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1 In vitro activity of ceftolozane-tazobactam against Enterobacter cloacae complex clinical

- 2 isolates with different β-lactam resistance phenotypes
- 3
- 4 <u>Running title:</u> Activity of TOL-TAZ against ECC clinical isolates
- 5
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- 23
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28 Abstract

The study evaluated the *in vitro* activity of ceftolozane-tazobactam (C/T) against 94 unique clinical isolates of *Enterobacter cloacae* complex (ECC). No difference was observed according to the ECC cluster. The *in vitro* activity greatly varied depending on the β lactamase-producing profile: 100%, 67% and 19% of wild-type, ESBL-producing, and AmpCoverproducing strains were susceptible to C/T, respectively. The use of C/T could be of interest for the treatment of some infections caused by ESBL-producing AmpC-nonoverexpressing ECC isolates. 36 The species belonging to the Enterobacter genus are responsible for 5-10% of infections among patients hospitalized in intensive care units (ICUs) and primarily due to the members 37 of the Enterobacter cloacae complex (ECC) (1,2). Actually, ECC is composed of 13 clusters 38 among which three (C-III, VI and VIII) are the most frequently recovered from human clinical 39 40 specimens (3,4). All ECC members intrinsically harbour a chromosomal *ampC* gene coding for a cephalosporinase (2,5-7). Among these third generation cephalosporin (TGC)-resistant 41 isolates, approximately one third has acquired plasmid-mediated extended-spectrum β -42 43 lactamases (ESBLs) while the remaining two thirds express a high-level production of cephalosporinase (HL-CASE) caused by ampC derepression that results from chromosomal 44 mutations (6). 45

Ceftolozane-tazobactam (C/T) is a novel TGC combined with a classical inhibitor of β -46 lactamase (ratio of 2:1), which has recently been approved for the treatment of complicated 47 intra-abdominal and urinary tract infections (8). Although ceftolozane has been developed to 48 be more stable than other TGCs against natural AmpC produced by P. aeruginosa (9), much 49 50 less is known about its activity against other intrinsically AmpC-producing species, such as ECC. Indeed, previous studies have mainly described the in vitro activity of C/T against 51 Enterobacter spp. with no distinction of species and/or phenotypes of resistance (10-13). In 52 addition, no data is available about the in vitro activity of C/T according to the ECC cluster. 53 The purpose of the study was then to 1) evaluate the *in vitro* activity of C/T against a 54 55 collection of ECC clinical isolates representing relevant clusters and exhibiting various

phenotypes of β-lactam susceptibility profiles; and 2) compare it to those of commonly-used
 β-lactams.

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60	total of 93 ECC clinical isolates (representing 12 clusters) collected from university hospital of
61	Caen were included in the study (3). Note that the strains were identified by MALDI-TOF
62	mass spectrometry (Microflex LT; Bruker Daltonics, Bremen, Germany) and ECC members
63	were clustered by hsp60 sequencing as previously described (7). MICs of C/T (C provided by
64	Cubist Pharmaceuticals and T purchased from Abcam Biochemicals), piperacillin-tazobactam
65	(TZP), cefotaxime (CTX), ceftriaxone (CRO), ceftazidime (CAZ), cefepime (FEP), ertapenem
66	(ETP) and imipenem (IMP) were determined by the broth microdilution reference method in
67	accordance with EUCAST guidelines (<u>http://www.eucast.org/</u>). ECC isolates were classified
68	into four β -lactam susceptibility phenotyes: wild-type [WT] (no resistance to TGCs), ESBL
69	(resistance to at least one TGC with a positive double-disk synergy test), HL-CASE (resistance
70	to at least one TGC with a negative double-disk synergy test and a significant difference in
71	TGC-mediated inhibition with or without cloxacillin 250 mg/L), and ESBL+HL-CASE (resistance
72	to at least one TGC with a positive double-disk synergy test and a significant difference in
73	TGC-mediated inhibition with or without cloxacillin 250 mg/L). To confirm the HL-CASE
74	phenotype (especially in isolates producing ESBLs), we quantified the levels of expression of
75	the chromosomal <i>ampC</i> gene by RT-qPCR using specific primers (Table S1). Total RNAs were
76	extracted as previously described (7). Transcript levels were determined by the DeltaDelta Ct
77	method using the <i>rpoB</i> gene as housekeeping control gene (Table S1), and the fold change
78	(FC) of expression was calculated between TGC-resistant strains and WT strains of the same
79	cluster. HL-CASE was defined if the FC was higher than 2. ESBLs were characterized as
80	previously described (14-16).
Q 1	

Besides the reference strain of E. cloacae subsp. cloacae ATCC 13047 (belonging to C-XI), a

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Twelve of the 13 clusters were represented in the study (**Table S2**). Among them, C-III (21%, 20/94), C-VI (20%, 19/94) and C-VIII (28%, 26/94) were predominant, as previously described (**Table S2**) (4). Note that none of the studied clusters expressing a WT phenotype exhibited an intrinsic resistance to the C/T in spite of the genetic variability of the *ampC* gene (7).

Among the 94 isolates, four antimicrobial susceptibility phenotypes were distinguished: WT 86 34% (32/94), ESBL alone 10% (9/94), ESBL+HL-CASE 20% (19/94) and HL-CASE 36% (34/94) 87 (Tables 1 and S2). By using the disk method with or without cloxacillin (250 mg/L), the HL-88 89 CASE phenotype was not highlighted in 21% of isolates (4/19) presenting an ESBL+HL-CASE 90 combined phenotype. By contrast, the expression of *ampC* allowed to accurately discriminate between all ESBL and ESBL+HL-CASE phenotypes (P < 0.0001) (Figure 1). Among 91 the 28 isolates expressing an ESBL phenotype (ESBL alone and ESBL+HL-CASE), four genes 92 encoding such β -lactamases were identified: $bla_{CTX-M-15}$ (17/28, 61%), bla_{SHV-12} (9/28, 32%), 93 bla_{CTX-M-9} (2/28, 7%) and bla_{TEM-15} (1/28, 4%). Note that one isolate co-produced bla_{CTX-M-15} 94 and bla_{SHV-12} genes (Table S3). The distribution of ESBLs was similar to that recently 95 96 described in French E. cloacae isolates (CTX-M-15, 52%; SHV-12, 38%; CTX-M-9, 10%) (17). Besides ESBL production, plasmid-mediated AmpC β-lactamase genes were also identified in 97 two isolates (bla_{CMY-4} and bla_{DHA-1}) and one strain harboured the acquired OXA-48-like 98 carbapenemase OXA-204 (Table S3). 99

For the 32 isolates with a WT phenotype, all were categorized as susceptible for all tested β lactams except one strain that was not susceptible to CAZ (MIC = 2 mg/L) according to EUCAST breakpoints (**Table 1**). MICs of C/T ranged from 0.12 to 0.5 mg/L with MIC₅₀ and MIC₉₀ at 0.25 and 0.5 mg/L, respectively (**Table 1**). These MIC values were identical to MIC₅₀ (0.25 mg/L) and MIC₉₀ (0.5 mg/L) published for ceftazidime-susceptible *Enterobacter* strains (12,18).

106 For the nine isolates expressing an ESBL phenotype, all were resistant to TGCs (CTX, CRO and CAZ) while TZP and FEP retained an activity against 22% and 44% of strains, respectively 107 (Table 1). Six isolates (67%) were categorized as susceptible to C/T, with MICs comprised 108 109 between 0.25 and 4 mg/L (**Table 1**). MIC_{50} and MIC_{90} were at 1 and 2 mg/L, which is similar to 110 values (2 and 4 mg/L, respectively) reported in a previous study on 15 ESBL-producing Enterobacter strains (19). Also, a recent study reports a proportion at 85% (40/47) of 111 Enterobacter isolates susceptible to C/T (20). This is in accordance with the fact that 112 113 tazobactam inhibits most of class A β -lactamases (including ESBLs) and that C/T remains 114 active against >80% of ESBL-producing *Escherichia coli* clinical isolates (11-13,18).

All the 53 isolates showing a HL-CASE phenotype, including 19 that co-produced an ESBL, 115 were categorized as resistant to TGCs (CTX, CRO and CAZ) and only 19% were susceptible to 116 117 C/T (Table 1). The percentages of susceptible strains were comparable between ESBL+HL-118 CASE and HL-CASE isolates for TZP (0 vs 3%), ETP (53 vs 47%) and IMP (95 vs 100%) but 119 different for FEP (11 vs 35%) (Table 1). MIC₅₀ and MIC₉₀ of C/T were higher for ECC isolates with an ESBL+HL-CASE phenotype (8 and 128 mg/L, respectively) than those for HL-CASE 120 strains (4 and 16 mg/L, respectively) (Table 1). Consequently, eight isolates (24%) were 121 122 categorized as susceptible to C/T among HL-CASE isolates whereas only two (11%) remained susceptible to the combination in the group of ESBL+HL-CASE strains (Table 1). As compared 123 to ESBL producers, this poorer activity of C/T against HL-CASE ECC isolates is due to the fact 124 125 that tazobactam is not effective against AmpC β -lactamases (8). In this subgroup (HL-CASE ECC), the percentage of strains inhibited by $\leq 1 \text{ mg/L}$ (corresponding to the EUCAST 126 breakpoint) of C/T varied between 14 and 36% (11-13,18), which is similar to our results. 127 Surprisingly, for the two studies where resistance mechanisms were specified (12,20), 50 to 128 75% of HL-CASE strains remained susceptible to C/T, which is much higher that proportions 129

- reported here. Interestingly, 30% (28/94) of ECC isolates were not susceptible to ETP
 (including one not susceptible to IMP) of which only two were susceptible to C/T (MIC = 1
 mg/L), suggesting that C/T is likely not a good option for the treatment of caused by non-CPE
 strains showing reduced carbapenem susceptibility.
- 135 In summary, there is no difference in β -lactamase-producing profile to C/T according to the
- 136 ECC cluster. By contrast, the in vitro activity of C/T greatly varies depending of the β -lactam
- 137 susceptibility profile.
- 138
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223 Legend of the figure

224	Figure 1. Fold change of expression of the <i>ampC</i> chromosomal gene according to the
225	resistant phenotype: production of an extended-spectrum β -lactamase (ESBL), AmpC
226	overproduction (HL-CASE), ESBL+HL-CASE. The fold change (expressed as Log_{10} values) was
227	calculated between resistant strains and wild-type strains of the same cluster. HL-CASE was
228	defined if the fold change was higher than 2.

Table 1. M	IICs of	different	β-lactams	against	a	collection	of 9	94 strains	(93	clinical	isolates	and
ATCC13047) of Ent	terobacter	<i>cloacae</i> co	mplex (I	ECC) accordin	g to	resistance	phe	notypes	;	

ECC clinical isolates (no.)		MIC (m	g/L)	EUCAST susceptibility	% of susceptible strains	
	MIC ₅₀	MIC ₅₀ MIC ₉₀ Range		breakpoint (mg/L)	•	
All ECC (94)						
Ceftolozane-tazobactam	1	16	0.12-128	≤1	51	
Imipenem	0.25	0.5	0.12-4	≤2	99	
Ertapenem	0.25	2	0.01-32	≤0.5	70	
Cefepime	0.5	16	0.03->256	<1	54	
Ceftazidime	64	256	0.25->256	<1	33	
Cefotaxime	64	>256	0.25->256	 <1	34	
Ceftriaxone	128	>256	0.25->256	<u>_</u> 1	34	
Piperacillin-tazobactam	64	256	2-256	<8	37	
Wild-type ECC (32)						
Ceftolozane-tazobactam	0.25	0.5	0.12-0.5	≤1	100	
Imipenem	0.25	0.5	0.12-0.5	<2	100	
Ertapenem	0.06	0.12	0.01-0.25	<0.5	100	
Cefepime	0.03	0.06	0.03-0.06	<1	100	
Ceftazidime	0.5	1	0.25-2	<1	97	
Cefotaxime	0.5	1	0.25-1	 <1	100	
Ceftriaxone	0.5	1	0.25-1	<u></u>	100	
Piperacillin-tazobactam	2	4	2-8	< <u>1</u>	100	
FSBL alone (9)				20		
Ceftolozane-tazobactam	1	2	0 25-4	<1	67	
Iminenem	0.25	0.5	0.12-0.5	 < 2	100	
Ertanenem	0.125	0.5	0.03-1	<u></u>	89	
Cefenime	4	256	0.06-64	≤0.5	44	
Ceftazidime	64	128	32-128	<u> </u>	0	
Cefotaxime	256	>256	4->256		0	
Ceftriaxone	256	>256	2->256	12	Ĵ	
Piperacillin-tazobactam	64	128	8-128	<u>≤1</u>	22	
FSBI +HI -CASE (19)				20		
Ceftolozane-tazobactam	8	128	1-128	<1	11	
Iminenem	05	1	0 25-4	<2	95	
Frtanenem	0.5	8	0.12-32	<u></u>	53	
Cefenime	4	256	0.12->256	<u>≤</u> 0.5	11	
Ceftazidime	128	256	32->256	<u></u>	0	
Cefotaxime	256	>256	64->256	51	Û	
Ceftriaxone	256	>256	128->256	12	0	
Piperacillin-tazobactam	128	256	32->256	≤1 <0	0	
HI-CASE (34)				20		
Ceftolozane-tazobactam	4	16	0.25-32	<1	24	
Imipenem	0.25	0.5	0.12-1	<2	100	
Frtapenem	1	2	0.03-4	<0.5	47	
Cefepime	2	8	0.12-16	0.5 <1	35	
Ceftazidime	128	256	2->256	<u></u>	0	
Cefotaxime	256	>256	16->256	<u>>1</u>	ů 0	
Ceftriaxone	256	>256	32->256	<u><u></u></u>	0	
Piperacillin-tazobactam	128	256	8-256	<u>≤1</u>	3	
			00	≤8	5	

ESBL, Extended-spectrum β -lactamase; HL-CASE, High-level production of cephalosporinase.



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