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ESBLs and resistance to ceftazidime/avibactam and ceftolozane/tazobactam combinations in *Escherichia coli* and *Pseudomonas aeruginosa*

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Objectives: To evaluate the efficacy of the recently launched β-lactam/β-lactamase inhibitor combinations ceftazidime/avibactam and ceftolozane/tazobactam against ESBL-producing *Escherichia coli* and *Pseudomonas aeruginosa* strains.

Methods: A series of ESBL-encoding genes (bla_{TEM}, bla_{SHV}, bla_{CTX-M}, bla_{VEB}, bla_{PER}, bla_{GES} and bla_{BEL}) was cloned and expressed in *E. coli* or *P. aeruginosa* recipient strains. Cultures of *E. coli* TOP10 harbouring recombinant plasmids and therefore producing the different ESBLs tested were grown in order to perform measurements of catalytic activities, using benzylpenicillin, ceftazidime and ceftolozane as substrates. IC_{50}s were additionally determined for clavulanic acid, tazobactam and avibactam.

Results: We showed here an overall better activity of ceftazidime/avibactam compared with ceftolozane/tazobactam toward ESBL-producing *E. coli* and *P. aeruginosa*. Several ESBLs of the GES, PER and BEL types conferred resistance to ceftolozane/tazobactam in *E. coli* and *P. aeruginosa*. For GES-6 and PER-1 producers, resistance to ceftolozane/tazobactam could be explained by a high hydrolysis of ceftolozane and a low activity of tazobactam as an inhibitor. On the other hand, PER-producing *P. aeruginosa* also exhibited resistance to ceftazidime/avibactam.

Conclusions: Altogether, the results show that the ESBL PER-1, which is widespread worldwide, may be a source of resistance to both ceftolozane/tazobactam and ceftazidime/avibactam. Excellent activity of ceftazidime/avibactam was highlighted for both ESBL-producing *E. coli* and ESBL-producing *P. aeruginosa*.

Introduction

MDR including resistance to broad-spectrum cephalosporins and ultimately to carbapenems is nowadays commonly observed worldwide in Enterobacteriaceae and in *Pseudomonas aeruginosa*.[1] Resistance to broad-spectrum cephalosporins may occur by different mechanisms, including permeability defects and efflux overproduction. However, production of broad-spectrum β-lactamases is the most significant mechanism leading to such resistance.[2] Hence, resistance to cephalosporins may occur through overproduction of AmpC β-lactamases, for which corresponding genes may be intrinsic in some species (such as *P. aeruginosa*, *Enterobacter cloacae* and *Serratia marcescens*) or acquired in *Klebsiella pneumoniae* or *Escherichia coli*, or by the acquisition of ESBLs.

Recently, two novel drug combinations have been launched, namely ceftazidime/avibactam and ceftolozane/tazobactam.[3] Avibactam is a non-β-lactam β-lactamase inhibitor with activity against Ambler class A ESBLs and class C AmpCs, thus potentiating the activity of ceftazidime.[4] Interestingly, it is active against KPC-type enzymes, which are weakly inhibited by clavulanic acid and tazobactam.[4] However, some KPC variants have been identified, such as KPC-31 or KPC-35, that were shown to confer resistance to ceftazidime/avibactam in clinical *K. pneumoniae* isolates.[5,6]

Ceftolozane is a novel cephalosporin derivative of ceftazidime with intrinsic broad activity, which is furthermore not hydrolysed by most broad-spectrum β-lactamases, namely ESBLs and AmpCs.[7] The ceftolozane/tazobactam association is particularly active against MDR (including carbapenem-resistant) *P. aeruginosa*.[8]

Although the most commonly identified ESBLs encountered in *E. coli* are CTX-M-type enzymes, along with TEM and SHV derivatives, those mainly encountered in *P. aeruginosa* are SHV-, VEB-,

GES- and PER-like enzymes, and BEL-, TEM- and CTX-M-like enzymes have been rarely identified.[9] Furthermore, the carbapenem-hydrolysing KPC enzymes possess an ESBL hydrolysis spectrum, but additionally hydrolyse carbapenems. They are increasingly identified in Enterobacteriaceae (mainly *K. pneumoniae*, but also *E. coli*), but remain rarely identified in *P. aeruginosa*.[10] One specific group of β-lactamases quite frequently identified in *P. aeruginosa* corresponds to GES enzymes, with GES-1 being an ESBL-sparing carbapenem,[11] whereas variants GES-2 and GES-5 possess significant carbapenemase activity.[12–14] GES-6, also possessing significant carbapenemase activity and originally identified in a *K. pneumoniae* isolate from Greece,[15] has recently been identified in *P. aeruginosa*.[16] We recently showed that GES-6 production is the source of acquired resistance to ceftolozane/tazobactam while not affecting the susceptibility to ceftazidime/avibactam.[17]

The objective of our study was therefore to evaluate the relative impact of different ESBLs on susceptibility to ceftolozane/tazobactam and to ceftazidime/avibactam, either in *E. coli* or in *P. aeruginosa* backgrounds. Those two bacterial species were retained since *E. coli* represents a common community-acquired pathogen and *P. aeruginosa* a common hospital-acquired pathogen.

Materials and methods

Bacterial isolates

A series of ESBL-encoding genes was cloned and expressed in *E. coli* or *P. aeruginosa* recipient strains. Those ESBLs were derivatives of bla_{TEM}, bla_{SHV}, bla_{CTX-M}, bla_{VEB}, bla_{PER}, bla_{GES} and bla_{BEL}. Donor strains are listed in Table 1. In addition, the two carbapenemase genes bla_{KPC-2} and bla_{VIM-2} were added. *P. aeruginosa* PAO1 and *E. coli* TOP10 were used as recipient strains.[17]

Cloning experiments

Cloning of all ESBL-encoding genes was performed in the shuttle and broad-host range pUCp24 using PCR amplicons encompassing the entire coding sequence of all respective genes.[18] Primers used for PCR amplification of the ESBL genes are listed in Table S1 (available as Supplementary data at *JAC* Online). Electroporation of the recombinant plasmids and expression of the respective ESBL genes were performed in the *E. coli* TOP10 background.[17] Subsequently, electroporation of those same recombinant plasmids was performed in the *P. aeruginosa* PAO1 background. Selection was made on plates containing 100 mg/L ticarcillin and 30 mg/L gentamicin for both *E. coli* and *P. aeruginosa* transformants.

Susceptibility testing

MIC values were determined in triplicate by Etest (AB bioMérieux; Solna, Sweden) or broth microdilution (for ceftolozane alone) as previously described.[17] Interpretation was based on EUCAST breakpoints, ceftazidime/avibactam resistance being defined as >8 mg/L for Enterobacteriaceae and *P. aeruginosa*, and ceftolozane/tazobactam resistance as >1 mg/L for Enterobacteriaceae and >4 mg/L for *P. aeruginosa*.[19] In order to further evaluate the respective contributions of the β-lactam agent (ceftazidime or ceftolozane) and that of the β-lactamase inhibitor (avibactam or tazobactam, respectively), MICs were determined not only at the usual ratio concentrations (inhibitor concentrations at 4 mg/L), but also at higher concentrations (8 and 16 mg/L, respectively).

β-Lactamase activities

Cultures of *E. coli* TOP10 harbouring recombinant plasmids and therefore producing the different ESBLs tested were grown overnight at 37°C in 1 L of brain heart infusion medium with amoxicillin (100 mg/L). The bacterial suspension was pelleted, resuspended in 10 mL of 100 mM phosphate buffer (pH 7), disrupted by sonification (20 min for 30 s of sonication and 50 s of rest at 20 kHz) with a Vibra Cell 75186 (Thermo Fisher) and centrifuged for 1 h at 11000 **g** and 4°C. β-Lactamase crude extracts were used for specific activity measurements performed at 30°C in 100 mM sodium phosphate (pH 7.0). The initial rates of hydrolysis were determined with a GENESYS 10S UV-Vis Spectrophotometer (Thermo Scientific). The following wavelengths/absorption coefficients were used: benzylpenicillin, 232 nm/−1100 $M^{-1}cm^{-1}$; ceftazidime, 260 nm/−8660 $M^{-1}cm^{-1}$; and ceftolozane, 254 nm/−6810 $M^{-1}cm^{-1}$. IC_{50}s were determined for clavulanic acid, tazobactam and avibactam. Various concentrations of these inhibitors were pre-incubated with the crude extract of the enzyme for 5 min at 30°C to determine the concentrations that reduced the hydrolysis rate of 100 μM nitrocefin by 50%. Results are expressed in micromolar units. The total protein content was measured using a Bradford assay.

Results

Among the 11 ESBL-producing *P. aeruginosa* clinical isolates, 10 were resistant to ceftolozane/tazobactam whereas most remained susceptible to ceftazidime/avibactam (Table 1). The ESBL-producing *E. coli* (CTX-M-15) was susceptible to both combinations. Notably, the KPC-2-producing *P. aeruginosa* isolate was susceptible to ceftazidime/avibactam, but resistant to ceftolozane/tazobactam.

Expression of the ESBL genes in *E. coli* TOP10 gave variable MIC values of ceftazidime/avibactam that remained in the susceptibility range (Table 2). However, the MIC of ceftazidime/avibactam for the bla_{PER-1}-positive recombinant strain was 8 mg/L, which actually corresponds to the breakpoint value. This latter result was probably the consequence of a very high MIC of ceftazidime conferred by PER-1 activity, which was confirmed by the lowest MIC observed when combining ceftazidime with a higher concentration of avibactam (Table 2). MICs of ceftolozane/tazobactam for those recombinant *E. coli* strains were also variable, but, notably, several values remained in the resistance range, including those for the GES-1 and GES-6 producers, as well as for the PER-1, BEL-1 and BEL-2 producers (Table 2). Notably, some high MIC values observed could be clearly explained by a combination of two features, namely a high hydrolysis rate toward ceftolozane and a lower inhibition by tazobactam, as for GES-6 and PER-1 (Table 3). Increasing the concentration of tazobactam significantly decreased the MIC of ceftazidime for the GES-1 and GES-5 producers, but not for the GES-6 and PER-1 producers (Table 2). The two carbapenemase-producing *E. coli* recombinant strains (KPC-2 and VIM-2) were resistant to ceftolozane/tazobactam, regardless of the tazobactam concentration used (Table 2).

Expression of the ESBL genes in *P. aeruginosa* PAO1 did not confer resistance to ceftazidime/avibactam except for the PER-1 producer (Table 4). This latter recombinant strain was the one with the highest MIC of ceftazidime, correlating with the poor activity of avibactam as an inhibitor toward PER-1 compared with other ESBLs (Table 3), this feature being confirmed by the still high MICs of ceftazidime observed even with higher concentrations of avibactam (Table 2). MICs of ceftolozane/tazobactam for recombinant *P. aeruginosa* strains varied considerably, with an overall high resistance rate observed for the GES-6, CTX-M-2, PER-1, BEL-1, BEL-2 and SHV-2a producers (Table 4). Of note was the discrepancy observed between the GES-1 and GES-2 producers on the one

Table 1. MICs for clinical isolates

Strain	MIC (mg/L)											
	IPM	MEM	CAZ	C/A[a]	C/A[b]	C/A[c]	COZ	C/T[d]	C/T[e]	C/T[f]	PIP	TZP[d]
P. aeruginosa (GES-1) R1189	1	2	16	2	2	2	64	**32**	**32**	**16**	128	16
P. aeruginosa (GES-2) R184	16	32	64	4	2	2	32	**16**	**8**	4	256	128
P. aeruginosa (GES-5) R186	32	128	64	**16**	8	8	16	**8**	**8**	**8**	256	128
P. aeruginosa (GES-6) R3451	16	64	32	2	2	2	32	**32**	**32**	**32**	512	64
P. aeruginosa (CTX-M-2) R1188	1	8	32	8	4	4	16	4	1	1	256	32
E. coli (CTX-M-15) R1818	0.12	<0.06	128	0.5	0.03	0.03	256	1	1	0.5	>512	32
P. aeruginosa (PER-1) R1192	0.5	1	>512	**64**	**16**	**16**	512	**512**	**128**	**64**	256	64
P. aeruginosa (BEL-1) R1185	1	2	32	4	2	2	16	**8**	**8**	**8**	128	64
P. aeruginosa (BEL-2) R1187	0.5	2	128	8	4	2	64	**32**	**32**	**32**	128	128
P. aeruginosa (VEB-1) R1205	8	16	256	8	4	4	64	**64**	**32**	**32**	128	32
P. aeruginosa (TEM-4) R1217	1	0.5	8	2	1	1	4	0.5	0.5	0.5	32	4
P. aeruginosa (SHV-2a) R136	1	2	32	4	4	2	8	4	4	2	512	256
P. aeruginosa (KPC-2) R96	>128	>128	256	8	4	2	64	**64**	**32**	**32**	>512	512
P. aeruginosa (VIM-2) R166	128	64	64	**64**	**64**	**64**	256	**256**	**256**	**256**	256	32

IPM, imipenem; MEM, meropenem; CAZ, ceftazidime; C/A, ceftazidime/avibactam; COZ, ceftolozane; C/T, ceftolozane/tazobactam; PIP, piperacillin; TZP, piperacillin/tazobactam.
MIC values indicated in bold for C/A and C/T are those corresponding to a categorization of resistance.
[a]Avibactam at 4 mg/L.
[b]Avibactam at 8 mg/L.
[c]Avibactam at 16 mg/L.
[d]Tazobactam at 4 mg/L.
[e]Tazobactam at 8 mg/L.
[f]Tazobactam at 16 mg/L.

hand and the GES-5 and GES-6 producers on the other hand. This result is in accordance with recent results that we have obtained showing that a serine residue at position 170 in either β-lactamase GES-5 or GES-6 (while GES-1 and GES-2, respectively, possess glycine and asparagine residues at that position) is responsible for a decreased sensitivity to tazobactam. Increasing the tazobactam concentration did not significantly modify the MICs of ceftolozane, except for the GES-1 recombinant strain (Table 2). As expected, the two carbapenemase-producing *P. aeruginosa* recombinant strains (KPC-2 and VIM-2) were also resistant to ceftolozane/tazobactam.

Overall, two of the ESBL-producing *P. aeruginosa* recombinant strains were resistant to both ceftazidime/avibactam and ceftolozane/tazobactam combinations, namely those producing CTX-M-2 and PER-1.

Discussion

We showed here that ESBL acquisition may significantly counteract the activity of recently launched cephalosporin/β-lactamase inhibitor combinations. Our data showed that ceftazidime/avibactam seems to be a very effective option, not only against ESBL-producing *E. coli* as reported in other studies, but also against ESBL-producing *P. aeruginosa*. In a recent study performed by Livermore *et al.*,[20] the prevalence of ESBL-producing (unknown determinant) Enterobacteriaceae susceptible to ceftazidime/avibactam was 99.7%, among which only 3.8% initially remained susceptible to ceftazidime, thus showing an extremely high efficacy of the inhibitor. Looking at the susceptibility to ceftolozane/tazobactam among ESBL-producing *E. coli* recovered from healthcare infections in Latin America, Pfaller *et al.*[21] found a rate of 9.2% of resistance, whereas it was very high (61.8%) in ESBL-producing *K. pneumoniae.* Here we showed that production of ESBLs in *E. coli* does not impact the susceptibility to ceftolozane/tazobactam, which is mainly due to the excellent activity of ceftolozane as an antibiotic.

In that same study from Pfaller *et al.*,[21] the rate of resistance to ceftolozane/tazobactam among ceftazidime-resistant *P. aeruginosa* was found to be 34.8%, for which the mechanism of resistance to ceftazidime was not determined (probably corresponding to a majority of AmpC overproducers). In a recent surveillance programme among isolates from US hospitals and assessing the susceptibility of Enterobacteriaceae to ceftolozane/tazobactam, a rate of 87.5% of susceptibility was found among non-carbapenemase- and ESBL-producing isolates. Here we showed that production of an ESBL in *P. aeruginosa* may significantly affect the efficacy of ceftolozane/tazobactam, although susceptibility to ceftazidime/avibactam was preserved.

Of note, the very interesting activity of avibactam as a β-lactamase inhibitor was evidenced here with ceftazidime as substrate, but may also be extremely valuable by enhancing the β-lactam activity of various β-lactams. By increasing the concentrations of the respective inhibitors in the ceftazidime/avibactam and ceftolozane/tazobactam combinations, we observed some significant drops in terms of MIC values for only some recombinant strains, namely both the GES-1- and GES-5-producing *E. coli* and

Table 2. MICs for *E. coli* recombinant isolates

	MIC (mg/L)											
Strain	IPM	MEM	CAZ	C/A[a]	C/A[b]	C/A[c]	COZ	C/T[d]	C/T[e]	C/T[f]	PIP	TZP[d]
E. coli TOP10	0.03	0.03	0.125	0.25	0.25	0.12	0.5	0.125	0.125	0.125	2	0.5
E. coli TOP10 + GES-1	0.12	0.03	8	0.25	0.25	0.12	32	**8**	0.5	0.25	16	1
E. coli TOP10 + GES-2	0.25	0.03	4	0.25	0.25	0.12	2	0.25	0.25	0.25	8	2
E. coli TOP10 + GES-5	1	0.5	8	0.5	0.25	0.12	8	**8**	0.25	0.25	32	16
E. coli TOP10 + GES-6	0.25	0.12	64	1	1	1	128	**64**	**32**	**16**	256	32
E. coli TOP10 + CTX-M-2	0.25	0.03	8	0.5	0.25	0.12	2	0.5	0.5	0.25	16	2
E. coli TOP10 + CTX-M-15	0.25	0.03	4	0.25	0.25	0.12	4	0.25	0.25	0.25	4	2
E. coli TOP10 + PER-1	0.5	0.03	512	8	0.25	0.25	512	**128**	**128**	**64**	8	4
E. coli TOP10 + BEL-1	0.25	0.03	16	0.5	0.25	0.12	8	4	2	1	8	8
E. coli TOP10 + BEL-2	0.25	0.03	32	0.5	0.25	0.12	16	**8**	2	2	16	2
E. coli TOP10 + VEB-1	0.25	0.03	16	0.5	0.25	0.12	0.5	0.5	0.25	0.25	128	16
E. coli TOP10 + TEM-4	0.25	0.03	2	0.25	0.25	0.12	0.5	0.25	0.25	0.25	8	2
E. coli TOP10 + SHV-2a	0.25	0.03	8	0.25	0.25	0.12	4	1	0.25	0.25	16	4
E. coli TOP10 + KPC-2	8	2	16	0.5	0.25	0.12	8	**8**	**8**	**8**	64	32
E. coli TOP10 + VIM-2	8	4	64	**32**	**16**	**16**	512	**128**	**128**	**128**	32	32

IPM, imipenem; MEM, meropenem; CAZ, ceftazidime; C/A, ceftazidime/avibactam; COZ, ceftolozane; C/T, ceftolozane/tazobactam; PIP, piperacillin; TZP, piperacillin/tazobactam.
MIC values indicated in bold for C/A and C/T are those corresponding to a categorization of resistance.
[a]Avibactam at 4 mg/L.
[b]Avibactam at 8 mg/L.
[c]Avibactam at 16 mg/L.
[d]Tazobactam at 4 mg/L.
[e]Tazobactam at 8 mg/L.
[f]Tazobactam at 16 mg/L.

Table 3. Specific β-lactamase activities and IC_{50}s for β-lactamase inhibitors toward ESBLs

	Specific activity (μmol min^{-1} mg^{-1})			IC_{50} (μM)		
Enzyme	benzylpenicillin	ceftazidime	ceftolozane	avibactam	tazobactam	clavulanic acid
GES-1	2.8	0.4	0.4	1.9	0.7	3.5
GES-2	2.4	0.3	0.2	0.8	0.3	1.5
GES-6	3.2	0.3	0.7	3.7	7.3	78
GES-5	1.9	0.3	0.1	3.2	9	80
CTX-M-2	12	0.2	0.06	0.1	0.1	0.06
CTX-M-15	2	0.3	0.2	0.5	0.3	0.7
PER-1	7	0.6	2.2	10.4	4.2	4.7
BEL-1	10	0.3	0.1	0.1	2.6	0.4
BEL-2	2.5	0.4	0.3	0.35	0.4	0.4
VEB-1	17	0.2	0.07	0.01	0.05	0.3
TEM-4	5	0.2	0.09	0.5	0.01	0.1
SHV-2a	15	0.3	0.2	0.01	0.04	0.05
KPC-2	12	0.3	0.3	3.9	50	130
VIM-2	6	0.4	1	>500	>500	>500

P. aeruginosa strains, respectively, while they remained almost unchanged for all the others, including the GES-6 producers. This highlights further that high MICs of the two drug combinations conferred by GES-1 and GES-5 might be counteracted by increased concentrations of the inhibitor. However, pharmacokinetic/pharmacodynamic data measured for those drug combinations show that the respective β-lactam/β-lactamase inhibitor ratio remains stable in urine.[22,23]

Table 4. MICs for *P. aeruginosa* recombinant isolates

Strain	MIC (mg/L)										
	IPM	MEM	CAZ	C/A[a]	C/A[b]	C/A[c]	COZ	C/T[d]	C/T[e]	C/T[f]	PIP	TZP[d]
PAO1	0.5	0.5	1	1	1	1	2	0.5	0.5	0.5	4	1
PAO1+GES-1	1	0.5	64	1	1	1	32	**16**	**8**	2	64	4
PAO1+GES-2	1	1	16	2	2	2	8	2	0.5	0.5	16	8
PAO1+GES-5	4	16	32	2	2	2	8	**16**	2	2	32	16
PAO1+GES-6	4	8	64	1	1	1	64	**32**	**32**	**32**	128	32
PAO1+CTX-M-2	1	0.5	128	8	4	2	32	**8**	**8**	**8**	512	256
PAO1+CTX-M-15	1	0.5	8	2	2	2	4	1	1	0.5	16	8/4
PAO1+PER-1	1	1	>512	**64**	**32**	**16**	>512	>**512**	**256**	**128**	128	64
PAO1+BEL-1	1	0.5	16	2	2	2	16	**8**	**8**	**8**	128	64
PAO1+BEL-2	1	0.5	128	4	4	2	64	**16**	**16**	**16**	64	32
PAO1+VEB-1	1	0.5	2	1	1	1	0.5	0.5	0.25	0.25	128	16
PAO1+TEM-4	1	1	4	2	1	1	4	0.5	0.25	0.25	16	4
PAO1+SHV-2a	1	1	64	4	2	2	16	**8**	**8**	**8**	256	128
PAO1+KPC-2	32	128	256	4	2	2	64	**64**	**64**	**64**	256	256
PAO1+VIM-2	32	32	32	**32**	**32**	**32**	512	**256**	**256**	**256**	64	64

IPM, imipenem; MEM, meropenem; CAZ, ceftazidime; C/A, ceftazidime/avibactam; COZ, ceftolozane; C/T, ceftolozane/tazobactam; PIP, piperacillin; TZP, piperacillin/tazobactam.
MIC values indicated in bold for C/A and C/T are those corresponding to a categorization of resistance.
[a]Avibactam at 4 mg/L.
[b]Avibactam at 8 mg/L.
[c]Avibactam at 16 mg/L.
[d]Tazobactam at 4 mg/L.
[e]Tazobactam at 8 mg/L.
[f]Tazobactam at 16 mg/L.

Further work is now needed to evaluate the respective activities of ceftazidime/avibactam and ceftolozane/tazobactam toward a large collection of ESBL-producing *E. coli* and *P. aeruginosa* clinical isolates.

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Transparency declarations

None to declare.

Supplementary data

Table S1 is available as Supplementary data at *JAC* Online.

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