

Unravelling ceftazidime/avibactam resistance of KPC-28, a KPC-2 variant lacking carbapenemase activity

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33 SYNOPSIS (238) **Backgrounds:** KPC-like carbapenemases have spread worldwide with more than 34 thirty variants identified and differing by single or double amino-acid substitutions. 35 36 Here, we describe the steady-state kinetic parameters of KPC-28 that differs from KPC-2 by a H274Y substitution and a two amino-acids deletion (Δ 242-GT-243). 37 38 **Materials/Methods**: The *bla*_{KPC-2}, *bla*_{KPC-3}, *bla*_{KPC-14} and *bla*_{KPC-28} genes were cloned 39 into pTOPO vector for susceptibility testing or into pET41b for over-expression, 40 purification and subsequent kinetic parameters (K_m , k_{cat}) determination. Molecular 41 docking experiments were performed to explore the role of the amino-acid changes in 42 the carbapenemase activity. 43 **Results:** Susceptibility testing revealed that *E. coli* producing KPC-28 displayed MICs 44 that were lower for carbapenems and higher for ceftazidime and ceftazidime/avibactam

as compared to KPC-2. The catalytic efficiencies of KPC-28 and KPC-14 for imipenem

were 700-fold and 200-fold lower, respectively, than those of KPC-2, suggesting that

the ∆242-GT-243 in KPC-28 and KPC-14 is responsible for reduced carbapenem

hydrolysis. Similarly, the H274Y substitution resulted in a 50-fold increase in

ceftazidime hydrolysis that was strongly reversed by clavulanate. **Conclusion:** Here, we have shown that KPC-28 is lacking carbapenemase activity,
has increased ceftazidime hydrolytic activity, and is strongly inhibited by clavulanate.
KPC-28-producing *E. coli* isolates display an avibactam-resistant ESBL profile, that
may be wrongly identified by molecular and immunochromatographic assays as a
carbapenemase. Accordingly, confirmation of carbapenem hydrolysis will be

55 mandatory with assays based solely on bla_{KPC} gene or gene product detection.

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INTRODUCTION

58 In Gram negative bacteria, acquired resistance to β-lactams may be mediated by various non-enzymatic mechanisms such as decreased permeability of the outer-59 60 membrane, or active efflux, but enzymatic inactivation by β -lactamases is the main 61 mechanism. These enzymes, which are able to hydrolyse β -lactams, are divided into 62 4 molecular classes based on structural homologies (Ambler classification). Ambler's 63 classes A, C and D are β-lactamases possessing a serine in their active site whereas 64 the class B enzymes are metallo- β -lactamases (MBLs) that use divalent Zn²⁺ ion(s) for their hydrolytic activity. During the last decade, the rising use of carbapenems 65 66 (imipenem and meropenem) for the treatment of infections caused by multidrug 67 resistant Gram-negative pathogens has been suggested to have contributed to the 68 emergence of carbapenem-resistant bacteria that may be the result of outer membrane permeability alteration associated with overproduction of a cephalosporinase and/or 69 70 production ESBL or of production of carbapenem-hydrolysing β -lactamases, called 71 carbapenemases.¹ These enzymes belong to Ambler's classes A, B and D². Within class A, several enzymes were described in Enterobacteriaceae (NMC-A, IMI-1, SME-72 1, GES-2, FRI-1) but Klebsiella pneumoniae carbapenemase (KPC) is the most 73 74 prevalent in the world. KPC is considered as the most worrisome class A 75 carbapenemase because of its location on self-conjugative plasmids, and its frequent 76 association with a highly successful K. pneumoniae clone, the clonal complex (CC) 77 258.³ Until 2005, the geographic distribution of KPC-producing Enterobacteriaceae remained limited to the east of United States.⁴ Nowadays, KPC-producers have 78 79 disseminated worldwide and are endemic in the United States, South America, 80 Greece, Italy, Poland, China and Israel. Since it's initial description in a K. pneumoniae 81 clinical isolate from North Carolina, ⁵ thirty-nine KPC-variants have been reported

(www.bldb.eu).⁶ Among these variants, a mono-substitution (KPC 3-6 and 9-11) 82 83 resulted in increased ceftazidime hydrolysis, with carbapenem hydrolysis being unaffected.7-12 Recently, in vitro selection of ceftazidime/avibactam (CAZ-AVI) 84 resistance in Enterobacteriaceae with KPC-3 carbapenemase revealed modifications 85 in the Ω loop that are also responsible for carbapenem susceptibility. The most 86 prevalent modification occurs at position 179 of the KPC enzyme,^{13,14} but few other 87 88 substitutions at different positions were also involved such as V240G and T243A.¹³ 89 Ceftazidime/avibactam resistance also occurred in vivo usually following 90 ceftazidime/avibactam treatment for prolonged periods (> 2 weeks). ^{15–18} The D179 91 modification is always reported in case of in vivo selection of ceftazidime/avibactam resistant KPC-producing isolates.^{15,17} Recently, KPC-28, a new variant of KPC-2 with 92 a two amino-acid deletion (Δ 242-GT-243, according to the ABL numbering scheme)¹⁹ 93 and a substitution H274Y, was reported. ²⁰ In this study, a KPC-28-producing clone 94 was found to be more resistant to ceftazidime as compared to KPC-2- or KPC-3-95 96 producers, but fully susceptible to carbapenems, suggesting a complete loss of 97 carbapenemase activity. Here, we report the susceptibility profile and the steady-state 98 kinetic characterization of the KPC-28 β-lactamase (a variant of KPC-3 deleted with 2 amino acid ∆242-GT-243) in comparison with KPC-2, KPC-3 and KPC-14. 99

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MATERIAL AND METHODS

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103 Bacterial strains.

The clinical strain *Escherichia coli* WI2 expressing the KPC-28 β-lactamase was used for cloning of the bla_{KPC-28} gene. ²⁰ *E. coli* TOP10 (Invitrogen, Saint-Aubin, France) was used for cloning and mutagenesis experiments, and *E. coli* BL21 Rosetta-gamiTM DE3 107 (Novagen-Merck, Fontenay-sous-Bois, France) was used for overexpression108 experiments.

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110 **Susceptibility testing.**

Antimicrobial susceptibilities were determined by the disk diffusion technique on Mueller-Hinton agar (Bio-Rad, Marnes-La-Coquette, France) and interpreted according to the EUCAST breakpoints, updated in May 2018 (http://www.eucast.org). MICs were determined using the Etest technique (bioMérieux, La Balme les Grottes, France).

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117 PCR, cloning experiments, site-directed mutagenesis and DNA sequencing.

Whole-cell DNAs of E. coli isolates producing KPC-2, KPC-3, and KPC-28 were 118 119 extracted using the QIAamp DNA minikit (Qiagen, Courtaboeuf, France) and were then 120 used as a template to amplify the *bla*_{KPC-2-like} genes. The gene encoded for KPC-14 121 was obtained by site-directed mutagenesis (QuikChange II Site-Directed Mutagenesis 122 Technologies), primer KPC-Y274H (5'-Kit, Agilent using the CTAACAAGGATGACAAGCACAGCGAGGCCGTCATC-3') and the plasmid pTOPO-123 *bla*_{KPC-28} as PCR, 124 а template. The using the primers Kpc-rbs (5'-125 CTCCACCTTCAAACAAGGAAT-3') (5'and Kpc-rev ATCTGCAGAATTCGCCCTTCGCCATCGTCAGTGCTCTAC-3'), was able to amplify 126 127 bla_{KPC-3} and bla_{KPC-28} genes. The amplicons obtained were then cloned into the pCR-128 Blunt II-Topo plasmid (Invitrogen) downstream from the pLac promoter, in the same 129 orientation for the phenotypic studies. The recombinant pTOPO-KPC- plasmids were 130 electroporated into the E. coli TOP10 strain. For protein production, the sequences 131 without the peptide signal of the bla_{KPC-2} , bla_{KPC-3} , bla_{KPC-14} and bla_{KPC-28} genes were

132 obtained PCR amplification Ndel-KPC-2₃₀₋₂₉₃ (5'by using primers 133 CATATGGCGGAACCATTCGCTAAC-3') and KPC-2 (5'-**ASTOP** 134 CTCGAGCTGCCCGTTGACGCCAAT-3') and were then inserted into plasmid pET41b 135 (Novagen). The recombinant plasmids were transformed into E. coli BL21 Rosetta-136 gami[™] DE3 (Novagen). All the recombinant plasmids were sequenced using a T7 137 promoter and M13 reverse primers or T7 terminator depending of the plasmid with an automated sequencer (ABI Prism 3100; Applied Biosystems). The nucleotide 138 139 sequences were analysed using software available at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov). 140

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142 **Detection of KPC producers and carbapenemase activity.**

143 The detection of the KPC-variants was performed with *E. coli* TOP10 harbouring the 144 recombinant vector pTOPO-KPC. The detection of the carbapenemase activity was 145 done by using six techniques: The Carba NP test as previously described, ²¹ the CIM test,²² the Hodge test,²³ the β-CARBA[™] test (BioRad)²⁴, the RAPIDEC® CARBA NP 146 (bioMérieux)²⁵ and the MBT STAR[®]-Carba IVD Kit (Bruker Daltonics, Bremen, 147 Germany)²⁶ according to the manufacturer's recommendations. Molecular tests were 148 performed with the kit Xpert[®] Carba-R (Cepheid, Sunnyvale, USA)²⁷ and by standard 149 150 in-house PCR using the primers KPC-A (5'-CTGTCTTGTCTCTCATGGCC-3') and 151 KPC-B (5'-CCTCGCTGTGCTTGT-CATCC-3'). Immunoenzymatic tests were carried out with two techniques: NG- CARBA 5 (NG Biotech, Guipry, France)²⁸ and the K-Set 152 Resist-4 O.K.N.V. (CORIS BioConcept, Gembloux, Belgium).²⁹ 153

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155 **Protein purification**

156 An overnight culture of *E. coli* BL21 Rosetta-gami[™] DE3 harbouring recombinant

157 pET41b-KPC- plasmids was used to inoculate 2 L of LB medium broth containing 50 158 mg/L kanamycin and 30 mg/L chloramphenicol. Bacteria were cultured at 37°C until 159 an OD of 0.6 at 600 nm was reached. The expression of the β -lactamase genes was 160 carried out overnight at 22°C with 1 mM IPTG as inducer. Cultures were centrifuged at 161 6000 g for 15 min and then the pellets were resuspended with the binding buffer (10 162 mM Imidazole, 25mM Sodium phosphate pH 7.4 and 300 mM NaCl). Bacterial cells 163 were disrupted by sonication and the bacterial pellet was removed by two consecutive 164 centrifugation steps at 10,000 g for 1h at 4°C and then the supernatant was centrifuged 165 at 96 000 g for 1h at 4°C. The soluble fractions were filtered and then passed through a HisTrap[™] HP column (GE Healthcare), proteins were eluted with the elution buffer 166 167 (500 mM Imidazole, 25mM Sodium phosphate pH 7.4 and 300 mM NaCl). Finally, a 168 gel filtration step was performed with 100 mM sodium phosphate buffer pH 7 and 150 169 mM NaCl with a Superdex 75 column (GE Healthcare). The protein purity was 170 estimated by SDS-PAGE and the pooled fractions were dialyzed against 10 mM Tris-171 HCI pH 7.6 and concentrated using Vivaspin columns. The concentrations were 172 determined by measuring the optical density at 280 nm and with the extinction 173 coefficients obtained from the ProtParam tool (Swiss Institute of Bioinformatics online 174 resource portal).³⁰

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176 Steady state kinetic parameters

Kinetic parameters were determined using purified KPC-2, KPC-3, KPC-14 and KPC-28 β-lactamases in 100 mM sodium phosphate buffer (pH 7). The k_{cat} and K_m values were determined by analysing β-lactam hydrolysis under initial-rate conditions with an ULTROSPEC 2000 UV spectrophotometer and the SWIFT II software (GE Healthcare, Velizy-Villacoublay, France) using the Eadie-Hoffstee linearization of the Michaelis-

182 Menten equation. The different β-lactams were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France). For some cephalosporins, saturation could not be reached. 183 184 Thus, the values for the catalytic efficiency (k_{cat}/K_m) of the enzymes KPC-2 and KPC-185 3 against these substrates were evaluated with the lower limits for the k_{cat} and K_m 186 determined. Fifty percent inhibitory concentration (IC50) for the β-lactamase inhibitors 187 : clavulanic acid and Avibactam was determined in 100 mM sodium phosphate buffer 188 (pH 7) and with 100 µM of piperacillin and 100µM of ceftazidime (respectively) as a 189 reporter substrate.

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191 Molecular Modelling

192 Molecular models of KPC-3, KPC-14 and KPC-28 were generated by comparative 193 modelling using MODELLER version 9.16³¹ with the KPC-2 structure³² (PDB code 194 5UJ3) as template. Three-dimensional structures of the ligands were generated using 195 CORINA version 3.60 (Molecular Networks GmbH, Erlangen, Germany, 196 http://www.molecular-networks.com). Covalent docking calculations were carried out 197 using GOLD version 5.2³³ and the GoldScore scoring function. The binding site was 198 defined as a sphere with a 20 Å radius around the OG atom of the Ser70 residue. The 199 covalent connection was made between the OG atom of the Ser70 residue and the 200 open form of the β -lactam ring in order to generate the acyl-enzyme complex. 201 Molecular modelling images were generated using UCSF CHIMERA.³⁴

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RESULTS

204 Clinical isolate

E. coli WI2 was recovered from a fecal sample from a Portuguese patient upon admission at a French hospital subsequent to a medical repatriation. This isolate was resistant to broad-spectrum cephalosporins, aminoglycosides and colistin and of reduced susceptibility to carbapenems.²⁰ As previously described, this isolate possessed the bla_{OXA-48} and bla_{KPC-28} genes and the acquired colistin resistance determinant *mcr-1*.²⁰ KPC-28 is a variant of KPC-3 with a deletion of 2 amino acids (Δ 242-GT-243) and KPC-14 is a variant of KPC-2 with the same deletion of the 2 amino acids (Figure 1).

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Antimicrobial susceptibilities of transformants with KPC-2, KPC-3, KPC-14 and KPC-28.

216 To evaluate and compare the antimicrobial susceptibility profiles conferred by 217 KPC-28, the *bla_{KPC-2}*, *bla_{KPC-3}*, *bla_{KPC-14}* and *bla_{KPC-28}* genes were cloned into pTOPO 218 vector and expressed in E. coli TOP10 (Table 1). Although the different KPC variants 219 exhibited similar MICs for amoxicillin, temocillin, cefixime and cefepime, KPC-28- and 220 KPC-14-producing E. coli TOP10 possessed lower MICs for cefotaxime and carbapenems (being fully susceptible) but exhibited higher MIC values for ceftazidime 221 222 as compared to KPC-2- and KPC-3-producers (Table 1). Of note, the highest MICs for 223 cefotaxime and aztreonam were found with the KPC-3-producing E. coli TOP10 clones. 224 The addition of clavulanic acid restored susceptibility to amoxicillin for KPC-28- and 225 KPC-14-producing *E. coli* clones, while inhibition by avibactam seemed to be less 226 efficient, as both KPC-28- and KPC-14-producing E. coli isolates were resistant to the 227 association ceftazidime/avibactam. Thus, MIC values suggested that the $\triangle 242$ -GT-243 228 (in KPC-14 and KPC-28) resulted in the loss of carbapenemase activity but in 229 increased hydrolytic activity towards ceftazidime. This increased ceftazidime hydrolytic 230 activity was potentiated by the H274Y substitution in KPC-28, as already shown for 231 KPC-3, but in the latter case, carbapenem-resistance was not affected. ¹²

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233 Biochemical properties of KPC-28

234 To characterize and compare kinetic parameters of KPC-28, the four variants 235 have been purified and kinetic studies were performed (Table 2). KPC-2 was used as 236 control for comparison. Overall, the steady-state kinetics revealed three patterns. KPC-237 3 exhibited a higher hydrolytic activity towards expanded-spectrum cephalosporins 238 (cefotaxime, ceftazidime and cefepime) but a similar hydrolysis rate for carbapenems 239 as compared to KPC-2. In comparison, KPC-14 and KPC-28 possessed a higher 240 affinity for ceftazidime resulting in increased catalytic efficiencies of 40-fold and 50-241 fold, respectively, with however reduced carbapenemase activity, as revealed by the 242 200-fold and 700-fold reduction in imipenem catalytic efficiencies, respectively, as 243 compared to KPC-2. Thus, the reduced carbapenem hydrolysis can be linked to the 244 Δ 242-GT-243 that results in a 10-fold increase in affinity but also a 1000-fold decrease in k_{cat} values. Similarly, KPC-14 and KPC-28 lost completely cefoxitine hydrolytic 245 246 activity. IC50 values of clavulanic acid for KPC-2, KPC-3, KPC-14 and KPC-28 were 247 47 µM, 16 µM, 0.1 µM and 0.09 µM, respectively. Taken together, our results suggest 248 that KPC-28 displays a clavulanic acid inhibited ESBL profile and not that of a 249 carbapenemase anymore. Finally, our data suggest that the increased ceftazidime 250 hydrolysis of KPC-28 is due to the H274Y substitution (Table 2) confirming previous results,¹² but is potentiated by the 2 amino-acid deletion (Δ 242-GT-243). The H274Y 251 252 substitution does not affect carbapenem-hydrolysis, unlike the ∆242-GT-243 deletion 253 (Table2). The activity of avibactam against the different KPC has been determined by 254 IC50 measurments. The IC50 values for KPC-2, KPC-3, KPC-14 and KPC-28 were 230 nM, 350 nM, 107 nM and 586 nM, respectively. These values are very similar, 255 which suggests that the deletion of 2 amino acids GT242-243 have no impact on the 256

257 inhibition properties of avibactam.

258 Molecular modelling

259 We performed a molecular modelling study to identify the structural 260 determinants that could explain the experimentally-determined differences between 261 the hydrolytic profiles of KPC-2, KPC-3, KPC-14 and KPC-28. In the absence of structural data for the KPC-2 variants, we have generated molecular models of KPC-262 3, KPC-14 and KPC-28 by comparative modelling using MODELLER version 9.16³¹ 263 264 with the KPC-2 structure (PDB code 5UJ3) as template (Figure 2A, 2B).³² The resulting models showed that the \triangle 242-GT-243 deletion didn't modify the overall structure of the 265 266 protein, but resulted in a shorter 238-243 loop, giving rise to a 2.4 Å shift of A244 in KPC-14 and KPC-28 (Figure 2A). This new position of A244 led to a clash between 267 268 the side chains of A244 and of the residue at position 274 (H and T for KPC-14 and 269 KPC-28, respectively) that may expand the active site and allow a better access for 270 the substrates. In addition, the covalent complex of KPC-2 with imipenem obtained by 271 docking showed a stabilizing interaction between H274 and the positively charged R2 substituent of imipenem (Figure 2B). In the case of KPC-14 and KPC-28, the above-272 273 mentioned clash may prevent this stabilizing interaction with imipenem. In these 274 conditions, the substrate may interact with the binding site in a slightly different mode and therefore explain the 10-fold increase in K_m and the 1000-fold decrease in k_{cat} , 275 276 which ultimately lead to the loss of the carbapenemase activity in these mutants. 277 Additional theoretical calculations, and especially molecular dynamics simulations, will 278 be needed to understand these details.

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280 **Detection methods for KPC variants**

Four carbapenemase detection tests based on imipenem hydrolysis were evaluated in

282 respect to their ability to detect these four variants when expressed in E. coli TOP10 (Table 3). Thus, the Carba NP Test²¹, the RAPIDEC[®]CARBA NP, the MBT STAR[®]-283 284 Carba IVD and the β-CARBA[™] test were able to detect KPC-2 and KPC-3, but failed to detect KPC-14 and KPC-28.^{21–26} These results are in line with the kinetic studies, 285 which showed a loss of carbapenemase activity for KPC-14 and KPC-28. On the other 286 hand, immunochromatographic assays RESIST-4 O.K.N.V kit (CORIS BioConcept, 287 288 Gembloux, Belgium) and the NG-Test CARBA 5 (NG Biotech, Guipry, France) and 289 molecular Xpert[®] Carba-R test (Cepheid, Sunnyvale, USA) and in-house PCR were positive enzymes. 27–29,35 290 for all four The positive results of the 291 immunochromatographic assay for KPC-14 and KPC-28 confirmed our molecular 292 modelling results, suggesting that the $\triangle 242$ -GT-243 did not affect the global 293 conformation of these proteins.

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DISCUSSION

KPC-producing Enterobacteriaceae are now endemic in the United State and have 296 297 spread worldwide. So far, thirty-nine variants of KPC have been reported⁶, with KPC-2 and KPC-3 appearing to be the most prevalent.³⁶ In this study, we have characterized 298 299 the biochemical properties of KPC-28, which shared with KPC-3 the same substitution 300 H274Y and possesses in addition a \triangle 242-GT-243 deletion previously identified in KPC-301 14. Steady-state kinetics of KPC-2, KPC-3, KPC-14 and KPC-28 β-lactamases revealed that the unique substitution of KPC-3 (H274Y) conferred a 20-fold increase 302 303 of the catalytic efficiency towards ceftazidime as compared with KPC-2. This 304 substitution was also been identified in other variants such as KPC-7 (M49I; H274Y), 305 KPC-8 (V240G; H274Y), KPC-9 (V240A; H274Y) and KPC-10 (P104R; H274Y), which are associated with increased ceftazidime resistance.⁹ The study of KPC-5 showed 306

307 that substitution of the residues 104 can also confer an increased ceftazidime 308 hydrolysis.¹¹ These studies highlighted that a single or a double substitution can affect 309 the hydrolysis rate of cephalosporins but do not change significantly the 310 carbapenemase activity. More recently, Shields et al. reported that T243A substitution 311 increased ceftazidime hydrolysis of the KPC-3 enzyme with little impact on carbapenemase activity.¹³ Here, we demonstrated that the ∆242-GT-243 deletion also 312 313 led to an increase of the ceftazidime hydrolysis, but unlike T243A substitution, has a 314 drastic impact on carbapenemase activity. For example, compared to KPC-2, the 315 catalytic efficiencies (k_{cat}/K_m) towards imipenem were 700-fold and 200-fold lower for 316 KPC-28 and KPC-14, respectively. This deletion is linked with a 10-fold higher affinity 317 (lower K_m) but a 1000-fold decreased k_{cat} for carbapenems. The loss of the 318 carbapenemase activity can be explained by the loss of the interaction between the 319 residue H274 in KPC-14 and Y274 in KPC-28 and carbapenems due to a 2Å shift of 320 the A244. Additionally, the 50% inhibitory concentration (IC50) of clavulanate was 500-321 fold lower with KPC-28 as compared to KPC-2. The most worrisome result is that this 322 2 AA deletion has also an impact on ceftazidime/avibactam susceptibility. Indeed, while 323 KPC-2 and **KPC-3-producing** Ε. coli isolates remain susceptible to 324 ceftazidime/avibactam, KPC-14 and even more so KPC-28-producing E. coli isolates are resistant. Several mutations have been identified to yield ceftazidime/avibactam 325 326 resistance with KPC enzymes. The most common in vivo mutation described is D179Y especially when associated with the H279Y mutation that yields in increased 327 ceftazidime hydrolysis.^{14,37} Other mutations, with minor phenotypic expressions have 328 329 also been identified, such as S130G, T243A or T243M.³⁸ In KPC-28, T243 has been 330 deleted and may thus play also an additional role in avibactam resistance. It is very likely that avibactam resistance in KPC-28 is the result of the increased of the catalytic 331

efficiency of the ceftazidime since no clashes were evidenced between avibactam and
the homology models of the KPC-3, KPC-14 and KPC-28 variants (Figure 2C). This
hypothesis is reinforced by the fact that the IC50 values for avaibactam of the different
KPC variants are similar identical.

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CONCLUSION

337 This study underlines that KPC-type β -lactamases are more complex and diverse than expected. As exemplified by KPC-28 and KPC-14, they are not all true 338 339 carbapenemases, a scenario well known for OXA-48-like enzymes. ³⁹ Unfortunately, molecular detection assays and immunochromatographic tests are not able to 340 341 discriminate KPC variants with carbapenem hydrolytic capacities from those lacking any carbapenemase activity. Therefore, the first-line screening of carbapenemase 342 producers in Enterobacteriaceae have to include a test able to detect a 343 344 carbapenemase activity, such as biochemical tests (e.g. Carba NP test and derivatives, β-CARBA[™] test), or MALDI-TOF-based assays (e.g. STAR[®]-Carba IVD 345 346 kit, Bruker). Finally, as KPC producing organisms cause infection with a high morbidity and mortality, avibactam was designed as a powerful inhibitor of KPC enzymes.^{40–43} 347 However, several studies report now resistance to ceftazidime/avibactam of KPC-348 349 producing isolates as a consequence of selection of point mutant derivatives.⁴³ KPC-350 28-producing bacterial isolates, are resistant to ceftazidime/avibactam as a 351 consequence of increased ceftazidime hydrolysis, rather than intrinsic avibactam 352 resistance. Finally, as KPC-28 is lacking carbapenemase activity, has increased 353 ceftazidime hydrolytic activity, and is strongly inhibited by clavulanate, KPC-28-354 producing bacterial isolates may be identified as ESBL producers.

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365	LD is co-inventor of the Carba NP Test, which patent has been licenced to bioMérieux (La
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- 512
- 513

		MIC (mg/L	.)			
	E. coli TOP10	E. coli TOP10	E. coli TOP10	E. coli TOP10		
Antimicrobial agent	рТОРО КРС-2	ptTOPO KPC-3	pTOPO KPC-14	pTOPO KPC-28	E.COILTOP10	
Amoxicillin	> 256	> 256	> 256	> 256	6	
Amoxicillin + CLA ^a	24	48	6	6	6	
Temocillin	12	16	16	16	6	
Ceftriaxone	12	48	6	4	0.032	
Cefotaxime	8	> 32	6	4	0.064	
Ceftazidime	4	> 256	> 256	> 256	0.125	
Ceftazidime + Avibactam ^b	0.38	0.75	24	12	0.125	
Cefixime	3	12	12	8	0.38	
Cefepime	2	6	4	4	0.0064	
Aztreonam	24	> 256	32	24	0.047	
Imipenem	8	8	0.25	0.25	0.25	
Meropenem	3	3	0.032	0.032	0.032	
Ertapenem	1	1	0.006	0.008	0.004	

Table 1. MIC profile of *E. coli* TOP10 expressing KPC-2, KPC-3, KPC-14 and KPC-28 β-lactamases determined by E-tests.

^a CLA, clavulanic acid at a fixed concentration of 4 mg/L, ^b Avibactam at a fixed concentration of 4 mg/L

	K <i>m</i> (μM)					k _{cat}			k _{cat} /Km			
Substrate	KPC-2	KPC-3	KPC-14	KPC-28	KPC-2	KPC-3	KPC-14	KPC-28	KPC-2	KPC-3	KPC-14	KPC-28
Piperacillin	137	255	6	11	59	38	0.8	0.95	434	150	127	86
Cefoxitine	>1000	>1000	NH	NH	>12	>4	ND	ND	2	1.6	ND	ND
Cefotaxime	>1000	335	77	75	>163	403	4	5	95	1202	52	67
Ceftazidime	>1000	656	41	125	>16	9	1	4	0.6	14	24	32
Cefepime	>1000	491	34	30	>48	14	1.7	2.5	13	29	50	83
Imipenem	198	235	5	18	47	31	0.006	0.006	237	131	1.2	0.34
Meropenem	45	18	7	NH	3	2	0.003	ND	67	103	0.4	ND
Ertapenem	30	25	5	9	4	3	0.002	0.004	133	114	0.47	0.42

Table 2. Steady state kinetic parameters for hydrolysis of β-lactam substrates by KPC-2 and KPC-2-like β-lactamases

ND, not determined; NH, no detectable hydrolysis.

Data are the mean of three independent experiments. Standard deviations were within 10% of the mean value.

				Bio	chemical test	S	Mole	ecular tests	Immunoenzymatic tests		
	Carba NP test	RAPIDEC [®] CARBA NP	CIM Test	Modified Hodge Test	β CARBA™	MBT STAR- Carba IVD Kit	Standard <i>bla_{KPC}</i> PCR	Xpert Carba-R	NG-Test CARBA 5	Coris Resist-4 O.K.N.V. K-Set	
KPC-2	+	+	+	+	+	+	+	+	+	+	
KPC-3	+	+	+	+	+	+	+	+	+	+	
KPC-14	-	-	-	-	-	-	+	+	+	+	
KPC-28	-	-	-	-	-	-	+	+	+	+	

 Table 3. Diagnostic tests performed on E. coli TOP10 harbouring the vector pTOPO expressing KPC-2, KPC-3, KPC-14 and KPC-28.

+, positive test; -, negative test

LEGENDS OF THE FIGURES

Figure 1. Sequence alignments of KPC variants.

Alpha helixes are indicated by dashed lines and beta strand by continuous black lines. Cysteines involved in disulfide bond are indicated by black triangles. Key residues known to be implicated in ceftazidime/avibactam resistance are indicated in red. Conserved residues among class A ß-lactamass are indicated by boxes.

Figure 2. Models of interaction of KPC-3 variant (KPC-14 and KPC-28) with ceftazidime and imipenem.

A. Superposition of the molecular model of KPC-3 and KPC-28 with ceftazidime. KPC-3 is in light green, KPC-28 in dark green and ceftazidime in magenta.

B. Crystal structure of KPC-2 (PDB code 5UJ3, green) superposed with homology models of KPC-3 (orange), KPC-14 (cyan) and KPC-28 (magenta). The imipenem conformation obtained by covalent docking on KPC-2 is shown as grey sticks. The steric clashes between the side chains of A244 and of the residue at position 274 are highlighted in light green.

C. Crystal structure of KPC-2 in complex with avibactam (**a**, PDB code 4ZBE, green) superposed with the KPC-3 (**b**, orange), KPC-14 (**c**, cyan) and KPC-28 (**d**, magenta) homology models, showing no significant clashes between the protein and the ligand.

			α1	β1	β2
KPC-2	MSLYRRLVLLSCLSW	PLAGFSATALTNLV	AEPFAKLEODF	GGSIGVYAMDTGS	GATVSYR
KPC-3					
KPC-14					
KPC-28					
	70-73	α2	<u>مع</u>	α4	<u>م</u> 5
	V				<u>u</u> 5
KPC-2	AEERFPL CSSFK GFL	AAAVLARSQQQAGL	LDTPIRYGKNA	LVPWSPISEKYLT	IGMTVAE
KPC-3					
KPC-14					
KPC-28					
	130-132	α6		₁₆₆ α7	179
KPC-2	LSAAAVOY SDN AAAN	LLLKELGGPAGLTA	FMRSIGDTTFR	LDRWELELNSAIP	GDARDTS
KPC-3					
KPC-14					
KPC-28					
	α8	α9		α10 β3 ₂₂₄	226 240
KPC-2	SPRAVTESLOKITIC	SALAAPOROOFVDW	LKCNTTCNHRT		
KPC-3					
KPC = 14					
KPC-28					
	242-243 B4	65	274	α11	
VDC 2					
KPC-2	GTANDIAVVWPTGRA	ET VLAV I TRAPNKD	VALDEAV LAAA	ALTATEGTGANGŐ	
KPC 14			T		
KPC 29	••				
NPC-20	•••				

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