Fidelity 2X Master Mix. KPC-F-EcoRI (5'-CCATGAT TACGAATTGTGCGCGGAACCCCTATTTG-3') and KPC-R-BamH (5'-CGACTCTAGAGGATCCAATAGAT GATTTTCAGCCTTAC-3') were used as primer pairs. The cycling parameters were: 98 °C for 30s, 98 °C for 30s, 63 °C for 30s, and 72 °C for 60s for 30 cycles. The plasmid pHSG398 cloning site was exposed using restriction endonuclease and recombinantly reacted with purified PCR products using 2X Hieff Clone Enzyme Premix; then, the recombinant plasmid was transferred into the recipient *E. coli* DH5 $\alpha$  strain via chemical transformation. The potential transformants were selected using MHA plates containing 50 mg/L of chloramphenicol (for pHSG398). PCR and sanger sequencing were then used to confirm the results.

## Whole Genome Sequencing and Data Analysis

The Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA) was used to extract genomic DNA. The whole genome sequence was submitted to next-generation sequencing (NGS) with the Illumina NovaSeq 6000 system (Oxford Genomics Centre, Oxford, UK) with 2×150 base pair (bp) paired-end reads and long-read high-throughput sequencing (LRS) on a PacBio Sequel II system.<sup>20</sup> The long-reads generated by the PacBio Sequel II system were assembled using the Canu genome assembler<sup>21</sup> and combined with the short-reads from the Illumina NovaSeq 6000 sequencing using Pilon<sup>22</sup> to obtain the whole genome and complete plasmid sequences. The coding sequence (CDS) of the assembled sequences was predicted using the microbial-gene finding system Glimmer (<http://ccb.jhu.edu/software/glimmer/index.shtml>).<sup>23</sup> Multi-locus sequence typing (MLST) was conducted using the MLST 2.0 software (<https://cge.food.dtu.dk/services/MLST/>).<sup>24</sup> Plasmid replicon types were determined directly using the PlasmidFinder tool.<sup>25</sup> Antimicrobial resistance genes were identified using ResFinder 4.0 (<https://cge.cbs.dtu.dk/services/ResFinder-4.0/>)<sup>26</sup> and the Comprehensive Antibiotic Resistance Database 1.1.3 (<https://card.mcmaster.ca/>).<sup>27</sup> Comparative genomic circle maps were constructed using the Basic Local Alignment Search Tool BLAST Ring Image Generator (BRIG) (<http://sourceforge.net/projects/brig>).<sup>28</sup> The insertion sequences (IS) and genomic islands (GI) on the plasmids were predicted using ISfinder ([www-is.biotoul.fr](http://www-is.biotoul.fr))<sup>29</sup> and IslandViewer 4 (<https://www.pathogenomics.sfu.ca/islandviewer/upload/>),<sup>30</sup> respectively. Covariance analysis of the plasmids was performed using Easyfig (<http://easyfig.sourceforge.net/>).<sup>31</sup> The autonomous transmissibility of plasmids was analysed using oriTfinder (<https://tool-mml.sjtu.edu.cn/oriTfinder/instruction.html>).<sup>32</sup>

## Results

### Antimicrobial Susceptibility in Parental and Mutant Strains

The parental strain (K1) was of the sequence type 859 (ST859) and carried the bla<sub>KPC-2</sub> gene. It exhibited resistance to aminoglycosides, quinolones, cephalosporins, and carbapenems. The MIC values of imipenem and meropenem were 128 mg/L and above 128 mg/L, respectively. K1 was still susceptible to tigecycline, polymyxin B, and ceftazidime–avibactam. The ceftazidime–avibactam-resistant mutant (K2) was isolated after incubating K1 on MHA plates containing 16/4 mg/L ceftazidime–avibactam overnight. Although K2 exhibited an eight-fold reduction in the MIC of IMP and above a two-fold reduction in the MIC of MEM compared with that of K1, it was still resistant to both antibiotics. Importantly, K2 was also resistant to ceftazidime–avibactam with a MIC value of 64 mg/L (Table 1).

**Table 1** Antimicrobial Susceptibility Results by Micro-Broth Dilution for KPC-Producing *K. pneumoniae* Isolates, KPC-Producing *E. coli* Transformants (pKPC-2-TM, pKPC-49-TM) and the *E. coli* DH5α-pHSG398

Antimicrobials	MIC (mg/L)				
	K1	K2	<i>E. coli</i> pKPC-2-TM	<i>E. coli</i> pKPC-49-TM	<i>E. coli</i> DH5α-pHSG398
Imipenem	128	16	16	0.125	0.125
Meropenem	>128	64	4	0.125	≤0.06
Ceftazidime	>32	>32	16	64	0.5
Ceftazidime–avibactam	8	64	0.125	32	0.125
Cefpodoxime	>128	128	4	2	0.125
Amikacin	>128	>128	0.5	0.5	1
Polymyxin B	0.25	0.25	0.5	0.5	0.5
Tigecycline	0.5	0.5	0.125	0.125	0.125
Sulfamethoxazole	≤0.25	≤0.25	≤0.25	≤0.25	0.25
Ciprofloxacin	>8	>8	≤0.06	≤0.06	≤0.06
Aztreonam	>128	>128	128	32	0.125

**Abbreviations:** MIC, minimum inhibitory concentration; K1, KPC-2-producing *K. pneumoniae* strain; K2, second KPC-producing *K. pneumoniae* strain.



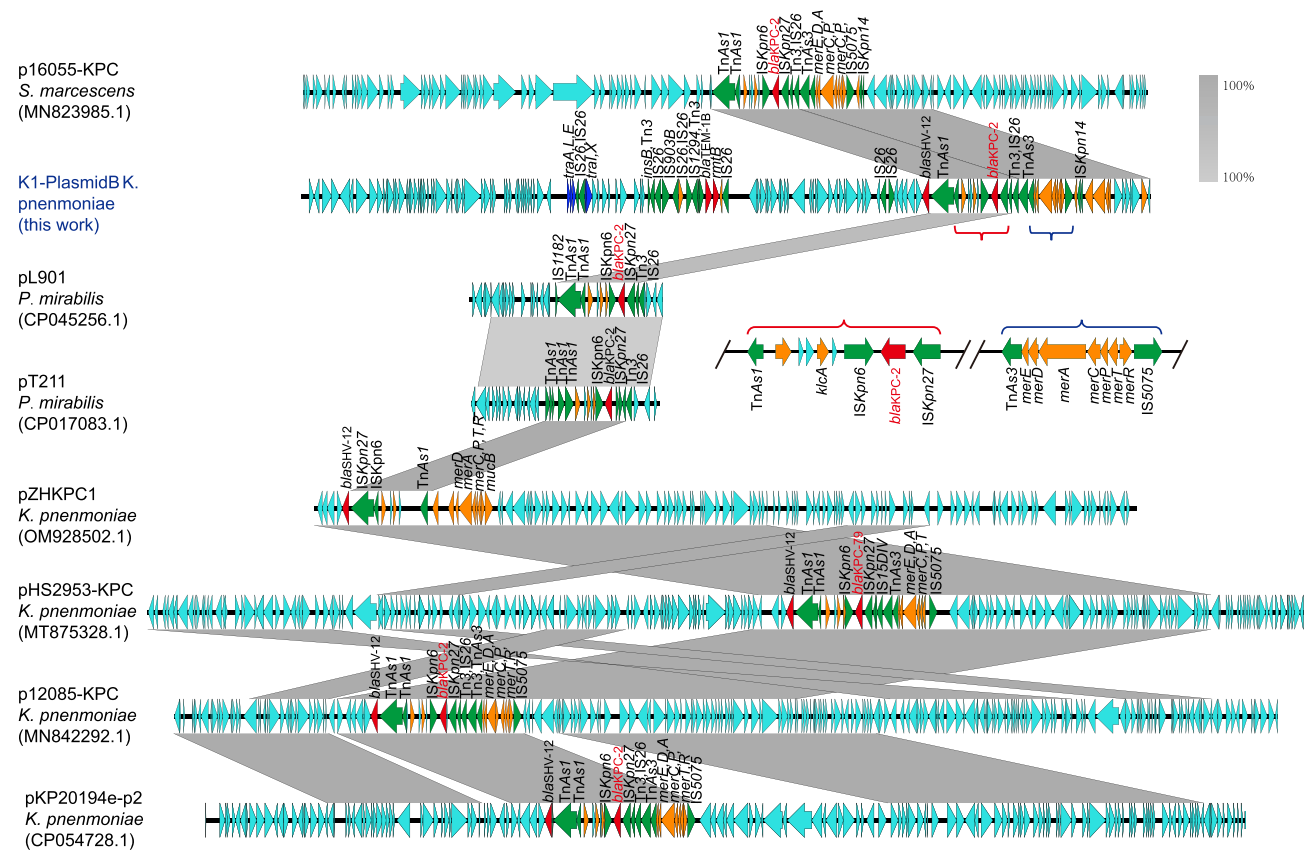
high similarity to PKP048 in the region spanning between the *bla<sub>SHV-12</sub>* and *holE* genes, and *bla<sub>KPC-2</sub>* was in this region. According to the alignment results on a BLAST search, all plasmids under comparison were from *K. pneumoniae*, excluding p16055-KPC, which was derived from *S. marcescens*.

As shown in Figure 2, plasmid B contained two genomic islands (GIs) between 84,004 and 99,319 bp and the critical carbapenem-resistance gene, *bla<sub>KPC-2</sub>*, was carried on the first of the two GIs (GI 1). Upstream of *bla<sub>KPC-2</sub>* were *TnAs1*, *ISKpn6*, a gene encoding a replication protein, *klcA*, and three genes encoding hypothetical proteins, whilst the *ISKpn27* insertion sequence was located downstream. The structure was “*TnAs1*-orf-orf-orf-orf-orf-*ISKpn6*-*bla<sub>KPC-2</sub>*-*ISKpn27*” from 84,004 to 90,918 bp. Furthermore, GI 2 was 5749 bp in length, spanning from 9,3570 to 99,319 bp, with *TnAs3* and *IS5075* flanking the two ends. It also included seven gene clusters encoding mercury resistance in the sequence of *merE-merD-merA-merC-merP-merT-merR*. The GI 1 region was very similar to the IncFII/IncR plasmids of *K. pneumoniae*, including pZHKPC1 (OM928502.1), pHS2953-KPC (MT875328.1), p12085-KPC (MN842292.1) and pKP0294e-p2 (CP054728.1). It was also present in the same plasmid type, p16055-KPC (MN823985.1), from *S. marcescens*. Similar structures were also found in the IncN plasmids, pL901 (CP045256.1) and pT211 (CP017083.1), isolated from *Proteus mirabilis*.

The oriTfinder tool was used to determine whether horizontal transfer elements, including the origin of transfer site (*oriT*), relaxase gene, type IV coupling protein (T4CP), and type IV secretion system (T4ss) were present in plasmid B. Importantly, plasmid B expressed part of the T4ss but not *oriT*, relaxase, or T4CP. Given that plasmid B did not express all four MGEs, it was concluded that plasmid B is a non-mobile plasmid.

## Results of Cloning Experiments

We successfully obtained pKPC-2 and pKPC-49 recombinant plasmids through cloning experiments and transferred them into *E. coli* DH5α strains to produce the transformants, pKPC-2-TM and pKPC-49-TM. The comparative results



**Figure 2** Detailed structure and comparison of the *bla<sub>KPC-2</sub>* gene clusters within the strains. Green arrow indicates transposase genes and red arrows show resistance genes. Arrows indicate the direction of translation of the coding genes. The larger picture depicts a genome-wide comparison of plasmid B with the near-source plasmids, and the smaller picture shows two conserved genomic islands (Islands 1 and 2).

indicated that the MIC values of pKPC-49-TM against carbapenems (imipenem and meropenem) were reduced, whereas the MIC value of ceftazidime–avibactam was significantly increased. The variations in the susceptibility profiles of the donors and transformants are listed in Table 1.

## Discussion

In the present study, we have identified a novel KPC-2 variant, sequence matched as KPC-49 by in-vitro single-step mutant selection. This variant was formerly identified as the KPC-3 variant.<sup>36</sup> The newly discovered variant in ST859 conferred resistance to both ceftazidime–avibactam and several carbapenems, distinguishing it from the KPC-49 variant in *E. coli* mentioned above. The KPC-49 variant in *E. coli* regained susceptibility to imipenem and meropenem. However, strain K2 was still resistant to imipenem and meropenem, and the pKPC-49-TM was susceptible to IMP and MEM in our study. According to the cloning experiment results, the MICs of imipenem and meropenem in the K1 and K2 strains were higher than those of the transformants (pKPC-2-TM and pKPC-49-TM). The reason for this phenomenon might be the existence of other carbapenem resistance mechanisms, including high expression of KPC, over-production of efflux pumps, and loss of outer membrane porins (OMPs), which can render bacteria more resistant to carbapenems. The enzyme inhibition enhancement assay and bioinformatics analysis supported these possibilities. The enzyme inhibition enhancement test of strain K2 suggested that the diameter of the imipenem inhibition circle was expanded by  $\geq 5$  mm after adding phenylboronic acid to strain K2 compared with that of the single drug. The KPC-49 variant likely allows bacteria to retain the hydrolytic ability of carbapenemase, and the high expression of KPC-49 in strain K2 is responsible for its resistance to carbapenems.

We also found that the K1 and K2 strains did not encode OmpK35 and OmpK36, two major OMPs of *K. pneumoniae*. The absence or deficiency of OMPs, along with the production of extended-spectrum beta-lactamases and/or AmpC, contribute significantly to carbapenem resistance in *K. pneumoniae*. Notably, this is also the mechanism of carbapenem resistance in *K. pneumoniae* that do not produce carbapenemases.<sup>37</sup>

In addition, bioinformatic investigation of antimicrobial resistance genes indicated that K1 and K2 strains expressed efflux pumps, including AcrAB-TolC from the resistance-nodulation-division family, KpnGH from the major facilitator superfamily, and KpnEF from the small multidrug resistance family. The genes *marA* and *ramA* positively control the efflux pump of AcrAB-TolC.<sup>38,39</sup> Eliminating the AcrAB-TolC pump of *K. pneumoniae* could impact resistance, fitness, and virulence.<sup>40</sup> High resistance to quinolones and decreased susceptibility to carbapenems have been linked to the efflux pump AcrAB-TolC in *K. pneumoniae*.<sup>41</sup> Srinivasan et al reported that the insertion of an inactivated *kpnGH* gene fragment increased the susceptibility of *K. pneumoniae* to antibiotics such as ceftazidime, ciprofloxacin, and ertapenem.<sup>42</sup> They used specific efflux genes *kpnE* and *kpnF* to encode the efflux pump KpnEF, which recognises a variety of substrates such as ceftriaxone, erythromycin, and tetracycline in *K. pneumoniae* strain NTUH-K2044. When the *kpnEF* gene was knocked out, the sensitivity of the KpnEF pump to the previously mentioned antibiotics was reduced.<sup>43</sup> *marA* and *ramA* both promote expression of AcrAB, thereby enhancing the ability of *K. pneumoniae* to eliminate antibiotics via the AcrAB-TolC efflux pump.<sup>38,39</sup>

Our analysis revealed that the KPC-49 variant was carried on an IncFII/IncR type *bla*<sub>KPC</sub>-bearing plasmid. IncFII/IncR type plasmids are frequently isolated from *K. pneumoniae*, including the pZHKPC1,<sup>34</sup> pHS2953-KPC, and p12085-KPC plasmids, as well as from other gram-negative bacteria, including the p16055-KPC plasmid from *S. marcescens*.

Plasmid B, on which *bla*<sub>KPC</sub> was expressed, was analysed using oriTfinder to determine its capacity for autonomous propagation. According to relevant research, plasmids can be classified into three categories<sup>32</sup>: 1) Conjugative plasmids containing relaxase, T4CP, and T4SS; 2) mobilisable plasmids containing only relaxase; and 3) non-mobilisable plasmids which lack relaxase. An analysis of the autonomous transmissibility of plasmid B using oriTfinder found that plasmid B did not have oriT, relaxase, and T4CP. Plasmid B can be categorised as a non-mobile plasmid since it lacks the necessary components for a mobile plasmid. A plasmid lacking a splice structure can transfer transposons, integrins, and its related resistance genes to another recipient bacterium with the help of another plasmid or by phage transduction or transformation.

In addition to plasmids, other MGEs, including insertion sequences, transposons, integrins, GIs, and integrated splice elements, play a critical role in the acquisition and dissemination of drug-resistant genes.<sup>44</sup> GI 1 of plasmid B contained

the carbapenem resistance gene, *bla*<sub>KPC-2</sub>, in the *TnAs1*-orf-orf-orf-orf-orf-*ISKpn6*-*bla*<sub>KPC-2</sub>-*ISKpn27* unit, which showed high similarity to the IncFII/IncR-type replicon of *K. pneumoniae* and was also present in the IncFII/IncR-type replicons of *S. marcescens* and IncN-type replicons of *P. mirabilis*. This suggests that GI 1 may not only be transferred horizontally between different bacterial species via plasmids but also in a transposable manner, binding to other types of replicons to achieve transfer of the drug resistance gene *bla*<sub>KPC</sub>. In addition to *bla*<sub>KPC</sub>, the plasmid encoded additional resistance genes, including *bla*<sub>TEM-1B</sub>, *rmtB*, and *bla*<sub>SHV-12</sub>. These resistance genes were surrounded by a range of insertion sequences and transposon elements, including the Tn3-family transposon, including *TnAs1*, *TnAs3*, IS26, IS5-IS903B, S91-IS1294, IS1182-*ISKpn6*, IS481-*ISKpn27*, IS110-IS5075, and IS1-*ISKpn14*, and they may also be transmitted through these MGEs.

In the present study, we analysed the development of resistance to ceftazidime–avibactam and carbapenems, including imipenem and meropenem, in *K. pneumoniae* expressing a novel KPC-2 variant (KPC-49) through bioinformatics analysis and cloning assays. Of concern is that strain K2 remains resistant to imipenem and meropenem. It was found that factors associated with this situation may involve high expression of KPC49, efflux pumps, and absence of associated OMPs, with one or more contributing to the outcome. We can further clarify this by relevant experiments in future studies.

## Conclusion

The over-prescription and inappropriate use of antibiotics in clinical care has led to the emergence of multiple drug-resistant strains that are difficult to eradicate and slow progress in the treatment of related diseases. Resistance genes can be transmitted through multiple mobile components. These have contributed to the global antibiotic resistance crisis. Technological innovations, such as WGS and NGS combined with bioinformatics analysis, provide valuable analytical tools for studying antimicrobial resistance. By combining traditional detection techniques with modern technologies, we have gained a more comprehensive and specific understanding of the drug-resistant phenotype, resistance mechanism, and transmission risk of the new KPC subtype *K. pneumoniae*. This will play a key role in early and precise clinical anti-infective treatment. It will also help to further combat microbial drug resistance.

## Ethics Approval

The Qingdao Municipal Hospital's Ethics Committee approved the whole plan for this study's research. The authors of this paper have not performed any studies on humans or animals. The need for informed consent was waived because this study was about bacteria and did not involve patients.

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## Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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## Disclosure

In this work, the authors state that they have no conflicts of interest.

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