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In vivo evolution of resistant subpopulations of KPC-producing *Klebsiella pneumoniae* during ceftazidime/avibactam treatment

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Objectives: KPC-producing *Klebsiella pneumoniae* (KPC-Kp) represent a serious problem worldwide. Herein, we describe the evolution of ceftazidime/avibactam resistance by sequencing longitudinal clinical isolates from a patient with KPC-Kp bloodstream infection undergoing ceftazidime/avibactam treatment.

Methods: WGS was performed on one ceftazidime/avibactam-susceptible KPC-Kp (BOT-CA-S) and two phenotypically different ceftazidime/avibactam-resistant KPC-Kp with low (BOT-CA-R) and high (BOT-EMO) carbapenem MICs. The population diversity was assessed by the frequency of allele mutations and population analysis profiles (PAPs).

Results: Phylogenetic analysis demonstrated clonal relatedness of the KPC-Kp isolates, all belonging to the clone ST1519. The D179Y mutation in bla_{KPC-3} was detected in both of the ceftazidime/avibactam-resistant KPC-Kp, whereas it was absent in the ceftazidime/avibactam-susceptible isolate. The mutation emerged independently in the two ceftazidime/avibactam-resistant isolates and was associated with a significant reduction in carbapenem MICs in BOT-CA-R, but not in BOT-EMO. WGS analysis revealed that the frequency of the D179Y mutation was 96.32% and 51.05% in BOT-CA-R and BOT-EMO, respectively. PAP results demonstrated that carbapenem resistance in BOT-EMO was due to the coexistence of mixed subpopulations harbouring WT and mutated bla_{KPC-3}. A bacterial subpopulation with high ceftazidime/avibactam resistance for BOT-EMO KPC-Kp showed low carbapenem MICs, whereas a subpopulation with high meropenem resistance had a low MIC of ceftazidime/avibactam.

Conclusions: Our analysis indicates that mixed subpopulations of ceftazidime/avibactam-resistant KPC-Kp emerge after ceftazidime/avibactam treatment. The evolution of different subpopulations that are highly resistant to ceftazidime/avibactam likely contributes to treatment failure, thereby highlighting the need for combination treatment strategies to limit selection of ceftazidime/avibactam-resistant KPC-Kp subpopulations.

Introduction

In the last decade, carbapenemase-producing Enterobacteriaceae (CPE) have spread worldwide. The dissemination of CPE has largely been owing to KPC, which represents the most common carbapenemase in *Klebsiella pneumoniae*.[1]

Avibactam is a novel non-β-lactam β-lactamase inhibitor that inactivates class A, class C and some class D carbapenemases. In combination with ceftazidime, avibactam restores ceftazidime activity against KPC-producing Enterobacteriaceae.[2] Initial clinical studies have demonstrated that ceftazidime/avibactam is effective for the treatment of infections caused by carbapenem-resistant Enterobacteriaceae.[3,4] However, emergence of ceftazidime/avibactam resistance in CPE has been recently reported.[4–6] Resistance to ceftazidime/avibactam has been linked to specific mutations in the bla_{KPC} gene,[5,6] porin deficiency combined with high ceftazidime hydrolysis,[7] or porin inactivation with or without increased expression of the bla_{KPC} gene.[8]

The major impact on ceftazidime/avibactam resistance has been observed to be mutations within the KPC Ω–loop, which were hypothesized to prevent binding of avibactam.[5,6] Interestingly, the mutations mediating ceftazidime/avibactam resistance are linked to reversion to carbapenem susceptibility in KPC-producing *K. pneumoniae* (KPC-Kp) strains.[5,6]

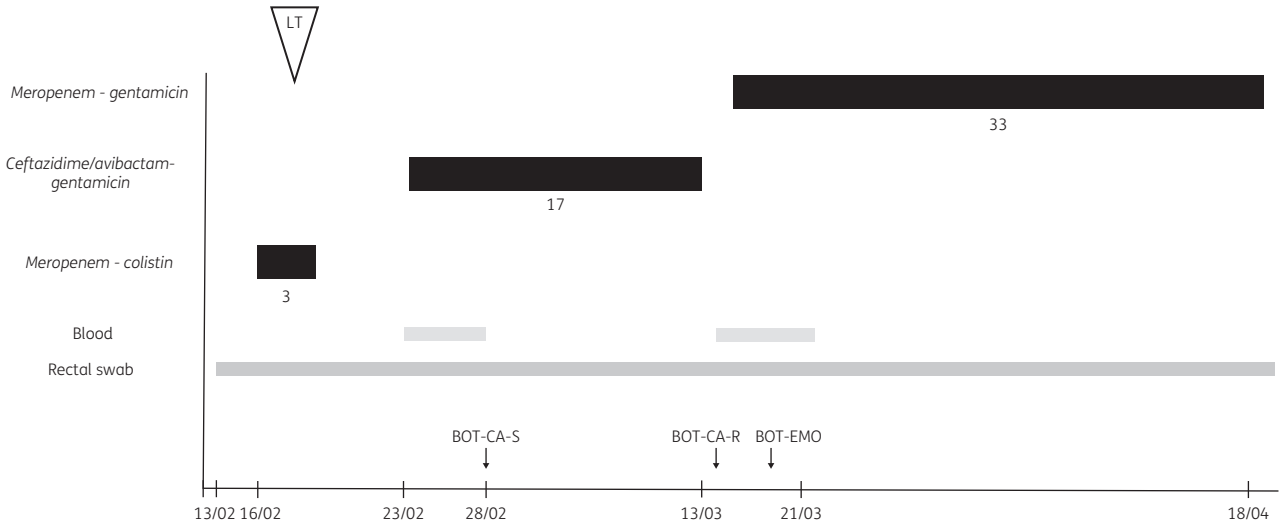

Figure 1. History of the isolation and antimicrobial treatments of a patient with KPC-Kp infections. Black bars represent the antimicrobial treatments and numbers indicate the days of therapy. Grey bars represent the duration of bacteraemia episodes and persistent colonization by KPC-Kp. The triangle indicates the time of liver transplant (LT) and arrows show the isolation times of BOT-CA-S, BOT-CA-R and BOT-EMO.

Herein, we describe a case of *in vivo* emergence of ceftazidime/avibactam resistance in KPC-Kp strains isolated from an Italian patient treated with ceftazidime/avibactam-based therapy.

Materials and methods

Bacterial identification and susceptibility testing

Three *K. pneumoniae* isolates were consecutively recovered from patient samples (see Table 1). MIC results were interpreted following EUCAST clinical breakpoints v7.1.[9] Carbapenemase production was detected with the KPC/MBL and OXA-48 Confirm kit (Rosco Diagnostica) and the presence of KPC and ESBL genes was investigated by PCR and sequencing.[10]

WGS and genetic analysis

WGS was performed as previously described.[11] A core genome SNP phylogeny was generated using Parsnp software[12] with available draft genomes of KPC-Kp CC258 strains isolated in Italy.[11] SNPs and insertion-deletions (indels) between three KPC-Kp strains included in this study were investigated using PATRIC.[13] Gene content analysis and genome comparison of three KPC-Kp genomes against a reference genome (NJST258_1) was performed with the Proteome Comparison tool available in PATRIC.[13]

Bacterial population analysis for each KPC-Kp genome was analysed as previously described.[14] Briefly, reads were mapped using Burrows–Wheeler Aligner against the bla_{KPC-3} gene (Accession no. AF395881) and SNPs were identified by SAMtools and visualized using Tablet software.[15] The frequency of bla_{KPC} alleles was evaluated by comparing the ratio of reads with the mutant allele to the total number of reads using LoFreq software.[16]

Population analysis profile

To investigate the presence of meropenem and ceftazidime/avibactam heteroresistance, a population analysis profile (PAP) was performed as previously described.[17] For agar dilution, the commercial preparation of ceftazidime/avibactam (Avycaz, AstraZeneca) was used for serial doubling dilution in order to maintain the 4:1 ratio thus resembling doses of ceftazidime/avibactam that patients receive.[2] Three colonies of each KPC-Kp isolate that grew at the highest antibiotic concentrations were selected and subcultured on Mueller–Hinton agar plates without antibiotics and tested for meropenem and ceftazidime/avibactam MICs.

Accession number

The sequence genomes were deposited at EMBL/EBI under the EBI project PRJEB21871.

Results

Case report

A young man with HCV-related cirrhosis was admitted for a liver transplant (LT) following the development of autoimmune haemolytic anaemia (AIHA) and a spontaneous psoas muscle haematoma. Three days prior to transplantation, the patient tested positive for KPC-Kp carriage by rectal swab and remained colonized during the entire hospitalization period (Figure 1).[18] The post-operative course was complicated by fever, which prompted the administration of empirical therapy with colistin and high-dose meropenem. Empirical therapy was withdrawn after 72 h with the disappearance of fever and negative blood culture results. On day 8 after LT, the fever recurred associated with hypotension and leucopenia, with blood cultures growing a colistin-resistant KPC-Kp (BOT-CA-S). Combination therapy with ceftazidime/avibactam plus gentamicin was started. The patient improved on therapy with clearance of blood cultures within 72 h of the new regimen. Therapy was discontinued after 2 weeks. However, 2 days after treatment was stopped, fever recurred with blood and bronchoalveolar cultures yielding a KPC-Kp strain (BOT-CA-R). A combination regimen of high-dose extended-infusion meropenem and gentamicin was started. A repeat blood culture drawn 12 h after starting therapy grew KPC-Kp susceptible to colistin (BOT-EMO). The patient's clinical condition gradually improved and blood cultures were negative within 3 days. Treatment was continued for 30 days without evidence of relapse. However, the patient died 14 days

later due to a new episode of ceftazidime/avibactam-susceptible KPC-Kp ST1519 bloodstream infection.

Antimicrobial susceptibility and genome comparison of KPC-Kp isolates

Antimicrobial susceptibility profiles of the three KPC-Kp strains collected before and after ceftazidime/avibactam-based therapy are shown in Table 1. The first ceftazidime/avibactam-resistant KPC-Kp strain (BOT-CA-R) was resistant to colistin and had meropenem, ertapenem and imipenem MICs that were lower by 4-, 4- and 128-fold, respectively, compared with the ceftazidime/avibactam-susceptible strain. The second ceftazidime/avibactam-resistant KPC-Kp strain (BOT-EMO) was susceptible to colistin and displayed carbapenem resistance.

Genetic analysis demonstrated that all three KPC-Kp strains belonged to ST1519 and had identical capsular polysaccharide and porin genes (Table 1). Plasmid content analysis showed that all three KPC-Kp strains shared the same plasmid replicon types, whereas the BOT-EMO isolate harboured an additional plasmid replicon type. Analysis of β-lactam resistance genes showed that ceftazidime/avibactam-resistant KPC-Kp strains exhibited a single amino acid substitution at position 179 (D179Y).

Phylogenetic analysis of KPC-Kp, based on the core genome SNPs of the three KPC-Kp ST1519 strains and 48 genomes of KPC-Kp strains isolated in Italy, showed that the three strains included in this study clustered into a monophyletic group on the tree (Figure S1, available as Supplementary data at *JAC* Online).

Gene content analysis against the NJST258_1 genome showed that 187, 176 and 194 genes were absent in the BOT-CA-S, BOT-CA-R and BOT-EMO genomes, respectively. Most of these genes are hypothetical proteins and are located within plasmids and 50.3 kb prophage regions (Figure S2).

Comparative analysis of SNPs and indels between ceftazidime/avibactam-resistant KPC-Kp strains showed no significant difference. However, deep examination of reads aligning to the bla_{KPC-3} gene demonstrated that 96.32% (coverage depth 136) of aligned reads of the BOT-CA-R isolate displayed the D179Y mutation, while the BOT-EMO isolate displayed a frequency of 51.05% (coverage depth 237) of the mutated allele (Figure S3).

PAP of KPC-Kp strains

Population analysis showed that the BOT-CA-S and BOT-EMO strains contained a meropenem-resistant subpopulation capable of growing in the presence of up to 256 and 128 mg/L meropenem, respectively, (Figure S4) with frequencies of 10^{-4} to 10^{-5}, respectively (Table S1). The proportion of meropenem-resistant subpopulations was statistically different between BOT-CA-S versus BOT-EMO isolates ($P < 0.01$) (see Figure S5), thus suggesting the presence of two distinct subpopulations with different patterns of resistance to meropenem and ceftazidime/avibactam.

The MICs for subpopulations of the BOT-EMO isolate selected on meropenem plates containing 128 mg/L were ≥32 and 4–8 mg/L for meropenem and ceftazidime/avibactam, respectively. When BOT-EMO was grown on agar containing 16/4 mg/L ceftazidime/avibactam, subpopulation MICs of meropenem and ceftazidime/avibactam were 8 and ≥256 mg/L, respectively.

Table 1. Microbiological and genetic characteristics of KPC-Kp clinical isolates described in this study

Isolate	Source of isolation	Days of antimicrobial exposure	MIC (mg/L)				Capsular genes	bla_{KPC} allele	Genetic determinants			Porin genes			Plasmid replicons (IncC)	No. of prophage regions
			ETP	IPM	MEM	CAZ/AVI	GEN	CST	ST			β-lactam	aminoglycoside	fluoroquinolone	colistin	*ompK35*	*ompK36*	*ompK37*		
BOT-CA-S	blood	4[a]	≥32	≥32	≥32	8	2	>16	1519	*wzc*_916, *wzi*_154	bla_{KPC-3}	bla_{TEM-1A}, bla_{OXA-9}, bla_{SHV-11}	*aac(6')-Ib*, *aadA2*, *aph(3')-Ia*, *aac(6')Ib-cr*	*oqxA*, *oqxB*, *aac(6')Ib-cr*	*mgrB*[d]	truncated at aa 88	WT	WT	IncFIIK, IncFIB (pQIL), IncFIBK (Kpn3), IncFIB(pKPHS1), IncX3, ColRNAI	9
BOT-CA-R	broncho alveolar lavage	17[a]	8	0.25	8	≥256	2	>16	1519	*wzc*_916, *wzi*_154	bla_{KPC-3}[c]	bla_{TEM-1A}, bla_{OXA-9}, bla_{SHV-11}	*aac(6')-Ib*, *aadA2*, *aph(3')-Ia*, *aac(6')Ib-cr*	*oqxA*, *oqxB*, *aac(6')Ib-cr*	*mgrB*[d]	truncated at aa 88	WT	WT	IncFIIK, IncFIB (pQIL), IncFIBK (Kpn3), IncFIB(pKPHS1), IncX3, ColRNAI	8
BOT-EMO	blood	17[a]1[b]	≥32	≥32	≥32	≥256	2	0.5	1519	*wzc*_916, *wzi*_154	bla_{KPC-3}[c]	bla_{TEM-1A}, bla_{OXA-9}, bla_{SHV-11}	*aac(6')-Ib*, *aadA2*, *aph(3')-Ia*, *aac(6')Ib-cr*	*oqxA*, *oqxB*, *aac(6')Ib-cr*	–	truncated at aa 88	WT	WT	IncFIIK, IncFIB (pQIL), IncFIBK (Kpn3), IncFIB(pKPHS1), IncX3, ColRNAI, Col(BS512)	8

ETP, ertapenem; IPM, imipenem; MEM, meropenem; CAZ/AVI, ceftazidime/avibactam; GEN, gentamicin; CST, colistin.
[a]Days of exposure to CAZ/AVI and GEN combination.
[b]Days of exposure to MEM and GEN combination.
[c]D179Y mutation in bla_{KPC-3} gene.
[d]*mgrB* gene interrupted by insertion of IS*Kpn25* (ISL3).

Discussion

In this study, we described heteroresistance to ceftazidime/avibactam in a KPC-Kp clinical isolate. Using WGS, we found that genetic diversity within the host population of KPC-Kp occurred as an adaption response to prolonged ceftazidime/avibactam treatment within a patient with bloodstream infection due to KPC-Kp.

Our results showed that the ceftazidime/avibactam-resistant KPC-Kp strains emerged after 17 days of treatment with ceftazidime/avibactam and gentamicin combination therapy. Genetic analysis revealed that ceftazidime/avibactam resistance was associated with the D179Y substitution in the bla_{KPC-3} gene, similar to previous studies.[5,6,19] However, whole-genome and PAP analysis showed that the D179Y mutation in bla_{KPC-3} occurred with different frequencies among ceftazidime/avibactam-resistant strains, thus indicating the presence of mixed subpopulations.

All KPC-Kp strains included in this study belonged to ST1519, a rare ST in Italy.[11,20] Although phylogenetic analysis showed that all KPC-Kp clinical isolates were closely related, it is likely that resistance to ceftazidime/avibactam emerged independently in the BOT-CA-R and BOT-EMO isolates. In support of this hypothesis, antimicrobial susceptibility of the two ceftazidime/avibactam-resistant KPC-Kp strains showed that the BOT-CA-R KPC-Kp was resistant to colistin, while the BOT-EMO isolate retained colistin susceptibility. Therefore, it seems probable that the two ceftazidime/avibactam-resistant strains evolved from a common ancestor rather than longitudinal evolution of *K. pneumoniae* harbouring the mutated bla_{KPC-3} gene. In addition, it is unclear whether carbapenem resistance in ceftazidime/avibactam-resistant KPC-Kp emerged after meropenem-based therapy, or was selected during previous ceftazidime/avibactam treatment and resulted from a transposition event of a bla_{KPC-3}-carrying element.

In conclusion, we found evidence of two different subpopulations harbouring WT and polymorphic bla_{KPC-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. In this context, the presence of different KPC-mutated subpopulations in distinct anatomical sites (i.e. bloodstream and respiratory tract) suggest the possibility of harbouring distinct resistant subclones with high genetic diversity in response to antimicrobial treatment. At the same time, evolution of a specific mutation conferring resistance to ceftazidime/avibactam within self-transmissible plasmids suggests the possible horizontal transfer of mutated KPC plasmid to other microorganisms, thus increasing the risk of developing infections that are difficult to treat with available antimicrobial options.

Therefore, this study highlights the urgent need to establish novel strategies to preserve ceftazidime/avibactam activity and to reduce the risk of rapid emergence of resistance to this last-line antibiotic for KPC-Kp infection.

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Transparency declarations

None to declare.

Supplementary data

Figures S1 to S5 and Table S1 are available as Supplementary data at *JAC* Online.

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