KPC-50 Confers Resistance to Ceftazidime-Avibactam Associated with Reduced Carbapenemase Activity

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ABSTRACT KPC-50 is a KPC-3 variant identified from a *Klebsiella pneumoniae* clinical isolate recovered in Switzerland in 2019. Compared to KPC-3, KPC-50 shows (i) a three-amino-acid insertion (Glu-Ala-Val) between amino acids 276 and 277, (ii) an increased affinity to ceftazidime, (iii) a decreased sensitivity to avibactam, explaining the ceftazidime-avibactam resistance, and (iv) an association with a sharp reduction of its carbapenemase activity.

KEYWORDS KPC, ceftazidime-avibactam, *Klebsiella pneumoniae*

The occurrence of multidrug-resistant *Enterobacterales*, especially carbapenemase-producing isolates, is increasingly reported, leaving very few therapeutic options for treating related infections (1). Interestingly, the recently marketed ceftazidime-avibactam (CZA) drug combination offers novel perspectives (2). This β-lactam/β-lactamase inhibitor combination provides a therapeutic alternative for treating infections caused by KPC-like and OXA-48-like producers, whereas producers of carbapenemases of the metallo-β-lactamase type remain resistant to that combination (1, 2). Despite CZA being rarely prescribed worldwide, KPC-like-producing isolates resistant to this drug combination have already been reported (3–7). Several reports identified KPC variants exhibiting single-amino-acid substitutions in their omega-loop (amino acid positions 164 to 179), particularly the Asp179Tyr substitution, leading to enhanced affinity toward ceftazidime with a concomitant reduced binding to avibactam (AVI) (8–12). In addition, we recently identified KPC-41, possessing a three-amino-acid insertion in the KPC-3 protein sequence and being distantly located from the omega loop (namely, between positions 269 and 270), that conferred high levels of resistance to CZA in a clinical *Klebsiella pneumoniae* isolate recovered in Switzerland (13).

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K. pneumoniae isolate N869 was recovered from a patient repatriated from Greece to Switzerland after a traffic accident. In the Greek hospital, the patient developed ventilator-associated pneumonia, for which he received a treatment made of clindamycin, linezolid, and meropenem for 2 days, to which colistin was added on day 5. A few days later, he was transferred to Switzerland, where all antibiotics but meropenem (as monotherapy) were discontinued. Rectal swabs performed at admission grew *K. pneumoniae* isolate N859 using the Chrom ID Carba Smart selective plate (bioMérieux, La Balme-les-Grottes, France). According to the EUCAST 2020 breakpoints (14), *K. pneumoniae* isolate N859 was resistant to all β-lactams, including imipenem and

TABLE 1 MICs of β-lactams for *K. pneumoniae* clinical isolate N859, *E. coli* TOP10 recombinant strains producing KPC-50 and KPC-3, and *E. coli* TOP10 recipient strain

β-lactam(s)[a]	MIC (μg/ml)			
	K. pneumoniae N859	*E. coli* TOP10 (pTOPO-KPC-50)	*E. coli* (pTOPO-KPC-3)	*E. coli* TOP10
Amoxicillin	>1,024	>256	>128	8
Amoxicillin-clavulanic acid	>1,024	64	>128	8
Ticarcillin	>1,024	>256	>128	8
Ticarcillin-clavulanic acid	>1,024	64	>128	8
Piperacillin	>1,024	256	>128	2
Piperacillin-tazobactam	>1,024	128	>128	2
Cefalotin	>1,024	512	>128	16
Cefotaxime	8	8	>128	<0.125
Cefepime	8	8	128	<0.125
Ceftazidime	2,048	1,024	2,048	0.25
Ceftazidime-avibactam	>256	64	2	0.25
Ceftolozane-tazobactam	>256	64	>256	0.06
Cefoxitin	32	8	128	8
Aztreonam	32	16	64	<0.125
Ertapenem	1	0.25	32	<0.125
Imipenem	16	4	16	<0.125
Imipenem-relebactam	2	0.5	0.5	<0.125
Meropenem	2	0.5	16	<0.125
Meropenem-vaborbactam	<0.125	<0.125	0.5	<0.125

[a]Clavulanic acid was added at a fixed concentration of 2 μg/ml, tazobactam at 4 μg/ml, avibactam at 4 μg/ml, relebactam at 4 μg/ml, and vaborbactam at 8 μg/ml.

ertapenem, but remained susceptible to meropenem (Table 1). The carbapenemase activity was evaluated by using the Rapid Carba NP test, which gave a positive result (15).

K. pneumoniae N859 was also resistant to aminoglycosides (kanamycin, tobramycin, and netilmicin), to fluoroquinolones, and to colistin (MIC at 128 μg/ml). It remained susceptible to tetracycline, tigecycline, chloramphenicol, trimethoprim-sulfamethoxazole, and fosfomycin and was of intermediate susceptibility to amikacin and gentamicin. It also showed resistance to CZA (MIC of >256 μg/ml) and to a ceftolozane-tazobactam combination (>256 μg/ml), using inhibitor concentrations of 4 μg/ml.

PCR identified a bla_{KPC}-like gene, and sequencing of the corresponding amplicon identified a gene encoding a KPC variant possessing a three-amino-acid insertion (Glu-Ala-Val) between amino acids 276 and 277 (Ambler numbering), leading to a novel variant named KPC-50 (see Fig. S1 in the supplemental material). A search for additional β-lactamase resistance genes, as reported previously (16), identified a bla_{SHV}-like gene (intrinsic to *K. pneumoniae*) but no additional extended-spectrum β-lactamase gene. Mating-out assays performed using *K. pneumoniae* N859 as the donor and azide-resistant *Escherichia coli* J53 strain as the recipient (13) were successful and confirmed the plasmid location of the bla_{KPC-50} gene, being ca. 60 kb in size (data not shown). No other antibiotic marker was cotransferred along with the bla_{KPC-50} gene. PCR-based replicon typing showed that this plasmid belonged to the IncFIB incompatibility group (17). Multilocus sequence typing, performed as described previously (18), showed that isolate N859 belonged to sequence type ST258, which corresponds to the worldwide spread of the KPC-producing *K. pneumoniae* background (19, 20).

To confirm whether the amino acid substitutions identified within the KPC sequence was responsible for the CZA resistance phenotype observed in *K. pneumoniae* N859, the bla_{KPC-50} gene was cloned and expressed in *E. coli* TOP10. MIC values then were compared with those of the previously obtained KPC-3-producing *E. coli* TOP10 (13). In such an *E. coli* background, KPC-3 conferred resistance to all β-lactams, including ceftazidime, but remained susceptible to CZA, as previously shown (13). Conversely, although KPC-50 also conferred high-level resistance to ceftazidime, it additionally conferred high-level resistance to CZA (Table 1). It is worth noting that KPC-50 conferred much lower levels of resistance to cefoxitin, cefotaxime, and cefepime than KPC-3. One of the most marked features of KPC-50 compared to KPC-3 was that its

TABLE 2 Kinetic parameters of purified β-lactamases KPC-50 and KPC-3[a]

	Kinetic parameter									
	KPC-50			KPC-3			IC_{50} (μM)		K_i (μM)	
β-Lactam or inhibitor	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($mM^{-1}\cdot s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($mM^{-1}\cdot s^{-1}$)	KPC-50	KPC-3	KPC-50	KPC-3
β-Lactam										
Benzylpenicillin	<0.01	ND	ND	5.6	33	0.2				
Cefalotin	2.2	30	0.07	47	113.5	0.4				
Cefotaxime	1.7	55	0.003	34.9	532.8	0.065				
Ceftazidime	<0.01	ND	ND	>3.3	>700	>4.7E−3				
Aztreonam	<0.01	ND	ND	5	194.8	0.03				
Imipenem	2	85	0.02	4.7	71.5	0.07				
Meropenem	<0.01	ND	ND	0.47	18.5	0.03				
Ertapenem	<0.01	ND	ND	0.58	37	0.02				
Inhibitor										
Clavulanic acid							10	20	4	20
Tazobactam							10	50	1	10
Avibactam							4	1	2	1

[a]Data for KPC-3 correspond to those previously reported (13). The IC_{50} and kinetic inhibition parameters of β-lactamase inhibitors against KPC-50 and KPC-3 are shown. ND, not determinable due to a low initial rate of hydrolysis. k_{cat}, turnover; K_m, Michaelis constant (affinity); k_{cat}/K_m, specificity constant (hydrolysis). IC_{50} represents the concentration of a drug that is required for 50% inhibition of the enzymatic activity. K_i corresponds to the k_{off}/k_{on} relative to that of the inhibitor for the enzyme.

production did not lead to resistance to carbapenems (Table 1). Indeed, *E. coli* expressing the bla_{KPC-50} gene remained susceptible to ertapenem and meropenem (MICs of 0.25 μg/ml and 0.5 μg/ml, respectively), while the MIC of imipenem observed for the KPC-50-producing *E. coli* recombinant strain was 4 μg/ml (breakpoint value). Low MICs were observed when testing the new carbapenem–β-lactamase inhibitor combinations, such as imipenem-relebactam, and, more specifically, meropenem-vaborbactam showed an excellent capacity to inhibit the growth of KPC-50 producers (Table 1).

The enzymatic properties of KPC-50 were determined using purified extracts and compared to those of KPC-3 previously obtained under the same conditions (13). Kinetic data showed that KPC-50 has a lower hydrolysis activity of cefalotin, cefotaxime, aztreonam, and imipenem than those of KPC-3 (Table 2). Similar decreased hydrolytic properties toward β-lactams have been reported previously for those KPC variants conferring resistance to the CZA combination, such as KPC-41 (13) or the Asp179Tyr KPC-2 mutants (21–23). Furthermore, the activity of KPC-50 toward aztreonam was not detectable, in contrast to KPC-3 (Table 2) and also contrasting with data obtained for KPC-41 (13).

Kinetic activities toward ceftazidime were measured and compared for KPC-50 and KPC-3 enzymes. As expected, a significant hydrolysis rate was detected with KPC-3, but no hydrolysis could be detected with KPC-50 under normal conditions (measurement made during 5 min). Another assay was performed for 1 h, showing that ceftazidime was indeed hydrolyzed by KPC-50, but the hydrolysis rate was much lower than that of KPC-3 (Fig. 1). Therefore, we observed a paradoxical situation here, with KPC-50 conferring high-level resistance to ceftazidime once produced by a recombinant *E. coli* clone but a weak hydrolysis rate as measured by UV spectrophotometry. Thus, affinities of KPC-50 and KPC-3 compared to those of the ceftazidime substrate were measured using various concentrations of ceftazidime to inhibit the hydrolysis of a reporter substrate (nitrocefin), as published previously (10, 13). At the same ceftazidime concentrations, a higher inhibition level of nitrocefin was observed with KPC-50 than with KPC-3 (Fig. 1), showing that KPC-50 exhibited a higher affinity toward ceftazidime than KPC-3.

Comparative inhibitory activities of clavulanic acid, tazobactam, and AVI were determined for KPC-50 and KPC-3, showing a 4-fold lower inhibitory activity of AVI toward KPC-50 than KPC-3; conversely, those of tazobactam and clavulanic acid were higher toward KPC-50 than KPC-3 (Table 2).

Overall, these results indicated that the 276-Glu-Ala-Val-277 insertion observed in

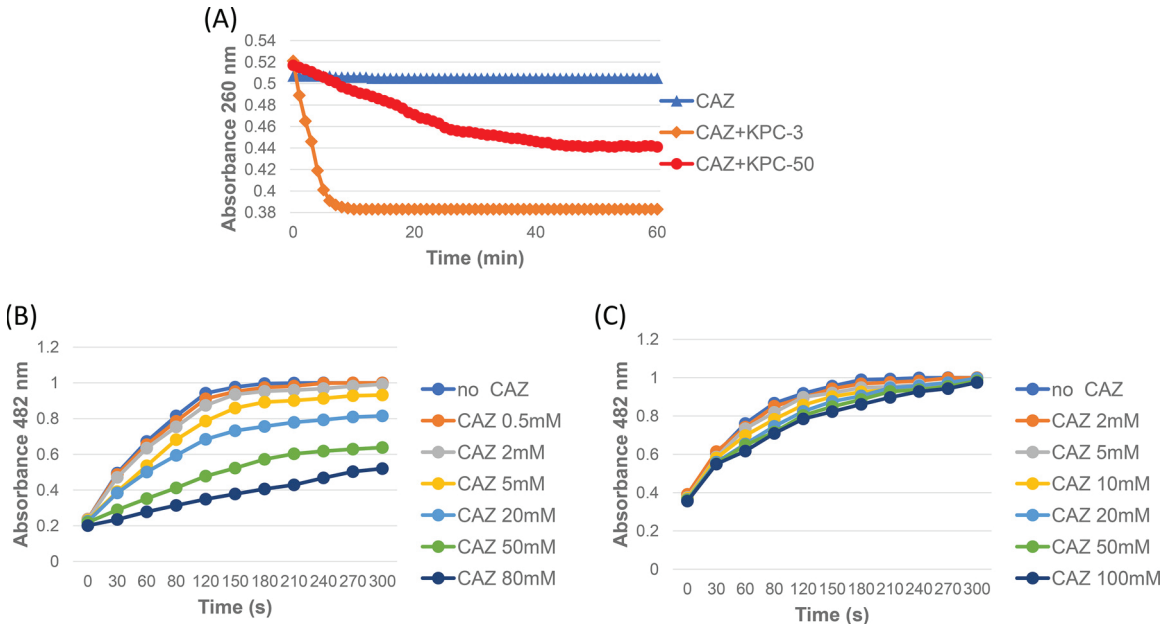

FIG 1 Analysis of ceftazidime hydrolysis. (A) KPC-50 and KPC-3 (1 μM enzyme) hydrolysis of 25 μM ceftazidime (CAZ) at room temperature. (B and C) Competitive inhibition curves determined with 50 μM nitrocefin and increasing concentrations of CAZ with 0.1 μM KPC-50 (B) and 0.1 μM KPC-3 (C) at room temperature. Nitrocefin absorbance was measured.

the KPC-50 sequence was responsible for the reduced hydrolysis of cefalotin, cefotaxime, and carbapenems, associated with a higher affinity toward ceftazidime and a reduced sensitivity to AVI.

Conclusions. A novel KPC-type enzyme conferring resistance to CZA was identified here from a multidrug-resistant *K. pneumoniae* isolate recovered in Switzerland but likely acquired in Greece, with no known history of treatment with CZA for the patient. Of note, and as already highlighted for KPC-41 and other KPC mutants conferring resistance to CZA, the overall decreased carbapenemase activity observed for KPC-50 might be considered good news. Furthermore, the newly developed carbapenem–β-lactamase inhibitor combinations (meropenem-vaborbactam and imipenem-relebactam) also showed an excellent efficacy against the KPC-50 producers (either the *K. pneumoniae* clinical isolate or the *E. coli* recombinant strain).

Data availability. The sequence of KPC-50 has been deposited in the NCBI database under GenBank accession number MN654342.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.04 MB.

ACKNOWLEDGMENTS

This work was financed by the University of Fribourg, Switzerland, the NARA, and the Swiss National Science Foundation (projects FNS-31003A_163432 and FNS-407240_177381).

L.P. and P.N. designed the study. S.M. provided the material. U.B.-G. and S.T. provided the clinical data. X.V., M.J., and A.M. performed the experiments. L.P. and P.N. wrote the manuscript.

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