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

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SYNOPSIS (238)

Backgrounds: KPC-like carbapenemases have spread worldwide with more than thirty variants identified and differing by single or double amino-acid substitutions. 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 ($\Delta 242$ -GT-243).

Materials/Methods: The *bla*_{KPC-2}, *bla*_{KPC-3}, *bla*_{KPC-14} and *bla*_{KPC-28} genes were cloned into pTOPO vector for susceptibility testing or into pET41b for over-expression, purification and subsequent kinetic parameters (K_m , k_{cat}) determination. Molecular docking experiments were performed to explore the role of the amino-acid changes in the carbapenemase activity.

Results: Susceptibility testing revealed that *E. coli* producing KPC-28 displayed MICs 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 $\Delta 242$ -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 mandatory with assays based solely on *bla*_{KPC} gene or gene product detection.

INTRODUCTION

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

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

MATERIAL AND METHODS

Bacterial strains.

The clinical strain *Escherichia coli* W12 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-gami^{TM} DE3

(Novagen-Merck, Fontenay-sous-Bois, France) was used for overexpression experiments.

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).

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

obtained by PCR amplification using primers NdeI-KPC-2₃₀₋₂₉₃ (5'-CATATGGCGGAACCATTCGCTAAC-3') and KPC-2_{ΔSTOP} (5'-CTCGAGCTGCCCCGTTGACGCCAAT-3') and were then inserted into plasmid pET41b (Novagen). The recombinant plasmids were transformed into *E. coli* BL21 Rosetta-gami™ DE3 (Novagen). All the recombinant plasmids were sequenced using a T7 promoter and M13 reverse primers or T7 terminator depending of the plasmid with an automated sequencer (ABI Prism 3100; Applied Biosystems). The nucleotide sequences were analysed using software available at the National Center for Biotechnology Information website (<http://www.ncbi.nlm.nih.gov>).

Detection of KPC producers and carbapenemase activity.

The detection of the KPC-variants was performed with *E. coli* TOP10 harbouring the recombinant vector pTOPO-KPC. The detection of the carbapenemase activity was 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 (bioMérieux)²⁵ and the MBT STAR®-Carba IVD Kit (Bruker Daltonics, Bremen, Germany)²⁶ according to the manufacturer's recommendations. Molecular tests were performed with the kit Xpert® Carba-R (Cepheid, Sunnyvale, USA)²⁷ and by standard in-house PCR using the primers KPC-A (5'-CTGTCTTGTCTCTCATGGCC-3') and 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 Resist-4 O.K.N.V. (CORIS BioConcept, Gembloux, Belgium).²⁹

Protein purification

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

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

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-

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

Molecular Modelling

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

RESULTS

Clinical isolate

E. coli W12 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).

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

To evaluate and compare the antimicrobial susceptibility profiles conferred by KPC-28, the *bla*_{KPC-2}, *bla*_{KPC-3}, *bla*_{KPC-14} and *bla*_{KPC-28} genes were cloned into pTOPO vector and expressed in *E. coli* TOP10 (Table 1). Although the different KPC variants exhibited similar MICs for amoxicillin, temocillin, cefixime and cefepime, KPC-28- and KPC-14-producing *E. coli* TOP10 possessed lower MICs for cefotaxime and carbapenems (being fully susceptible) but exhibited higher MIC values for ceftazidime as compared to KPC-2- and KPC-3-producers (Table 1). Of note, the highest MICs for cefotaxime and aztreonam were found with the KPC-3-producing *E. coli* TOP10 clones. The addition of clavulanic acid restored susceptibility to amoxicillin for KPC-28- and KPC-14-producing *E. coli* clones, while inhibition by avibactam seemed to be less efficient, as both KPC-28- and KPC-14-producing *E. coli* isolates were resistant to the association ceftazidime/avibactam. Thus, MIC values suggested that the Δ 242-GT-243 (in KPC-14 and KPC-28) resulted in the loss of carbapenemase activity but in increased hydrolytic activity towards ceftazidime. This increased ceftazidime hydrolytic activity was potentiated by the H274Y substitution in KPC-28, as already shown for 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 $\Delta 242$ -GT-243 that results in a 10-fold increase in affinity but also a 1000-fold decrease
245 in k_{cat} values. Similarly, KPC-14 and KPC-28 lost completely ceftazidime hydrolytic
246 activity. IC₅₀ values of clavulanic acid for KPC-2, KPC-3, KPC-14 and KPC-28 were
247 47 μ M, 16 μ M, 0.1 μ M and 0.09 μ M, 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
251 results,¹² but is potentiated by the 2 amino-acid deletion ($\Delta 242$ -GT-243). The H274Y
252 substitution does not affect carbapenem-hydrolysis, unlike the $\Delta 242$ -GT-243 deletion
253 (Table2). The activity of avibactam against the different KPC has been determined by
254 IC₅₀ measurements. The IC₅₀ values for KPC-2, KPC-3, KPC-14 and KPC-28 were
255 230 nM, 350 nM, 107 nM and 586 nM, respectively. These values are very similar,
256 which suggests that the deletion of 2 amino acids GT242-243 have no impact on the

inhibition properties of avibactam.

Molecular modelling

We performed a molecular modelling study to identify the structural determinants that could explain the experimentally-determined differences between 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-3, KPC-14 and KPC-28 by comparative modelling using MODELLER version 9.16³¹ with the KPC-2 structure (PDB code 5UJ3) as template (Figure 2A, 2B).³² The resulting models showed that the Δ 242-GT-243 deletion didn't modify the overall structure of the 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 the side chains of A244 and of the residue at position 274 (H and T for KPC-14 and KPC-28, respectively) that may expand the active site and allow a better access for the substrates. In addition, the covalent complex of KPC-2 with imipenem obtained by 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-mentioned clash may prevent this stabilizing interaction with imipenem. In these 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} , which ultimately lead to the loss of the carbapenemase activity in these mutants. Additional theoretical calculations, and especially molecular dynamics simulations, will be needed to understand these details.

Detection methods for KPC variants

Four carbapenemase detection tests based on imipenem hydrolysis were evaluated in

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®-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, which showed a loss of carbapenemase activity for KPC-14 and KPC-28. On the other hand, immunochromatographic assays RESIST-4 O.K.N.V kit (CORIS BioConcept, Gembloux, Belgium) and the NG-Test CARBA 5 (NG Biotech, Guipry, France) and molecular Xpert® Carba-R test (Cepheid, Sunnyvale, USA) and in-house PCR were positive for all four enzymes.^{27–29,35} The positive results of the immunochromatographic assay for KPC-14 and KPC-28 confirmed our molecular modelling results, suggesting that the Δ242-GT-243 did not affect the global conformation of these proteins.

DISCUSSION

KPC-producing Enterobacteriaceae are now endemic in the United State and have 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 the biochemical properties of KPC-28, which shared with KPC-3 the same substitution H274Y and possesses in addition a Δ242-GT-243 deletion previously identified in KPC-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 of the catalytic efficiency towards ceftazidime as compared with KPC-2. This substitution was also been identified in other variants such as KPC-7 (M49I; H274Y), 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

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
312 carbapenemase activity.¹³ Here, we demonstrated that the Δ 242-GT-243 deletion also
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 *E. coli* isolates remain susceptible to
324 ceftazidime/avibactam, KPC-14 and even more so KPC-28-producing *E. coli* isolates
325 are resistant. Several mutations have been identified to yield ceftazidime/avibactam
326 resistance with KPC enzymes. The most common *in vivo* mutation described is D179Y
327 especially when associated with the H279Y mutation that yields in increased
328 ceftazidime hydrolysis.^{14,37} Other mutations, with minor phenotypic expressions have
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
331 likely that avibactam resistance in KPC-28 is the result of the increased of the catalytic

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 avibactam of the different KPC variants are similar identical.

CONCLUSION

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 carbapenemases, a scenario well known for OXA-48-like enzymes.³⁹ Unfortunately, molecular detection assays and immunochromatographic tests are not able to discriminate KPC variants with carbapenem hydrolytic capacities from those lacking any carbapenemase activity. Therefore, the first-line screening of carbapenemase producers in *Enterobacteriaceae* have to include a test able to detect a 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 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} However, several studies report now resistance to ceftazidime/avibactam of KPC-producing isolates as a consequence of selection of point mutant derivatives.⁴³ KPC-28-producing bacterial isolates, are resistant to ceftazidime/avibactam as a consequence of increased ceftazidime hydrolysis, rather than intrinsic avibactam resistance. Finally, as KPC-28 is lacking carbapenemase activity, has increased ceftazidime hydrolytic activity, and is strongly inhibited by clavulanate, KPC-28-producing bacterial isolates may be identified as ESBL producers.

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TRANSPARENCY DECLARATIONS

LD is co-inventor of the Carba NP Test, which patent has been licenced to bioMérieux (La Balme les Grottes, France). All other authors: none to declare.

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

Antimicrobial agent	MIC (mg/L)				<i>E.coli</i> TOP10
	<i>E. coli</i> TOP10 pTOPO KPC-2	<i>E. coli</i> TOP10 ptTOPO KPC-3	<i>E. coli</i> TOP10 pTOPO KPC-14	<i>E. coli</i> TOP10 pTOPO KPC-28	
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

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

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

Substrate	K_m (μ M)				k_{cat}				k_{cat}/K_m			
	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

ND, not determined; NH, no detectable hydrolysis.

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

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

	Biochemical tests						Molecular tests		Immunoenzymatic tests	
	Carba NP test	RAPIDEC® CARBA NP	CIM Test	Modified Hodge Test	β CARBA™	MBT STAR-Carba IVD Kit	Standard <i>bla</i> _{KPC} 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	-	-	-	-	-	-	+	+	+	+

+, 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 β -lactamase 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.

1.

2. A.

2. B.

2. C.