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Different phenotypic expressions of KPC β -lactamases and challenges in their detection

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25 Sir,

26 Carbapenem-resistance among Enterobacteriaceae has become a major public health
27 issue owing to their rapid worldwide spread.¹ In Enterobacteriaceae this resistance is largely
28 due to the production of carbapenemases, the most prevalent throughout the world being
29 KPC-, VIM-, IMP-, NDM- and OXA-48 type variants. KPC-2, initially described in the USA in
30 2001,² went global in less than a decade likely as a result of being located on a transposon,
31 Tn4401 capable of high frequency transposition, itself inserted on self-conjugative plasmids,
32 and its frequent association with the highly successful *K. pneumoniae* clonal group 258.³
33 Today, more than 44 natural KPC variants have been described, but only a few of them have
34 been characterized in terms of β -lactam hydrolytic properties.⁴ It is crucial to understand the
35 impact of single amino-acid changes on the hydrolytic profile of KPC-variants and the ability
36 of commercially-available diagnostic tests to efficiently detect them.

37 In this work, we have compared the phenotypic expressions of 14 KPC variants either
38 natural or generated by site-directed mutagenesis and evaluated the performance of several
39 diagnostic tests to detect them. *Bla*_{KPC-2} and *bla*_{KPC-3} alleles were cloned into pTOPO vector
40 along with its natural ribosomal binding site (RBS) upstream, and electroporated into
41 electrocompetent *E. coli* TOP10 as previously described.⁵ The resulting plasmids were used to
42 construct 11 single *bla*_{KPC-2} mutants and one single *bla*_{KPC-3} mutant using the QuikChange II Site-
43 Directed Mutagenesis Kit (Agilent Technologies, Les Ulis, France) (Table 1). Thus among the
44 12 generated mutants (Figure S1A), four were natural variants of KPC-2 [KPC-5 (P104R)⁶, KPC-
45 6 (V240G)⁷, KPC-12 (L168M), and KPC-33 (D179Y)] and one of KPC-3 [KPC-31 (D179Y)]. Six
46 substitutions correspond to natural substitutions that occurred in KPC-2, but that were not
47 reported alone: V240A, G147K, A120L, D92G, W105A and W105G. Finally, the last mutant has
48 a substitution C238S, which is a conserved residue that establishes a di-sulfide bridge with the

C68. MICs were performed using the Etest (bioMérieux, Marcy-l'Etoile, France) and detection tests were performed according to manufacturers' recommendations. Phenotypic profiles and diagnostic testing results are summarized in Table 1. Increased MICs for aztreonam and cephalosporins without any modification of those for carbapenems have been observed with mutants in position 240. On the opposite substitutions at positions 168 and 92 led to low carbapenem MICs, but yet detectable carbapenem hydrolysis (as evidenced using biochemical tests, see Table 1). The mutants P104R and G147K led to increased MICs for aztreonam and cephalosporins, with slight decreased MICs for carbapenems. The mutations on residues 105 and 238 resulted in a global decrease in MICs for all β -lactams except amoxicillin, with a stronger impact observed for the C238S mutant. With all of these mutants (including those with highly increased MICs for ceftazidime, such as P104R, V240G and V240A) avibactam was able to restore ceftazidime susceptibility. Interestingly, KPC-31 and KPC-33 with a D179Y substitution, exhibit reduced MICs for all β -lactams, including aztreonam and amoxicillin, except for ceftazidime, for which increased MICs were observed. In addition, these two mutants led to MICs for the combination of ceftazidime/avibactam, that were limit to resistance according to CLSI and EUCAST. These results suggest that avibactam is not capable of inhibiting KPC-31 and KPC-33 efficiently. In order to confirm this hypothesis IC₅₀s were measured with purified proteins.⁵ The IC₅₀ values for KPC-31 (20 μ M) and KPC-33 (42 μ M) were ~100 fold higher than those of KPC-2 (230nM) and KPC-3 (350nM), further supporting that the residue D179 is important in the binding of avibactam.^{8,9,5}

Overall, all tested residues, except A120, have a direct impact on the phenotypic expression profile of KPC enzymes. Residues 168 and 179 are located on the Ω loop, which is known to play a crucial role in the substrate selectivity of the enzyme.⁹ Residues 105, 104 and 240 delimit the cavity of the active site. Accordingly, hydrolysis spectrum is influenced by the

nature of the residues at these positions. Since the two cysteines C238 and C68 (adjacent to the S70) form a disulfide bridge that stabilizes the KPC folding, it was expected to identify a dramatic impact of the C238S substitution on the catalytic activity of the enzyme, as shown for other class A carbapenemases such as SME and GES-5.^{10,11} Finally, although located at the opposite of the active site, residues 92 and 147 have also a crucial impact on KPC activity. Molecular modeling of the G147K mutant, performed with UCSF Chimera software,¹² shows a clash with G143 located on the helix α_6 , which is in vicinity of the active site (Figure S1B). This clash might lead to a switch of this helix that subsequently impact on the shape of the active site.

Several diagnostic tests based on imipenem hydrolysis (biochemical and MALDI-TOF-based), Lateral flow immunoassays and molecular techniques (PCR) were evaluated on these different mutants. Unlike molecular tests that were able to detect all mutants, biochemical tests based on imipenem hydrolysis: Carba NP test¹³, Rapidec CARBA NP¹³ (bioMérieux, Marcy l'Etoile, France) and β -CARBA¹⁴ (BioRad, Marnes la Coquette, France), and MALDI-TOF -based MBT STAR-Carba IVD¹⁵ (Bruker, Illkirch-Graffenstaden, France) were able to distinguish between KPC variants with carbapenem hydrolytic capacities from those lacking any carbapenemase activity (Table 1). Molecular assays, being an in-house KPC specific PCR or a commercially-available PCR (Xpert Carba, Cepheid, Maurens-Scopont, France) detected accurately all variants. NG-test Carba5 (NG Biotech, Guipry, France) and Resist-4 O.K.N.V. K-Set (Coris, Gembloux – Belgium) lateral flow immunoassays (LFIA)¹⁶ assays were also able to detect all KPC variants except KPC-31 and KPC-33.⁴ As the two latter variants lack carbapenemase activity, the non-detection does not lead to false negative results in terms of true carbapenemase detection. As these 2 variants lead to avibactam resistance, it would be nevertheless important that they are detected, but not classified as carbapenemases.

Whether, carbapenems might be used to treat infections with KPC-31 or KPC-33-producers, is still debatable, but according to EUCAST guidelines the answer is Yes, as the MICs for imipenem are in the susceptibility range.

Overall, our results demonstrate that KPC variants are not equal, probably selected by different antibiotic usages. This is the case for KPC-5 that leads to high level cefixime resistance. Some KPC variants cannot be considered as carbapenemases anymore. This is the case for KPC-31 and KPC-33 that have lost their carbapenemase activity in a similar manner to KPC-14 and KPC-28 variants.⁵ These peculiar variants are challenging for their detection, and will require complementary approaches combining molecular or LFIA together with carbapenem hydrolysis detection systems.

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Transparency declarations

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

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Table 1. MIC profiles of *E. coli* TOP10 expressing single KPC mutants and performances of various diagnostic tests.

AA changes	Variant	MICs (mg/L)											Carbapenem hydrolysis assays				PCR		LFIA ^a	
		AMX	ATM	CRO	CAZ	CAZ / AVI	CFX	CTX	FEP	IMP	MEM	ERT	Carba	RAPIDEC [®]	β-	MBT STAR-	In house	Xpert	NG-Test	Coris Resist-4
													NP test	CARBA NP	CARBA™	Carba IVD	<i>bla</i> _{KPC} PCR	Carba-R	CARBA 5	O.K.N.V. K-Set
	KPC-2	>256	32	16	4	0.38	4	8	2	8	3	1	+	+	+	+	+	+	+	+
H272Y	KPC-3	>256	>256	48	>256	0.75	12	>32	6	8	3	1	+	+	+	+	+	+	+	+
P104R	KPC-5	>256	>256	>256	>256	0.5	>256	8	2	1	0.25	0.19	+	+	+	+	+	+	+	+
V240G	KPC-6	>256	>256	48	32	0.75	16	>32	4	4	3	1	+	+	+	+	+	+	+	+
L168M	KPC-12	>256	12	6	4	0.38	3	4	1	0.75	0.25	0.125	+	+	+	+	+	+	+	+
D179Y	KPC-33	12	0.75	1	>256	8	6	1.5	1	0.25	0.032	0.008	-	-	-	-	+	+	-	-
H272Y, D179Y	KPC-31	6	0.75	2	>256	12	4	1.5	1	0.25	0.032	0.008	-	-	-	-	+	+	-	-
D92G		>256	64	12	4	0.25	4	4	2	1.5	0.5	0.38	+	+	+	+	+	+	+	+
W105A		>256	32	1	1	0.25	3	1.5	0.75	0.75	0.125	0.094	+	+	+	+	+	+	+	+
W105G		>256	24	1	1	0.25	2	0.75	0.25	1	0.19	0.125	+	+	+	+	+	+	+	+
A120L		>256	32	16	3	0.38	3	6	2	6	1.5	1.5	+	+	+	+	+	+	+	+
G147K		>256	64	32	6	0.38	6	24	6	6	1	0.75	+	+	+	+	+	+	+	+
C238S		>256	2	0.25	2	0.38	1	0.25	0.19	0.25	0.047	0.008	-	-	-	-	+	+	+	+
V240A		>256	>256	64	12	0.38	12	>32	4	8	2	1.5	+	+	+	+	+	+	+	+
<i>E. coli</i> TOP10		6	0.047	0.032	0.012	0.012	0.38	0.064	0.064	0.25	0.032	0.004	-	-	-	-	-	-	-	-

AMX: Amoxicillin, ATM: Aztreonam, CRO: Ceftriaxone, CAZ: Ceftazidime, AVI: Avibactam, CFX : Cefixime, CTX : Cefotaxime, FEP : Cefepime, IMP : Imipenem, MEM : Meropenem and ETP : Ertapenem
Dark grey, light grey and white boxes represent MICs in the resistance, intermediate and susceptible range, respectively. Breakpoints were those of the European Committee on Antimicrobial Susceptibility Testing
Breakpoint tables for interpretation of MICs and zone diameters Version 9.0, valid from 2019-01-01 (http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_9.0_Breakpoint_Tables.pdf; last accessed September 2019). Grey boxes represent negative test results.
^a LFIA: Lateral flow immunoassay

Supplementary data

A

B

Figure S1. **A** Positions of the substituted residues included in this study on the Crystal structure of KPC-2 (PDB code 5UJ3) complex with cefotaxime (green), **B.** Representation of the steric clashes (red lines) between the side chains of K147 and G143.