In vivo Selection of Imipenem Resistance Among Ceftazidime-Avibactam-Resistant, Imipenem-Susceptible Klebsiella pneumoniae Isolate With KPC-33 Carbapenemase

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We describe in vivo evolution of carbapenem and ceftazidime-avibactam resistance by analyzing four longitudinal Klebsiella pneumoniae clinical isolates from a patient with pneumonia following antimicrobial treatment. The patient had fever, cough associated with expectoration, and new infiltration was found on the chest CT. Antimicrobial susceptibility was determined, and whole genome sequencing (WGS) was performed to investigate its dynamic change of resistance phenotype. Population analysis profile was performed to investigate the population of Klebsiella pneumoniae. The infection started with a KPC-2-producing K. pneumoniae (ZRKP01, ceftazidime-avibactam-S/carbapenem-R). Then, after ceftazidime-avibactam treatment, the strain switched to D179Y mutant that is KPC-33 (ZRKP02, ceftazidime-avibactam-R/carbapenem-S), which restored carbapenem susceptibility. However, the restored carbapenem susceptibility in vivo was not stable and the subsequent use of imipenem against KPC-33-producing K. pneumoniae infection resulted in a reversion of KPC-2 producers (ZRKP03 and ZRKP04, ceftazidime-avibactam-S/carbapenem-R). Genetic analysis demonstrated that all four K. pneumoniae isolates belonged to sequence type 11and had identical capsular polysaccharide (KL47), identical porin genes, and same plasmid replicon types. Phylogenetic analysis indicated that four K. pneumoniae isolates showed a high degree of relatedness. Single nucleotide polymorphisms analysis indicated that the number of mutations observed in the KPC-33 isolate was more than in the wild-type KPC-2 isolates and the four KPC-Kp isolates evolved from a longitudinal evolution of K. pneumoniae harboring blaKPC-2 gene. This is the first report to observe the in vivo evolution of wild-type KPC-2 to KPC-33 and then the reversion to its original wild-type KPC-2. Through WGS, we demonstrated the role of selective pressure of antibiotic in the mutation and reversion...
INTRODUCTION

Ceftazidime-avibactam is a β-lactam/β-lactamase inhibitor combination that was approved for the treatment of complicated intra-abdominal infections, complicated urinary tract infections, hospital-acquired pneumonia, and ventilator-associated pneumonia (Falcone and Paterson, 2016). The agent is highly active against class A β-lactamases including Klebsiella pneumoniae carbapenemases, class C β-lactamases, and OXA-48 carbapenemase, but not metallo-β-lactamases such as NDM, VIM, and IMP (van Duin and Bonomo, 2016). Despite a limited use of ceftazidime-avibactam at a worldwide scale, ceftazidime-avibactam resistance has been reported either in patient with no history of ceftazidime-avibactam therapy (Humphries, 2015) or in patient after short periods of ceftazidime-avibactam exposure (Shields et al., 2016). Resistance to ceftazidime-avibactam has been linked to specific mutations in the blaKPC gene (Shields et al., 2017a), specific mutations in the blacTX-M gene (Both et al., 2017), porin deficiency combined with high ceftazidime hydrolysis (Shen et al., 2017; Galani et al., 2019), porin inactivation with or without expression of the blaKPC gene (Humphries and Hemarajata, 2017; Coppi et al., 2020), or transposition of KPC with porin deficiency (Nelson et al., 2017). The mechanism most often associated with the emergence of ceftazidime-avibactam resistance after treatment has been observed to be mutations in the blaKPC genes encoding for KPC enzymes, such as L169P, A177E, D179Y, D179N, V240G, Y241H, T243M, and H274N mutations in KPC (Shields et al., 2017a; Giddins et al., 2018; Hemarajata and Humphries, 2019; Sun et al., 2020). These mutational changes in KPC that emerged in vivo are often associated with fully or partially reversion to carbapenem susceptibility (Haidar et al., 2017; Shields et al., 2017a; Giddins et al., 2018). However, clinical significance of this observation is unclear, since subsequent exposure to carbapenems can restore resistance to them in vitro passage experiments (Shields et al., 2017b). In the present study, we observed the in vivo evolution of KPC-2 to KPC-33 and then the reversion to KPC-2, which leading to the dynamic emergence of resistance to ceftazidime-avibactam and carbapenems.

MATERIALS AND METHODS

Bacterial Strains and Susceptibility Testing
The patient underwent a routine culture of sputum or bronchoalveolar lavage fluid (BALF) over a 3-month hospitalization. A total of 19 respiratory tract specimens from the patient were collected, including 12 sputum and 7 bronchoalveolar lavage. Four of these specimens yield K. pneumoniae. Isolates were identified as K. pneumoniae by MALDI-TOF MS (Bruker Daltonics, Billerica, MA, United States), and antimicrobial susceptibility testing (AST) was performed using the VITEK-2 compact system (bioMérieux, Marcy-l’Etoile, France). AST was further performed by means of broth microdilution, which was performed and interpreted according to the guidelines established by the Clinical and Laboratory Standards Institute, United States (CLSI, 2019). Avibactam was tested at a fixed concentration of 4 mg/l in combination with increasing concentrations of ceftazidime.

DNA Sequencing, de novo Assembly, and Annotation
For whole genome sequencing (WGS), the genomic DNA of four isolates was subjected to both short- and long-read massively parallel sequencing. Short-read sequencing was performed on the Illumina HiSeq 2,500 sequencing platform (Illumina, San...
Population Analysis Profile (PAP) of Four KPC-Producing Klebsiella pneumoniae (KPC-Kp) Isolates

To investigate the presence of meropenem and ceftazidime/avibactam heteroresistance, population analysis profiles were determined by spiral plating 50-μl aliquots of the starting bacterial cell suspension on Mueller-Hinton agar plates without or with various concentrations of meropenem and ceftazidime-avibactam (0, 0.125, 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, 128, 256, and 512 mg/l) as previously described (Li et al., 2009; Genome Analysis Toolkit (Mckenna et al., 2010). All SNPs were manually checked. Subsequently, we constructed a maximum likelihood phylogenetic tree by using RAxML (Stamatakis, 2014) with general time reversible model of nucleotide substitution and a Gamma distribution of rate heterogeneity.

Emergence of an Apparent Phenotypic Change of K. pneumoniae Isolates During Infection

During the course of illness, four serial K. pneumoniae isolates were cultured from respiratory tract specimens on hospital days 32, 47, 61, and 67 days, respectively. Testing of susceptibility to ceftazidime-avibactam and carbapenems showed three distinct susceptibility phenotypes as shown in Table 1. Phenotype 1 (isolate ZRKPO1), collected at the baseline (under therapy with ceftazidime-avibactam for 3 days), was susceptible to ceftazidime-avibactam (MIC, 0.5 mg/l) and resistant to carbapenem (IMP, MIC 32 mg/l; MEM, MIC 128 mg/l). Phenotype 2 (isolate ZRKPO2), collected following 12 days ceftazidime-avibactam, displayed ceftazidime-avibactam resistance (MIC, 64 mg/l) and restored susceptibility to carbapenem (IMP, MIC 0.06 mg/l; MEM, MIC 2 mg/l). Of note, phenotype 3 (isolate ZRKPO3), collected after 15 days imipenem plus 6 days polymyxin B, reverted to carbapenem resistance (IMP, MIC 128 mg/l; MEM, MIC 512 mg/l) and restored susceptibility to ceftazidime-avibactam (MIC, 2 mg/l; although with elevated MIC compared with baseline isolate ZRKPO1). Phenotype 3 (isolate ZRKPO4), collected during imipenem, ceftazidime-avibactam plus amikacin combination therapy, was susceptible to ceftazidime-avibactam (MIC, 2 mg/l) and resistant to carbapenem (IMP, MIC 64 mg/l; MEM, MIC 256 mg/l; a lower MIC compared with ZRKPO3). These isolates displayed in an apparent phenotypic change from carbapenem-resistant to susceptible and then reverted to resistant for carbapenem, while the phenotypic change from ceftazidime-avibactam-susceptible to resistant then reverted to susceptible for ceftazidime-avibactam.
Genome Comparison of Four KPC-Kp Isolates

Genetic analysis demonstrated that all four KPC-Kp isolates belonged to sequence type (ST11) and had identical capsular polysaccharide (KL47; wzc: 47, wzi: 209), a common CRE species in China. All the four isolates had genes encoding KPC, SHV-182, and CTX-M β-lactamases. Other acquired resistance genes justifying the resistance phenotype and virulence genes are depicted in Figure 2. Analysis of outer membrane porin genes demonstrated that all the four isolates had a mutated ompK35 gene encoding truncated porin (premature stop codon at amino acid position 88) and had a mutated ompK36 gene encoding a porin OmpK36 with a GD insertion at amino acid position 134–135.

The genomic features of assemblies are presented in Table 2. The chromosome lengths of ZRKP01, ZRKP02, ZRKP03, and ZRKP04 were 5.435 Mbp, 5.438 Mbp, 5.434 Mbp, and 5.436 Mbp, respectively, similar in length to other K. pneumoniae genomes in public databases (range, 5.3–5.6 Mbp). They had an average G + C content of 56.9% and carried 25 ribosomal RNA genes and 85 transfer RNA genes. The four K. pneumoniae isolates harbored five similar plasmids referred as plasmid 01, plasmid 02, plasmid 03, plasmid 04, and plasmid 05. Plasmid content analysis showed that all four KPC-Kp isolates shared the same plasmid replicon types as shown in Table 2. Furthermore, the fusion was observed of IncFII and IncFIB in plasmid 01 and IncFII and IncR in plasmid 03. The plasmid 03 lengths of ZRKP01, ZRKP02, ZRKP03, and ZRKP04 were 91,097, 91,502, 91,097, and 86,253 base pairs, respectively. The $bla_{KPC}$ gene was located in plasmid 03 as shown in Figure 3. Ceftazidime-avibactam-susceptible isolates (ZRKP01, ZRKP03, and ZRKP4) carried $bla_{KPC-2}$, whereas ceftazidime-avibactam-resistant isolate (ZRKP02) carried mutant $bla_{KPC-2}$ ($bla_{KPC-33}$) encoding for KPC enzymes. Deep examination of reads aligning...
to the \textit{bla}_{KPC-2} gene demonstrated that 94% of aligned reads of the ZRKP01 isolate displayed the wild-type (KPC-2) and 6% displayed D179Y mutation (KPC-33). We did not find coexistence of wild-type KPC-2 and mutation D179Y (KPC-33) in the WGS data of the remaining three \textit{K. pneumoniae} isolates (ZRKP02-ZRKP04). Corresponding to the phenotype change, the \textit{K. pneumoniae} isolates displayed an genotype change from wild-type KPC-2 to variant KPC-2 (KPC-33) and then reversion to its original wild-type KPC-2.

Using the genome assembly of \textit{K. pneumoniae} isolate (ZRKP01) as a reference, we identified 17 substitutions among \textit{K. pneumoniae} isolates comprising 6 synonymous and 11 nonsynonymous substitutions. SNP analysis revealed that the isolate ZRKP02 (KPC-33) evolved by up to 12 SNPs/genome, the ZRKP03 evolved by 6 SNPs/genome, and the ZRKP04 evolved by 7 SNPs/genome over the patient’s hospitalization. Numbers and positions of SNPs for each pairwise comparison of isolates are shown in Figure 4. In our analysis of the evolutionary process in this case, we found that the number of mutations observed in the ZRKP02 (KPC-33) isolate was more than in the wild-type KPC-2 isolates (ZRKP01, ZRKP03, and ZRKP04). These mutations in ZRKP02 isolate accounted for 9 of 11 total nonsynonymous mutations. Of note, 6 of these targets (2 \textit{malt}, \textit{srIB}, \textit{nfJ}, \textit{ydiA}, and \textit{bla}_{KPC-33}) reverted to its original wild-type on further imipenem treatment; in particular, wild-type KPC-2 confer resistance to carbapenem. On the other hand, the remaining 3 mutant genes (\textit{rhtB}, \textit{manZ}, and a hypothetical protein) showed clonal succession (Figure 4) Therefore, it demonstrated that the four KPC-Kp isolates evolved from a longitudinal evolution of \textit{K. pneumoniae} harboring \textit{bla}_{KPC-2} gene.

PAP of Four KPC-Kp Isolates

PAP curves of the four KPC-Kp isolates are shown in Figure 5. Population analysis showed that both the meropenem-resistant subpopulation and ceftazidime-avibactam-resistant subpopulation were not found in all four isolates (CRKP01-CRKP04).

DISCUSSION

Here, we report the dynamic emergence of resistance to ceftazidime-avibactam and carbapenems in KPC-Kp during antimicrobial therapy. Using WGS of four longitudinal clinical isolates, we directly documented clonal succession of the \textit{bla}_{KPC-2} conferring carbapenems and \textit{bla}_{KPC-33} conferring ceftazidime-avibactam resistance.

The patient described in this study was intermittently exposed to 40 days of ceftazidime-avibactam. After 12 days

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**TABLE 2 | Genomic features of four \textit{Klebsiella pneumoniae} ST11 isolates.**

| Feature | ZRKP01 | ZRKP02 | ZRKP03 | ZRKP04 |
|---------|--------|--------|--------|--------|
| G+C content, % | 56.98 | 56.98 | 56.99 | 56.99 |
| Plasmids, no. | 5 | 5 | 5 | 5 |
| Size, base pairs | | | | |
| Chromosome | 5,433,561 | 5,433,841 | 5,433,420 | 5,436,990 |
| Plasmid 01 | 239,460 | 220,761 | 205,940 | 238,333 |
| Plasmid 02 | 110,529 | 110,529 | 110,529 | 110,529 |
| Plasmid 03 | 91,097 | 91,097 | 91,097 | 86,253 |
| Plasmid 04 | 10,060 | 10,060 | 10,060 | 10,060 |
| Plasmid 05 | 5,596 | 5,596 | 5,596 | 5,596 |
| Plasmid (Inc) | | | | |
| Plasmid 01 | IncFIB, IncFII | IncFIB, IncFII | IncFIB, IncFII | IncFIB, IncFII |
| Plasmid 02 | IncFIB | IncFIB | IncFIB | IncFIB |
| Plasmid 03 | IncFIB, IncR | IncFIB, IncR | IncFIB, IncR | IncFIB, IncR |
| Plasmid 04 | CoiRNAI | CoiRNAI | CoiRNAI | CoiRNAI |
| Plasmid 05 | NA | NA | NA | NA |
| Genes, no. | 5,182 | 5,182 | 5,182 | 5,182 |
| CDS, no. | 5,076 | 5,076 | 5,076 | 5,076 |
| Ribosomal RNA genes, no. | 25 | 25 | 25 | 25 |
| Transfer RNA genes, no. | 85 | 85 | 85 | 85 |
| Prophages, no. | 15 | 14 | 14 | 14 |
| IS elements, no. | 50 | 53 | 49 | 51 |
| Chromosome IS family (no.) | IS1 (4), IS3 (9), IS5 (25), IS6 (1), IS110 (2), IS11182 (1), IS1380 (3), ISNCY (6) | IS1 (3), IS3 (9), IS5 (25), IS6 (1), IS110 (2), IS11182 (1), IS1380 (3), ISNCY (6) | IS1 (3), IS3 (9), IS5 (25), IS6 (1), IS110 (2), IS11182 (1), IS1380 (3), ISNCY (6) | IS1 (3), IS3 (9), IS5 (25), IS6 (1), IS110 (2), IS11182 (1), IS1380 (3), ISNCY (6) |

CDS, coding DNA sequences; IS, insertion sequence.
FIGURE 3 | Major structural features of plasmid 03 identified in the four *K. pneumoniae* isolates. Light grey shading denotes shared regions of homology, and open reading frames (ORFs) are portrayed by arrows. Pink arrows represent antibiotic resistant genes, and green arrows represent transposon-related genes and insertion sequences. The plasmid sequence of sample ZRKP02 was reverse-complemented to get a better presentation.

FIGURE 4 | Summary of mutations present in four *K. pneumoniae* isolates and their effects on annotated coding sequences. The mutations in ZRKp 02 (KPC-33) isolate accounted for 9 of 11 total nonsynonymous mutations. Six of these targets (*matT*, *srnB*, *nlj*, *yvdA*, and *blaKPC-33*) reverted to its original wild-type, including wild-type KPC-2 confer resistance to carbapenem in ZRKp 03 and ZRKp04 isolates. The remaining 3 mutant genes (*rhtB*, *manZ*, and a hypothetical protein) showed clonal succession in ZRKp 03 and ZRKp04 isolates. Amino acid abbreviations follow the standard one-letter code. Red arrows represent the nonsynonymous substitutions, green arrows represent the synonymous substitutions, and blue arrows represent the reversion of the nonsynonymous substitutions.
ceftazidime-avibactam plus amikacin treatment, ceftazidime-avibactam-resistant KPC-33 (KPC-2 D179Y variant) producing K. pneumoniae strain appeared, as indicated from previous study (Shields et al., 2016; Haidar et al., 2017; Shields et al., 2017a; Giddins et al., 2018; Hemarajata and Humphries, 2019; Sun et al., 2020). Of note, KPC-33 producing K. pneumoniae (ceftazidime-avibactam-resistant) isolate was unable to preserve the resistance phenotype without the selective pressure of ceftazidime-avibactam and it reverted to susceptible phenotype on further selective pressure of imipenem. Importantly, the restored carbapenem susceptibility was not stable and subsequently reverted to its original carbapenem resistance phenotype on the selective pressure of imipenem. It should be noted that understanding the clinical significance of this observation is of critical importance, and reversion to carbapenem susceptibility would not imply a potential role for carbapenems monotherapy. In addition, the efficacy of dual ceftazidime-avibactam and carbapenem therapy in these settings is unclear. More studies are needed to establish the precise role of carbapenems in treating such infections and the durability of restored carbapenem susceptibility in vivo.

Similarly, the in vitro reversion of blakpc-3 mutations by K. pneumoniae isolates was recently described by Shields et al., 2017b; isolates with D179Y substitutions in KPC-3 when exposed to meropenem, blakpc-3 mutations reverted to wild type, were replaced by new mutations, or were retained (Shields et al., 2017b). This in vitro reversion of blakpc-3 mutation was also observed by Göttig et al. from Germany (Göttig et al., 2019). They found that isolate with D179Y substitution in KPC-3, blakpc-3 mutation reverted to wild type, and demonstrated the isolate with mutational change in KPC-3 under selection pressure are associated with ceftazidime-avibactam resistance, while imipenem resistance was solely due to reversion of KPC-3 D179Y to wild-type KPC-3. To the best of our knowledge, this is the first report to observe the in vivo evolution of wild-type KPC-2 to KPC-33 and then the reversion to its original wild-type KPC-2.

Mechanisms of horizontal gene spread among K. pneumoniae often considered to be the main mediators of antibiotic resistance, such as acquisition and loss of antibiotic resistance genes as previously described (Villa et al., 2013; Simner et al., 2018). However, mutational resistance also has primary clinical importance when considering resistance to particular antibiotics, especially to carbapenems and ceftazidime-avibactam (Haidar et al., 2017; Shields et al., 2017a; Giddins et al., 2018; Hemarajata and Humphries, 2019; Sun et al., 2020). In the present study, genetic analysis demonstrated that all four K. pneumoniae isolates belonged to sequence type 11, had identical capsular polysaccharide (KL47), identical porin genes, and same plasmid replicon types. Phylogenetic analysis indicated that four K. pneumoniae isolates showed a high degree of relatedness. PAPs showed no co-mixed infection population. These results demonstrate the four KPC-Kp isolates evolved from a longitudinal evolution of K. pneumoniae harboring blakpc-2 gene. Our result indicated that the variant KPC can be relatively unstable, which may result in KPC enzymes dynamic change through mutations or reversions to alter their spectra of activity under different selection pressure.

In the course of ceftazidime-avibactam treatment, the dynamic change of resistance to ceftazidime-avibactam and carbapenems has been documented in KPC-Kp clinical isolates (Gaibani et al., 2018; Sun et al., 2020). However, little is known regarding the mechanisms of dynamic change. Gaibani et al. found that two different subpopulations harboring wild-type and mutant blakpc-3 coexisting in the same KPC-Kp clinical isolate and the coexistence of different variants within a single isolate determining a hybrid phenotype resulting in resistance to both carbapenems and ceftazidime/avibactam (Gaibani et al., 2018). Heteroresistance to ceftazidime-avibactam was also observed in our previous study (Sun et al., 2020). We described coexistence of wild-type KPC-2 (29.4%) and mutation D179Y (KPC-33, 71.6%) within a single isolate, which may contribute resistance to both carbapenems and ceftazidime-avibactam. In the present study, through WGS, we demonstrated the role of selective pressure of antibiotic in the mutation and reversion of blakpc genes, which leading to the dynamic change of KPC enzymes and the dynamic emergence of resistance to ceftazidime/avibactam and carbapenems.

![Graphs showing the population analysis profile (PAP) curves of the four KPC-producing K. pneumoniae clinical isolates.](image-url)
It should be noted that K. pneumoniae with mutant bla_{KPC}
may be identified as ESBL producers (rather than KPC producers),
if carbapenemase screening is triggered by elevated carbapenem
MICs (Shields et al., 2017a). Targeted sequencing of bla_{KPC}
of isolates may prove to be powerful tools for rapidly identifying
loss or restored activity of ceftazidime-avibactam and carbapenems,
respectively.

**CONCLUSION**

This study described the plasticity and speed of evolutionary
changes in KPC-Kp strains and stressed the importance of
mutations and reversion of bla_{KPC} genes in dynamic change
of resistance to ceftazidime-avibactam and carbapenems.

**DATA AVAILABILITY STATEMENT**

The raw data supporting the conclusions of this article will
be made available by the authors to any qualified researcher.
The assembly genome sequences have been deposited on NCBI
with BioProject `PRJNA613645`.

**ETHICS STATEMENT**

Permission for using the information in the medical records of
the patients and the K. pneumoniae isolates for research
purposes was granted by the ethical committee of China-Japan
Friendship Hospital (2019-164-K113).

**AUTHOR CONTRIBUTIONS**

BC and YL conceived or designed the work. ZL, AS, and BL
collected the clinical data. BL and LS collected the laboratory
data and performed the tests. CW and JZ analyzed and interpreted the data. CW, JZ, YL, and BC drafted the manuscript.
All authors contributed to the article and approved the final
version of the manuscript to be published.

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