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MECHANISMS OF RESISTANCE

KPC-39-Mediated Resistance to Ceftazidime-Avibactam in a *Klebsiella pneumoniae* ST307 Clinical Isolate

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ABSTRACT Resistance to the ceftazidime (CAZ)-avibactam (AVI) combination is increasingly being reported. Here, we report a CAZ-AVI-resistant *Klebsiella pneumoniae* strain belonging to the high-risk sequence type 307 (ST307) clone and producing *Klebsiella pneumoniae* carbapenemase 39 (KPC-39), a single-amino-acid variant of KPC-3 (A172T). Cloning experiments, steady-state kinetic parameters, and molecular dynamics simulations revealed a loss of carbapenemase activity and increased affinity for CAZ. KPC-39 was identified in a patient without prior exposure to CAZ-AVI, suggesting silent dissemination in European health care settings.

KEYWORDS avibactam resistance, carbapenemase, KPC

Carbapenemase-producing *Enterobacterales* (CPE) represent a serious threat to human health (1). Among newly commercialized drugs, avibactam (AVI) is a non-β-lactam inhibitor of β-lactamases that has been successfully used for the treatment of infections caused by *Klebsiella pneumoniae* carbapenemase (KPC)-producing *Klebsiella pneumoniae* isolates (2). However, KPC-producing *K. pneumoniae* isolates that are resistant to ceftazidime (CAZ)-AVI are increasingly being reported. This resistance may be due to either (i) increased expression of KPC after transposition events (3) or increases in the copy number of the KPC-carrying plasmid (4), especially in strains with impaired outer membrane permeability, or (ii) amino acid (AA) substitutions in the KPC protein (5–8). Here, we report the identification of KPC-39, a KPC-3 variant that confers CAZ-AVI resistance to a *K. pneumoniae* sequence type 307 (ST307) clinical isolate.

K. pneumoniae 181E2 was recovered from a rectal swab sample from a patient upon admission to a French hospital. Because this isolate displayed reduced susceptibility to ertapenem on a disk diffusion antibiogram, it was sent to the French National Reference Center for Carbapenem-Resistant *Enterobacterales* for further investigations. MICs for β-lactams, determined by Etest (bioMérieux, Marcy-l'Étoile, France), revealed that *K. pneumoniae* 181E2 was resistant to expanded-spectrum cephalosporins and showed decreased susceptibility to ertapenem but was fully susceptible to imipenem and meropenem, according to EUCAST guidelines (9) (Table 1). Interestingly, *K. pneumoniae* 181E2 was resistant to CAZ-AVI, with a MIC of 12 μg/ml determined by Etest and a MIC of 16 μg/ml determined using broth microdilution (Sensititre; Thermo Fisher Scientific, Les Ulis, France). In addition, this isolate was resistant to fluoroquinolones but remained susceptible to aminoglycosides, cotrimoxazole, fosfomycin, nitrofurantoin, tigecycline, and colistin, as determined using broth microdilution (Sensititre; Thermo Fisher Scientific) (Table 1). Biochemical tests failed to detect a carbapenem-hydrolyzing enzyme. However, a lateral flow immunoassay (LFIA)

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TABLE 1 Antimicrobial susceptibility and carbapenemase confirmation test results for clinical isolate *K. pneumoniae* 181E2 (KPC-39), *E. coli* TOP10 transformants, and *E. coli* TOP10

Antibiotic or confirmation test	Antibiotic MIC (mg/liter) or confirmation test result			
	K. pneumoniae 181E2 (KPC-39)	*E. coli* TOP10 (pTOPO-KPC-3)	*E. coli* TOP10 (pTOPO-KPC-39)	*E. coli* TOP10
Antibiotics				
Amoxicillin[a]	>256	>256	>256	6
Amoxicillin-clavulanate[a]	24	48	24	6
Cefixime[a]	6	12	4	0.38
Cefotaxime[a]	4	>32	2	0.064
CAZ[a]	>256	>256	>256	0.125
CAZ-AVI[a]	12	0.75	4	0.125
Cefepime[a]	4	6	2	0.0064
Aztreonam[a]	>256	>256	>256	0.047
Ertapenem[a]	0.25	1	0.094	0.004
Imipenem[a]	0.5	8	1	0.25
Meropenem[a]	0.19	3	0.094	0.032
Imipenem plus relebactam[b]	0.12			
Meropenem plus vaborbactam[b]	<0.06			
Cefiderocol[b]	0.5			
Fosfomycin[b]	<16			
Tigecycline[b]	<0.5			
Co-trimoxazole[b]	0.125			
Colistin[b]	<0.5			
Amikacin[b]	<2			
Tobramycin[b]	<0.5			
Ciprofloxacin[b]	>16			
Nitrofurantoin[b]	<8			
Carbapenemase confirmation tests				
Biochemical[c]				
CarbaNP test	−	+	−	ND[d]
β CARBA test	−	+	−	ND
MBT Star-Carba IVD kit	−	+	−	ND
LFIA (NG-Test CARBA-5)[e]	+	ND	ND	ND
Molecular (Xpert Carba-R)[f]	+	ND	ND	ND

[a]MIC results obtained using Etests (bioMérieux).
[b]MIC results obtained by broth microdilution (Sensititre; Thermo Fisher Scientific).
[c]CarbaNP test (30), β-CARBA test (Bio-Rad, Marne-la-Coquette, France), and matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) MBT Star-Carba IVD kit (Bruker Daltonics, Bremen, Germany).
[d]ND, not determined.
[e]LFIA (NG-Test Carba 5) (NG Biotech, Guipry, France).
[f]Xpert Carba-R PCR assay (Cepheid, Maurens-Scopont, France).

detected the production of a KPC-like enzyme, and a PCR assay confirmed the presence of a $bla_{\text{KPC-like}}$ allele (Table 1).

Illumina-based whole-genome sequencing of *K. pneumoniae* 181E2 was performed as described previously (10). The resistome, as determined using ResFinder v4.1 (11), revealed the presence of four β-lactamase genes, i.e., the chromosomally encoded penicillinase $bla_{\text{SHV-28}}$ and three acquired genes, namely, $bla_{\text{TEM-1}}$, $\Delta bla_{\text{OXA-9}}$ (disrupted by a stop codon), and a new $bla_{\text{KPC-3}}$-derived allele named the $bla_{\text{KPC-39}}$ gene. KPC-39 differs from KPC-3 by a single-AA substitution at position 172 (A172T). While OmpK35 is likely not functional due to a premature stop codon, OmpK36 differed from that of *K. pneumoniae* ATCC 43816 by 25 AA substitutions, 4 AA deletions, and 2 AA insertions (12), which did not have a significant impact on carbapenem susceptibility. Unlike most KPC-producing *K. pneumoniae* strains, which belong to clonal group 258 (CG258), *K. pneumoniae* 181E2 belonged to ST307. The $bla_{\text{KPC-39}}$ gene was embedded in Tn*4401a* and was carried on a multireplicon IncFII_{K}-IncFI_{B} self-transferable plasmid.

The $bla_{\text{KPC-39}}$ gene was amplified using the primers KPC-RBS (5′-CTCCACCTTCAAAC AAGGAAT-3′) and KPC-REV (5′-ATCTGCAGAATTCGCCCTTCGCCATCGTCAGTGCTCTA

TABLE 2 Steady-state kinetic parameters of KPC-39, KPC-3, and KPC-31 for selected β-lactam substrates[a]

	K_m (μM)			k_{cat} (s^{-1})			k_{cat}/K_m (mM^{-1}/s^{-1})		
Substrate	**KPC-39**	**KPC-3**	**KPC-31**	**KPC-39**	**KPC-3**	**KPC-31**	**KPC-39**	**KPC-3**	**KPC-31**
Benzylpenicillin	57	40	NH[b]	4	16.7	ND[c]	70	417	ND
CAZ	>1,000[d]	>1,000[d]	>1,000[d]	>3.7[e]	>14[e]	>2.3[e]	>1.4[e]	>8[e]	>2.1[e]
Aztreonam	>1,200	>1,200	NH	>20	>134	ND	>15	>57	ND
Imipenem	137	184	NH	1.27	22.4	ND	9.2	122	ND
Ertapenem	6.5	22	NH	0.12	2.5	ND	17.7	115	ND
Meropenem	5.4	20	NH	0.04	2.5	ND	6.7	96	ND

[a]Data are the means of three independent experiments. Standard deviations were within 15% of the mean value.
[b]NH, no hydrolysis was observed with 0.7 μM purified enzyme and up to 1,000 μM substrate.
[c]ND, not determined.
[d]K_m values were above 1,000 μM and thus could not be determined experimentally.
[e]Because the exact K_m values could not be determined, k_{cat} values and catalytic efficiencies were extrapolated.

C-3′) and cloned into the pCR-Blunt II-Topo plasmid (Invitrogen, Villebon-sur-Yvette, France), and the resulting plasmid, pTOPO-KPC-39, was electroporated into *Escherichia coli* TOP10, as described previously for KPC-3 (13). Similar MICs for amoxicillin, cefixime, and cefepime were obtained for both transformants, but *E. coli* (pTOPO-KPC-39) was more susceptible to carbapenems, lacked carbapenem hydrolysis, as revealed by biochemical confirmation tests (Table 1), and was 5-fold more resistant to CAZ-AVI, compared to *E. coli* (pTOPO-KPC-3), suggesting that the A172T substitution is responsible for the resistance to CAZ-AVI and the loss of carbapenemase activity.

KPC-39 and KPC-31 were produced and purified to purity (as revealed by SDS–PAGE analysis) as described previously for KPC-3 (13). Steady-state kinetic parameters of KPC-39 were compared to those of KPC-3 and KPC-31, the most prevalent CAZ-AVI-resistant KPC-3 variant (D179Y). As expected, the catalytic efficiency for imipenem was reduced 13-fold for KPC-39 (Table 2). Globally, k_{cat} values for carbapenems and all tested β-lactams were reduced for KPC-39, suggesting that the A172T substitution led to overall reduced hydrolysis. A kinetic study of KPC-31 confirmed the absence of hydrolysis of selected β-lactams except for CAZ, as had been proposed by Oueslati et al. based on MIC determinations (5). Regarding CAZ hydrolysis, KPC-3, KPC-39, and KPC-31 exhibited high K_m values (>1,000 μM), making exact measurement of the catalytic efficiency impossible. AVI 50% inhibitory concentration (IC_{50}) values were 320 nM and 400 nM for KPC-3 and KPC-39, respectively, suggesting that the A172T substitution had no impact on the inhibition properties of AVI, in contrast to KPC-31, for which the IC_{50} value was about 50-fold higher (20 μM) (5). IC_{50} values of clavulanate and tazobactam for KPC-39 (46 μM and 31 μM, respectively) were comparable to those of KPC-3 (20 μM and 50 μM, respectively) (6).

In silico modeling and molecular dynamics of KPC-39 enzyme were studied to understand the mechanism of resistance to CAZ-AVI. The three-dimensional structure of KPC-39 was generated using the swapaa command implemented in UCSF Chimera (14), starting from the structure of KPC-3 (PDB code 6QWD) (15). Molecular dynamics simulations were performed in triplicate with GROMACS v2020.3 (16) using the Amber99SB-ILDN force field (17). The protein was centered in a cubic periodic box, with at least 1.0 nm on each side. The simulation box was then filled with TIP3P water molecules, and the system was neutralized with Na^+ and Cl^- ions until the physiological ionic strength (150 mM) was reached. Each system was energy minimized to convergence using a steepest-descents algorithm. Molecular dynamics, performed for 100 ns in each case, as described previously (18), showed overall good stability of the three-dimensional structure of KPC-39. Significant conformational changes occurred during the simulation time, compared to KPC-3, for the Ω-loop in the region containing the mutation A172T. Additionally, a correlated movement of β-strands β3, β4, β5,

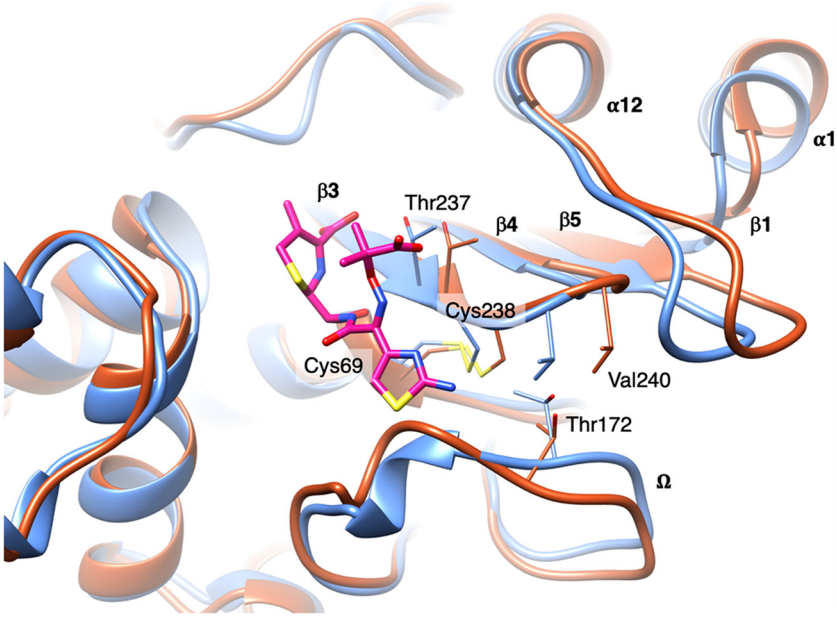

FIG 1 KPC-39 conformations at the beginning (blue) and at the end (red) of the molecular dynamics simulation. Covalently bound CAZ (PDB code 2ZQD) is represented as magenta-colored sticks.

and β1 (with C-α of Val240 shifted by 2.6 Å), which was propagated as far as the α-helix α1, was observed (Fig. 1).

K. pneumoniae 181E2 was isolated from a patient who had had no prior exposure to CAZ-AVI or to anticancer chemotherapies, which have also been suggested to contribute to the selection of CAZ-AVI-resistant KPC variants (19). It is likely that transmission from a CAZ-AVI-treated patient might have occurred. To date, KPC-39 producers have been reported from Italy (two *K. pneumoniae* ST512 strains from different patients) (20) and from Spain (a strain of unknown ST from one previously CAZ-AVI-treated patient) (21). These sporadic descriptions of KPC-39 may reflect silent dissemination in European hospitals or several concomitant selections. *K. pneumoniae* 181E2, described here, belongs to *K. pneumoniae* ST307, a high-risk clone associated with the spread of $bla_{\text{CTX-M-15}}$ and carbapenemase genes (22) and responsible for hospital outbreaks of CPE in Italy, Germany, Portugal, and France (22, 23).

KPC-39 differs from KPC-3 by one AA substitution at position 172, located in the Ω-loop. Changes in this loop are frequently involved in CAZ-AVI resistance among KPC variants (KPC-31, KPC-33, KPC-35, KPC-40, KPC-48, KPC-51, KPC-52, KPC-53, and KPC-57) or other class A β-lactamases such as SHV or CTX-M (24, 25). Increased hydrolysis of CAZ but loss of hydrolysis of most β-lactams, as observed for KPC-39, has also been reported for CTX-M-93, an L169Q variant of CTX-M-27 (26). Position 169 is conserved in most extended-spectrum β-lactamases (ESBLs), being either a leucine or a methionine. Nine single-AA polymorphisms were identified in the Ω-loop of the 172 CTX-M sequences present in the Beta-Lactamase Database (BLDB) (http://www.bldb.eu) (27). None of the nine substitutions introduced into CTX-M-15 led to CAZ-AVI resistance, but two produced increased enzymatic activity against CAZ, i.e., 4-fold (P167S) and 16-fold (L169Q) increases in the MIC of CAZ (25). However, no substitution at position 172 was evidenced.

CAZ-AVI resistance can be due to better affinity for CAZ (KPC-14, KPC-28 [13], KPC-33 [28], and KPC-41 [6]) or to an impaired capacity of AVI to bind the enzyme (KPC-31 and KPC-33 [5, 13]). Here, the CAZ-AVI resistance mechanism seems more complex. Since the maximum velocity for CAZ could not be reached experimentally, exact K_m values could not be determined. Molecular dynamics simulations showed important

changes in the shape of the active site induced by the A172T mutation; to avoid the steric hindrance of Thr172 with Val240 and Cys238, the Ω-loop is pushed back and the β-sheet containing β3, β4, β5, and β1 is shifted laterally (while being restrained by the Cys69-Cys238 disulfide bond), with the effect of this movement being propagated as far as the α-helix α1 (Fig. 1). Overall, this larger active site would accommodate CAZ with higher affinity, thus leading to the CAZ-AVI-resistant phenotype determined experimentally.

Accurate identification of KPC variants conferring CAZ-AVI resistance and carbapenem susceptibility to *Enterobacterales* strains is needed, especially since recent studies showed that carbapenem-based regimens may be used to effectively treat infections (21, 29). Combined use of carbapenemase detection assays (biochemical assays, LFIAs, or molecular assays) is needed to detect these KPC variants, which are increasingly being detected in Europe, even in patients with no prior exposure to CAZ-AVI.

Data availability. The draft genome of *K. pneumoniae* 181E2 was deposited in GenBank under accession number JAFFPJ000000000.

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