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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 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 β -
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 β -
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 β -
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 β -lactam susceptibility profiles; and 2) compare it to those of commonly-used
57 β -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 β -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 β -lactamases were identified: *bla*_{CTX-M-15} (17/28, 61%), *bla*_{SHV-12} (9/28, 32%),
94 *bla*_{CTX-M-9} (2/28, 7%) and *bla*_{TEM-15} (1/28, 4%). Note that one isolate co-produced *bla*_{CTX-M-15}
95 and *bla*_{SHV-12} 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 β -lactamase genes were also identified in
98 two isolates (*bla*_{CMY-4} and *bla*_{DHA-1}) 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 β -
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 MIC₅₀ and
103 MIC₉₀ at 0.25 and 0.5 mg/L, respectively (**Table 1**). These MIC values were identical to MIC₅₀
104 (0.25 mg/L) and MIC₉₀ (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**). MIC₅₀ and MIC₉₀ 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 β -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**). MIC₅₀ and MIC₉₀ 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 β -lactamases (8). In this subgroup (HL-CASE
126 ECC), the percentage of strains inhibited by ≤ 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 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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- 221
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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 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. MICs of different β -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 ₅₀	MIC ₉₀	Range		
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	≤ 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	≤ 1	100
Piperacillin-tazobactam	2	4	2-8	≤ 8	100
ESBL alone (9)					
Ceftolozane-tazobactam	1	2	0.25-4	≤ 1	67
Imipenem	0.25	0.5	0.12-0.5	≤ 2	100
Ertapenem	0.125	0.5	0.03-1	≤ 0.5	89
Cefepime	4	256	0.06-64	≤ 1	44
Ceftazidime	64	128	32-128	≤ 1	0
Cefotaxime	256	>256	4->256	≤ 1	0
Ceftriaxone	256	>256	2->256	≤ 1	0
Piperacillin-tazobactam	64	128	8-128	≤ 8	22
ESBL+HL-CASE (19)					
Ceftolozane-tazobactam	8	128	1-128	≤ 1	11
Imipenem	0.5	1	0.25-4	≤ 2	95
Ertapenem	0.5	8	0.12-32	≤ 0.5	53
Cefepime	4	256	0.12->256	≤ 1	11
Ceftazidime	128	256	32->256	≤ 1	0
Cefotaxime	256	>256	64->256	≤ 1	0
Ceftriaxone	256	>256	128->256	≤ 1	0
Piperacillin-tazobactam	128	256	32->256	≤ 8	0
HL-CASE (34)					
Ceftolozane-tazobactam	4	16	0.25-32	≤ 1	24
Imipenem	0.25	0.5	0.12-1	≤ 2	100
Ertapenem	1	2	0.03-4	≤ 0.5	47
Cefepime	2	8	0.12-16	≤ 1	35
Ceftazidime	128	256	2->256	≤ 1	0
Cefotaxime	256	>256	16->256	≤ 1	0
Ceftriaxone	256	>256	32->256	≤ 1	0
Piperacillin-tazobactam	128	256	8-256	≤ 8	3

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

