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**KPC-39-mediated resistance to Ceftazidime-Avibactam in a *Klebsiella pneumoniae* ST307 clinical isolate**

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32 **ABSTRACT (74/75)**

33

34 Resistance to ceftazidime–avibactam (CAZ-AVI) combination is being increasingly reported. Here, we report a  
35 CAZ-AVI resistant *Klebsiella pneumoniae* belonging to the high-risk ST307 clone and producing KPC-39, a single  
36 amino-acid variant of KPC-3 (A172T). Cloning experiments, steady state kinetic parameters and molecular  
37 dynamics simulations revealed a loss of carbapenemase activity and an increased affinity for ceftazidime. KPC-  
38 39 was identified in a patient without prior exposure to CAZ-AVI, suggesting silent dissemination in European  
39 healthcare settings.

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

51 *Kp* 181E2 was recovered from a rectal swab of a patient upon admission to a French hospital. As this  
52 isolate displayed reduced susceptibility to ertapenem on a disk diffusion antibiogram it was sent to the French  
53 National Reference Center for Carbapenem-resistant *Enterobacterales* for further investigations. MICs for  $\beta$ -  
54 lactams, determined by Etest (bioMérieux, Marcy-L-Etoile, France), revealed that *Kp* 181E2 was resistant to  
55 expanded-spectrum cephalosporins, showed decreased susceptibility to ertapenem but was fully susceptible to  
56 imipenem and meropenem according to EUCAST guidelines (9) (Table 1). Interestingly, *Kp* 181E2 was resistant  
57 to CAZ-AVI with a MIC of 12  $\mu$ g/mL determined by E-test and 16  $\mu$ g/mL using broth microdilution (Sensititre  
58 Thermofisher®, Les Ulis, France). In addition, this isolate was resistant to fluoroquinolones but remained  
59 susceptible to aminoglycosides, cotrimoxazole, Fosfomycin, nitrofurantoin, tigecyclin and colistin, using broth  
60 microdilution (Sensititre Thermofisher®)(Table 1). Biochemical tests failed to detect a carbapenem-hydrolyzing  
61 enzyme. However, lateral flow immunoassay (LFIA) detected the production of a KPC-like enzyme and PCR  
62 assay confirmed the presence of *bla*<sub>KPC-like</sub> allele (Table 1).

63 Illumina based whole genome sequencing of *Kp* 181E2 was performed as previously described (10) and  
64 the draft genome was deposited at Genbank under accession number JAFFPJ000000000. The resistome,  
65 determined using Resfinder v4.1 (11), revealed the presence of 4  $\beta$ -lactamase genes: the chromosomally-  
66 encoded penicillinase *bla*<sub>SHV-28</sub>, and three acquired genes: *bla*<sub>TEM-1</sub>,  $\Delta$ *bla*<sub>OXA-9</sub> (disrupted by a stop codon) and a  
67 new *bla*<sub>KPC-3</sub>-derived allele, named *bla*<sub>KPC-39</sub> gene. KPC-39 differs from KPC-3 by a single amino-acid (AA)  
68 substitution at position 172 (A172T). While *OmpK35* is likely not functional due to a premature stop codon,  
69 *OmpK36* differed from that of *Kp* ATCC 43816 by 25 AA substitutions, 4 AA deletions and 2 AA insertions (12),  
70 that did not have a significant impact on carbapenem susceptibility. Unlike most KPC-*Kps* that belong to CG-  
71 258, *Kp* 181E2 belonged to ST307. The *bla*<sub>KPC-39</sub> gene was embedded in *Tn4401a* and was carried on a multi-  
72 replicon *IncFII<sub>K</sub>-IncFI<sub>B</sub>* self-transferable plasmid.

73 The *bla*<sub>KPC-39</sub> gene was amplified using the primers KPC-RBS (5'- CTCCACCTCAAACAAGGAAT -3') and  
74 KPC-REV (5'- ATCTGCAGAATTCGCCCTTCGCCATCGTCAGTGCTCTAC -3'), cloned into pCR-Blunt II-Topo plasmid  
75 (Invitrogen, Villebon-sur-Yvette, France) and the resulting plasmid, pTOPO-KPC-39 was electroporated into *E.*  
76 *coli* TOP10, as previously described for KPC-3 (13). Similar MICs for amoxicillin, cefixime and cefepime were

77 obtained for both transformants, but *E. coli* (pTOPO-KPC-39) was more susceptible to carbapenems, lacked  
78 carbapenem-hydrolysis as revealed with biochemical confirmation tests (Table 1), and was 5-fold more  
79 resistant to CAZ-AVI as compared to *E. coli* (pTOPO-KPC-3), suggesting that the A172T substitution is  
80 responsible of the resistance to CAZ-AVI and the loss of carbapenemase activity.

81 KPC-39 and KPC-31 were produced and purified to purity (as revealed by SDS-PAGE analysis) as  
82 previously described for KPC-3 (13). Steady state kinetic parameters of KPC-39 were compared to those of KPC-  
83 3 and KPC-31, the most prevalent CAZ-AVI resistant KPC-3 variant (D179Y). As expected, catalytic efficiency for  
84 imipenem was reduced 13-fold for KPC-39 (Table 2). Globally,  $k_{cat}$  values for carbapenems and all tested  $\beta$ -  
85 lactams were reduced for KPC-39 suggesting that the A172T substitution led to an overall reduced hydrolysis.  
86 Kinetic study of KPC-31 confirmed the absence of hydrolysis of selected  $\beta$ -lactams except for ceftazidime, as it  
87 has been proposed by Oueslati *et al.* based on MIC determinations (5). Regarding ceftazidime hydrolysis, KPC-3,  
88 KPC-39 and KPC-31 exhibited high  $K_m$  value ( $>1000 \mu\text{M}$ ) making impossible the exact measure of the catalytic  
89 efficiency.  $IC_{50}$  values for avibactam were of 320 nM and 400 nM for KPC-3 and KPC-39, respectively, suggesting  
90 that the A172T substitution has no impact on the inhibition properties of avibactam, in contrast with KPC-31  
91 whose  $IC_{50}$  value was about 50-fold higher (20  $\mu\text{M}$ ) (5).  $IC_{50}$  values of clavulanate and tazobactam for KPC-39  
92 (46  $\mu\text{M}$  and 31  $\mu\text{M}$ , respectively) were comparable to those of KPC-3 (20  $\mu\text{M}$  and 50  $\mu\text{M}$  respectively) (6).

93 *In silico* modelling and molecular dynamics of KPC-39 enzyme was studied to understand the mechanism of  
94 resistance to CAZ-AVI. The three-dimensional structure of KPC-39 was generated using the *swappa* command  
95 implemented in UCSF CHIMERA (14) starting from the structure of KPC-3 (PDB code 6QWD) (15). Molecular  
96 dynamics simulations were performed in triplicate with GROMACS version 2020.3 (16) using the AMBER99SB-  
97 ILDN force field (17). The protein was centred in a cubic periodic box, with at least 1.0 nm on each side. The  
98 simulation box was then filled with TIP3P water molecules and the system neutralized with  $\text{Na}^+$  and  $\text{Cl}^-$  ions  
99 until reaching the physiological ionic strength (150 mM). Each system was energy-minimized until convergence  
100 using a steepest descents algorithm. Molecular dynamics, performed for 100 ns in each case as previously  
101 described (18), showed an overall good stability of the three-dimensional structure of KPC-39. Significant  
102 conformational changes occurred during the simulation time, as compared to KPC-3, for the  $\Omega$  loop in the  
103 region containing the mutation A172T. Additionally, a correlated movement of  $\beta$ -strands  $\beta_3$ ,  $\beta_4$ ,  $\beta_5$  and  $\beta_1$   
104 (with  $\text{C}\alpha$  of Val240 shifted by 2.6 Å), which was propagated as far as the  $\alpha$ -helix  $\alpha_1$ , was also observed (Figure  
105 1).

106 *Kp* 181E2 was isolated from a patient that had no prior exposure to CAZ-AVI nor to anticancer  
107 chemotherapies, as it has also been suggested to contribute to the selection of CAZ-AVI-resistant KPC variants  
108 (19). It is likely that transmission from a CAZ-AVI-treated patient may have occurred. To date, KPC-39-  
109 producers have been reported from Italy (two *Kp* ST512 from different patients) (20), and from Spain (unknown  
110 ST from one previously CAZ-AVI treated patient) (21). These sporadic descriptions of KPC-39 may reflect a silent  
111 dissemination in European hospitals or several concomitant selections. *Kp* 181E2 described here belong to *Kp*

112 ST307, a high-risk clone associated with the spread of *bla*<sub>CTX-M-15</sub> and carbapenemase genes (22) and  
113 responsible of hospital outbreaks of CPE in Italy, Germany, Portugal and France (22, 23).

114 KPC-39 differs from KPC-3 by one AA substitution at position 172 located in the  $\Omega$ -loop. Changes in this  
115 loop are frequently involved in CAZ-AVI resistance in KPC-variants (KPC-31; -33; -35; -40; -48; -51; 52; -53; -57)  
116 or in other class A  $\beta$ -lactamases such as SHV or CTX-M (24, 25). Increased hydrolysis of ceftazidime but loss of  
117 hydrolysis of most  $\beta$ -lactams, as observed for KPC-39, has also been reported for CTX-M-93, an L169Q variant  
118 of CTX-M-27 (26). Position 169 is conserved in most ESBLs being either a Leucine or a Methionine. Nine single  
119 AA polymorphisms were identified in the  $\Omega$  loop of the 172 CTX-M sequences present in the BLDB  
120 (<http://www.bldb.eu>) (27). None of the nine substitutions introduced to CTX-M-15 led to ceftazidime-  
121 avibactam resistance, but 2 exhibited an increased enzymatic activity against ceftazidime: 4-fold (P167S) and  
122 16-fold (L169Q) increase in the MIC of ceftazidime (25). However, no substitution at position 172 was  
123 evidenced.

124 CAZ-AVI resistance can be due to a better affinity for ceftazidime (KPC-14, KPC-28 (13), KPC-33 (28),  
125 KPC-41 (6) , or to an impaired capacity of avibactam to bind the enzyme (KPC-31, KPC-33) (5, 13). Here, the  
126 CAZ-AVI resistance mechanism seems more complex. Since the maximum velocity for ceftazidime could not be  
127 reached experimentally, exact *K<sub>m</sub>* values could not be determined. Molecular dynamics simulations showed  
128 important changes in the shape of the active site induced by the A172T mutation: to avoid the steric hindrance  
129 of Thr172 with Val240 and Cys238, the  $\Omega$  loop is pushed back and the  $\beta$ -sheet containing  $\beta$ 3,  $\beta$ 4,  $\beta$ 5 and  $\beta$ 1 is  
130 shifted laterally (while being restrained by the Cys69-Cys238 disulfide bond), the effect of this movement being  
131 propagated as far as the  $\alpha$ -helix  $\alpha$ 1 (Figure 1). Overall, this larger active site would accommodate ceftazidime  
132 with a higher affinity, thus leading to the CAZ-AVI resistant phenotype determined experimentally.

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

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143 Tackling mUlti-Resistance by acting on Alternative bacterial tARgets in Synergy with mEmbrane-disruptiNg  
144 Antimicrobial peptides" (ANR-19-AMRB-0004).

#### 145 **Transparency declarations**

146 L.D is a coinventor of the Carba NP test, the patent for which has been licensed to bioMérieux (La Balmes les  
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Table 1. Antimicrobial susceptibility and carbapenemase confirmation test results of *K. pneumoniae* 181E2 (KPC-39) clinical isolate, *E. coli* TOP10 transformants, and *E. coli* TOP10.

	MIC (mg/L)			
	<i>Kp</i> 181E2 (KPC-39)	<i>E. coli</i> TOP10 (pTOPO-KPC-3)	<i>E. coli</i> TOP10 (pTOPO-KPC-39)	<i>E. coli</i> TOP10
<b>Antibiotics</b>				
Amoxicillin <sup>a</sup>	>256	>256	>256	6
Amoxicillin/Clavulanate <sup>a</sup>	24	48	24	6
Cefixime <sup>a</sup>	6	12	4	0.38
Cefotaxime <sup>a</sup>	4	>32	2	0.064
Ceftazidime <sup>a</sup>	>256	>256	>256	0.125
Ceftazidime/Avibactam <sup>a</sup>	12	0.75	4	0.125
Cefepime <sup>a</sup>	4	6	2	0.0064
Aztreonam <sup>a</sup>	>256	>256	>256	0.047
Ertapenem <sup>a</sup>	0.25	1	0.094	0.004
Imipenem <sup>a</sup>	0.5	8	1	0.25
Meropenem <sup>a</sup>	0.19	3	0.094	0.032
Imipenem + Relebactam <sup>b</sup>	0.12			
Meropenem + Vaborbactam <sup>b</sup>	< 0.06			
Cefiderocol <sup>b</sup>	0.5			
Fosfomicin <sup>b</sup>	< 16			
Tigecyclin <sup>b</sup>	< 0.5			
Cotrimoxazole <sup>b</sup>	0.125			
Colistin <sup>b</sup>	< 0.5			
Amikacin <sup>b</sup>	< 2			
Tobramycin <sup>b</sup>	< 0.5			
Ciprofloxacin <sup>b</sup>	> 16			
Nitrofurantoin <sup>b</sup>	< 8			
<b>Carbapenemase confirmation tests</b>				
Biochemical <sup>c</sup>				
- CarbaNP test	-	+	-	NR <sup>d</sup>
- β CARBA™	-	+	-	NR
- MBT Star-Carba kit	-	+	-	NR
LFIA (NG-Test CARBA-5) <sup>e</sup>	+	NR	NR	NR
Molecular (Xpert Carba-R) <sup>f</sup>	+	NR	NR	NR

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<sup>a</sup> MIC results obtained using Etests (bioMérieux); <sup>b</sup> MIC results obtained by broth microdilution (Sensititre, Thermofischer); <sup>c</sup> Carba NP test (30), β-CARBA™ (Bio-Rad, Marne-la-Coquette, France) and MALDI-TOF MBT

245 STAR<sup>®</sup>-Carba IVD kit (Bruker Daltonics, Bremen, Germany);<sup>d</sup> NR: not realized;<sup>e</sup> LFIA NG-Test Carba 5 (NG  
246 Biotech, Guipry, France);<sup>f</sup> Xpert Carba-R PCR assay (Cepheid, Maurens-Scopont, France).

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Table 2. Steady-state kinetic parameters of KPC-39, KPC-3 and KPC-31 for selected  $\beta$ -lactam substrates<sup>a</sup>

Substrate	$K_m$ ( $\mu\text{M}$ )			$k_{\text{cat}}$ ( $\text{s}^{-1}$ )			$k_{\text{cat}}/K_m$ ( $\text{mM}^{-1}/\text{s}^{-1}$ )		
	KPC-39	KPC-3	KPC-31	KPC-39	KPC-3	KPC-31	KPC-39	KPC-3	KPC-31
Benzylpenicillin	57	40	NH <sup>d</sup>	4	16.7	ND	70	417	ND
Ceftazidime	>1000 <sup>b</sup>	>1000 <sup>b</sup>	>1000 <sup>b</sup>	>3.7 <sup>c</sup>	>14 <sup>c</sup>	>2.3 <sup>c</sup>	>1.4 <sup>c</sup>	>8 <sup>c</sup>	>2.1 <sup>c</sup>
Aztreonam	>1200	>1200	NH <sup>d</sup>	>20	>134	ND	>15	>57	ND
Imipenem	137	184	NH <sup>d</sup>	1.27	22.4	ND	9.2	122	ND
Ertapenem	6.5	22	NH <sup>d</sup>	0.12	2.5	ND	17.7	115	ND
Meropenem	5.4	20	NH <sup>d</sup>	0.04	2.5	ND	6.7	96	ND

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<sup>a</sup> Data are the mean of three independent experiments. Standard deviations were within 15% of the mean value.

<sup>b</sup>  $K_m$  values were above 1000, and thus not determinable experimentally

<sup>c</sup> As the exact  $K_m$  values could not be determined,  $k_{\text{cat}}$  values and catalytic efficiencies were extrapolated

<sup>d</sup> NH, no hydrolysis was observed with 0,7  $\mu\text{M}$  of purified enzyme and up to 1000  $\mu\text{M}$  of substrate

259 Figure legends

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261 **Figure 1.** KPC-39 conformations at the beginning (blue) and at the end (red) of the molecular dynamics simulation.

262 Covalently-bound ceftazidime (PDB code 2ZQD) is represented as sticks coloured in magenta.

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