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**In vitro activity of ceftolozane-tazobactam against  
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1 **In vitro activity of ceftolozane-tazobactam against *Enterobacter cloacae* complex clinical**  
2 **isolates with different  $\beta$ -lactam resistance phenotypes**

3  
4 Running title: Activity of TOL-TAZ against ECC clinical isolates

5  
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23

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26 Supplemental materials.

27

28 **Abstract**

29 The study evaluated the *in vitro* activity of ceftolozane-tazobactam (C/T) against 94 unique  
30 clinical isolates of *Enterobacter cloacae* complex (ECC). No difference was observed  
31 according to the ECC cluster. The *in vitro* activity greatly varied depending on the  $\beta$ -  
32 lactamase-producing profile: 100%, 67% and 19% of wild-type, ESBL-producing, and AmpC-  
33 overproducing strains were susceptible to C/T, respectively. The use of C/T could be of  
34 interest for the treatment of some infections caused by ESBL-producing AmpC-non-  
35 overexpressing ECC isolates.

36 The species belonging to the *Enterobacter* genus are responsible for 5-10% of infections  
37 among patients hospitalized in intensive care units (ICUs) and primarily due to the members  
38 of the *Enterobacter cloacae* complex (ECC) (1,2). Actually, ECC is composed of 13 clusters  
39 among which three (C-III, VI and VIII) are the most frequently recovered from human clinical  
40 specimens (3,4). All ECC members intrinsically harbour a chromosomal *ampC* gene coding for  
41 a cephalosporinase (2,5-7). Among these third generation cephalosporin (TGC)-resistant  
42 isolates, approximately one third has acquired plasmid-mediated extended-spectrum  $\beta$ -  
43 lactamases (ESBLs) while the remaining two thirds express a high-level production of  
44 cephalosporinase (HL-CASE) caused by *ampC* derepression that results from chromosomal  
45 mutations (6).

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

54 The purpose of the study was then to 1) evaluate the *in vitro* activity of C/T against a  
55 collection of ECC clinical isolates representing relevant clusters and exhibiting various  
56 phenotypes of  $\beta$ -lactam susceptibility profiles; and 2) compare it to those of commonly-used  
57  $\beta$ -lactams.

58

59 Besides the reference strain of *E. cloacae* subsp. *cloacae* ATCC 13047 (belonging to C-XI), a  
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 (<http://www.eucast.org/>). ECC isolates were classified  
68 into four  $\beta$ -lactam susceptibility phenotypes: 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 *ampC* 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 *rpoB* 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).

81

82 Twelve of the 13 clusters were represented in the study (**Table S2**). Among them, C-III (21%,  
83 20/94), C-VI (20%, 19/94) and C-VIII (28%, 26/94) were predominant, as previously described  
84 (**Table S2**) (4). Note that none of the studied clusters expressing a WT phenotype exhibited  
85 an intrinsic resistance to the C/T in spite of the genetic variability of the *ampC* gene (7).

86 Among the 94 isolates, four antimicrobial susceptibility phenotypes were distinguished: WT  
87 34% (32/94), ESBL alone 10% (9/94), ESBL+HL-CASE 20% (19/94) and HL-CASE 36% (34/94)  
88 (**Tables 1 and S2**). By using the disk method with or without cloxacillin (250 mg/L), the HL-  
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  
91 discriminate between all ESBL and ESBL+HL-CASE phenotypes ( $P < 0.0001$ ) (**Figure 1**). Among  
92 the 28 isolates expressing an ESBL phenotype (ESBL alone and ESBL+HL-CASE), four genes  
93 encoding such  $\beta$ -lactamases were identified: *bla*<sub>CTX-M-15</sub> (17/28, 61%), *bla*<sub>SHV-12</sub> (9/28, 32%),  
94 *bla*<sub>CTX-M-9</sub> (2/28, 7%) and *bla*<sub>TEM-15</sub> (1/28, 4%). Note that one isolate co-produced *bla*<sub>CTX-M-15</sub>  
95 and *bla*<sub>SHV-12</sub> genes (**Table S3**). The distribution of ESBLs was similar to that recently  
96 described in French *E. cloacae* isolates (CTX-M-15, 52%; SHV-12, 38%; CTX-M-9, 10%) (17).

97 Besides ESBL production, plasmid-mediated AmpC  $\beta$ -lactamase genes were also identified in  
98 two isolates (*bla*<sub>CMY-4</sub> and *bla*<sub>DHA-1</sub>) and one strain harboured the acquired OXA-48-like  
99 carbapenemase OXA-204 (**Table S3**).

100 For the 32 isolates with a WT phenotype, all were categorized as susceptible for all tested  $\beta$ -  
101 lactams except one strain that was not susceptible to CAZ (MIC = 2 mg/L) according to  
102 EUCAST breakpoints (**Table 1**). MICs of C/T ranged from 0.12 to 0.5 mg/L with MIC<sub>50</sub> and  
103 MIC<sub>90</sub> at 0.25 and 0.5 mg/L, respectively (**Table 1**). These MIC values were identical to MIC<sub>50</sub>  
104 (0.25 mg/L) and MIC<sub>90</sub> (0.5 mg/L) published for ceftazidime-susceptible *Enterobacter* strains  
105 (12,18).



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

115 All the 53 isolates showing a HL-CASE phenotype, including 19 that co-produced an ESBL,  
116 were categorized as resistant to TGCs (CTX, CRO and CAZ) and only 19% were susceptible to  
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**). MIC<sub>50</sub> and MIC<sub>90</sub> of C/T were higher for ECC isolates  
120 with an ESBL+HL-CASE phenotype (8 and 128 mg/L, respectively) than those for HL-CASE  
121 strains (4 and 16 mg/L, respectively) (**Table 1**). Consequently, eight isolates (24%) were  
122 categorized as susceptible to C/T among HL-CASE isolates whereas only two (11%) remained  
123 susceptible to the combination in the group of ESBL+HL-CASE strains (**Table 1**). As compared  
124 to ESBL producers, this poorer activity of C/T against HL-CASE ECC isolates is due to the fact  
125 that tazobactam is not effective against AmpC  $\beta$ -lactamases (8). In this subgroup (HL-CASE  
126 ECC), the percentage of strains inhibited by  $\leq 1$  mg/L (corresponding to the EUCAST  
127 breakpoint) of C/T varied between 14 and 36% (11-13,18), which is similar to our results.  
128 Surprisingly, for the two studies where resistance mechanisms were specified (12,20), 50 to  
129 75% of HL-CASE strains remained susceptible to C/T, which is much higher than proportions

130 reported here. Interestingly, 30% (28/94) of ECC isolates were not susceptible to ETP  
131 (including one not susceptible to IMP) of which only two were susceptible to C/T (MIC = 1  
132 mg/L), suggesting that C/T is likely not a good option for the treatment of caused by non-CPE  
133 strains showing reduced carbapenem susceptibility.

134

135 In summary, there is no difference in  $\beta$ -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  $\beta$ -lactam  
137 susceptibility profile.

138

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- 221
- 222

223 **Legend of the figure**

224 **Figure 1.** Fold change of expression of the *ampC* chromosomal gene according to the  
225 resistant phenotype: production of an extended-spectrum  $\beta$ -lactamase (ESBL), AmpC  
226 overproduction (HL-CASE), ESBL+HL-CASE. The fold change (expressed as  $\text{Log}_{10}$  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.** MICs of different  $\beta$ -lactams against a collection of 94 strains (93 clinical isolates and ATCC13047) of *Enterobacter cloacae* complex (ECC) according to resistance phenotypes

ECC clinical isolates (no.)	MIC (mg/L)			EUCAST susceptibility breakpoint (mg/L)	% of susceptible strains
	MIC <sub>50</sub>	MIC <sub>90</sub>	Range		
<b>All ECC (94)</b>					
Ceftolozane-tazobactam	1	16	0.12-128	$\leq 1$	51
Imipenem	0.25	0.5	0.12-4	$\leq 2$	99
Ertapenem	0.25	2	0.01-32	$\leq 0.5$	70
Cefepime	0.5	16	0.03->256	$\leq 1$	54
Ceftazidime	64	256	0.25->256	$\leq 1$	33
Cefotaxime	64	>256	0.25->256	$\leq 1$	34
Ceftriaxone	128	>256	0.25->256	$\leq 1$	34
Piperacillin-tazobactam	64	256	2-256	$\leq 8$	37
<b>Wild-type ECC (32)</b>					
Ceftolozane-tazobactam	0.25	0.5	0.12-0.5	$\leq 1$	100
Imipenem	0.25	0.5	0.12-0.5	$\leq 2$	100
Ertapenem	0.06	0.12	0.01-0.25	$\leq 0.5$	100
Cefepime	0.03	0.06	0.03-0.06	$\leq 1$	100
Ceftazidime	0.5	1	0.25-2	$\leq 1$	97
Cefotaxime	0.5	1	0.25-1	$\leq 1$	100
Ceftriaxone	0.5	1	0.25-1	$\leq 1$	100
Piperacillin-tazobactam	2	4	2-8	$\leq 8$	100
<b>ESBL alone (9)</b>					
Ceftolozane-tazobactam	1	2	0.25-4	$\leq 1$	67
Imipenem	0.25	0.5	0.12-0.5	$\leq 2$	100
Ertapenem	0.125	0.5	0.03-1	$\leq 0.5$	89
Cefepime	4	256	0.06-64	$\leq 1$	44
Ceftazidime	64	128	32-128	$\leq 1$	0
Cefotaxime	256	>256	4->256	$\leq 1$	0
Ceftriaxone	256	>256	2->256	$\leq 1$	0
Piperacillin-tazobactam	64	128	8-128	$\leq 8$	22
<b>ESBL+HL-CASE (19)</b>					
Ceftolozane-tazobactam	8	128	1-128	$\leq 1$	11
Imipenem	0.5	1	0.25-4	$\leq 2$	95
Ertapenem	0.5	8	0.12-32	$\leq 0.5$	53
Cefepime	4	256	0.12->256	$\leq 1$	11
Ceftazidime	128	256	32->256	$\leq 1$	0
Cefotaxime	256	>256	64->256	$\leq 1$	0
Ceftriaxone	256	>256	128->256	$\leq 1$	0
Piperacillin-tazobactam	128	256	32->256	$\leq 8$	0
<b>HL-CASE (34)</b>					
Ceftolozane-tazobactam	4	16	0.25-32	$\leq 1$	24
Imipenem	0.25	0.5	0.12-1	$\leq 2$	100
Ertapenem	1	2	0.03-4	$\leq 0.5$	47
Cefepime	2	8	0.12-16	$\leq 1$	35
Ceftazidime	128	256	2->256	$\leq 1$	0
Cefotaxime	256	>256	16->256	$\leq 1$	0
Ceftriaxone	256	>256	32->256	$\leq 1$	0
Piperacillin-tazobactam	128	256	8-256	$\leq 8$	3

ESBL, Extended-spectrum  $\beta$ -lactamase; HL-CASE, High-level production of cephalosporinase.



