In vitro activity of ceftolozane-tazobactam against Enterobacter cloacae complex clinical isolates with different β-lactam resistance phenotypes

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

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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 AmpC-overproducing 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-non-overexpressing ECC isolates.

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 of the *Enterobacter cloacae* complex (ECC) (1,2). Actually, ECC is composed of 13 clusters among which three (C-III, VI and VIII) are the most frequently recovered from human clinical 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 isolates, approximately one third has acquired plasmid-mediated extended-spectrum β-lactamases (ESBLs) while the remaining two thirds express a high-level production of cephalosporinase (HL-CASE) caused by *ampC* derepression that results from chromosomal mutations (6).

Ceftolozane-tazobactam (C/T) is a novel TGC combined with a classical inhibitor of β-lactamase (ratio of 2:1), which has recently been approved for the treatment of complicated intra-abdominal and urinary tract infections (8). Although ceftolozane has been developed to be more stable than other TGCs against natural AmpC produced by *P. aeruginosa* (9), much 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 *Enterobacter* spp. with no distinction of species and/or phenotypes of resistance (10-13). In addition, no data is available about the *in vitro* activity of C/T according to the ECC cluster.

The purpose of the study was then to 1) evaluate the *in vitro* activity of C/T against a 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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Besides the reference strain of *E. cloacae* subsp. *cloacae* ATCC 13047 (belonging to C-XI), a total of 93 ECC clinical isolates (representing 12 clusters) collected from university hospital of Caen were included in the study (3). Note that the strains were identified by MALDI-TOF mass spectrometry (Microflex LT; Bruker Daltonics, Bremen, Germany) and ECC members were clustered by *hsp60* sequencing as previously described (7). MICs of C/T (C provided by Cubist Pharmaceuticals and T purchased from Abcam Biochemicals), piperacillin-tazobactam (TZP), cefotaxime (CTX), ceftriaxone (CRO), ceftazidime (CAZ), cefepime (FEP), ertapenem (ETP) and imipenem (IMP) were determined by the broth microdilution reference method in accordance with EUCAST guidelines (http://www.eucast.org/). ECC isolates were classified into four β-lactam susceptibility phenotyes: wild-type [WT] (no resistance to TGCs), ESBL (resistance to at least one TGC with a positive double-disk synergy test), HL-CASE (resistance to at least one TGC with a negative double-disk synergy test and a significant difference in TGC-mediated inhibition with or without cloxacillin 250 mg/L), and ESBL+HL-CASE (resistance to at least one TGC with a positive double-disk synergy test and a significant difference in TGC-mediated inhibition with or without cloxacillin 250 mg/L). To confirm the HL-CASE phenotype (especially in isolates producing ESBLs), we quantified the levels of expression of the chromosomal *ampC* gene by RT-qPCR using specific primers (**Table S1**). Total RNAs were extracted as previously described (7). Transcript levels were determined by the DeltaDelta Ct method using the *rpoB* gene as housekeeping control gene (**Table S1**), and the fold change (FC) of expression was calculated between TGC-resistant strains and WT strains of the same cluster. HL-CASE was defined if the FC was higher than 2. ESBLs were characterized as previously described (14-16).

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 34% (32/94), ESBL alone 10% (9/94), ESBL+HL-CASE 20% (19/94) and HL-CASE 36% (34/94) (**Tables 1 and S2**). By using the disk method with or without cloxacillin (250 mg/L), the HL-CASE phenotype was not highlighted in 21% of isolates (4/19) presenting an ESBL+HL-CASE 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 the 28 isolates expressing an ESBL phenotype (ESBL alone and ESBL+HL-CASE), four genes encoding such β-lactamases were identified: $bla_{\text{CTX-M-15}}$ (17/28, 61%), $bla_{\text{SHV-12}}$ (9/28, 32%), $bla_{\text{CTX-M-9}}$ (2/28, 7%) and $bla_{\text{TEM-15}}$ (1/28, 4%). Note that one isolate co-produced $bla_{\text{CTX-M-15}}$ and $bla_{\text{SHV-12}}$ genes (**Table S3**). The distribution of ESBLs was similar to that recently 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 two isolates ($bla_{\text{CMY-4}}$ and $bla_{\text{DHA-1}}$) and one strain harboured the acquired OXA-48-like carbapenemase OXA-204 (**Table S3**).

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 MIC_{50} and MIC_{90} at 0.25 and 0.5 mg/L, respectively (**Table 1**). These MIC values were identical to MIC_{50} (0.25 mg/L) and MIC_{90} (0.5 mg/L) published for ceftazidime-susceptible *Enterobacter* strains (12,18).

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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 (**Table 1**). Six isolates (67%) were categorized as susceptible to C/T, with MICs comprised 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 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 *Enterobacter* isolates susceptible to C/T (20). This is in accordance with the fact that tazobactam inhibits most of class A β-lactamases (including ESBLs) and that C/T remains 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, were categorized as resistant to TGCs (CTX, CRO and CAZ) and only 19% were susceptible to C/T (**Table 1**). The percentages of susceptible strains were comparable between ESBL+HL-CASE and HL-CASE isolates for TZP (0 *vs* 3%), ETP (53 *vs* 47%) and IMP (95 *vs* 100%) but different for FEP (11 *vs* 35%) (**Table 1**). MIC_{50} and MIC_{90} 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 strains (4 and 16 mg/L, respectively) (**Table 1**). Consequently, eight isolates (24%) were 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 to ESBL producers, this poorer activity of C/T against HL-CASE ECC isolates is due to the fact that tazobactam is not effective against AmpC β-lactamases (8). In this subgroup (HL-CASE ECC), the percentage of strains inhibited by ≤1 mg/L (corresponding to the EUCAST breakpoint) of C/T varied between 14 and 36% (11-13,18), which is similar to our results. Surprisingly, for the two studies where resistance mechanisms were specified (12,20), 50 to 75% of HL-CASE strains remained susceptible to C/T, which is much higher that proportions

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.

In summary, there is no difference in β-lactamase-producing profile to C/T according to the ECC cluster. By contrast, the in vitro activity of C/T greatly varies depending of the β-lactam susceptibility profile.

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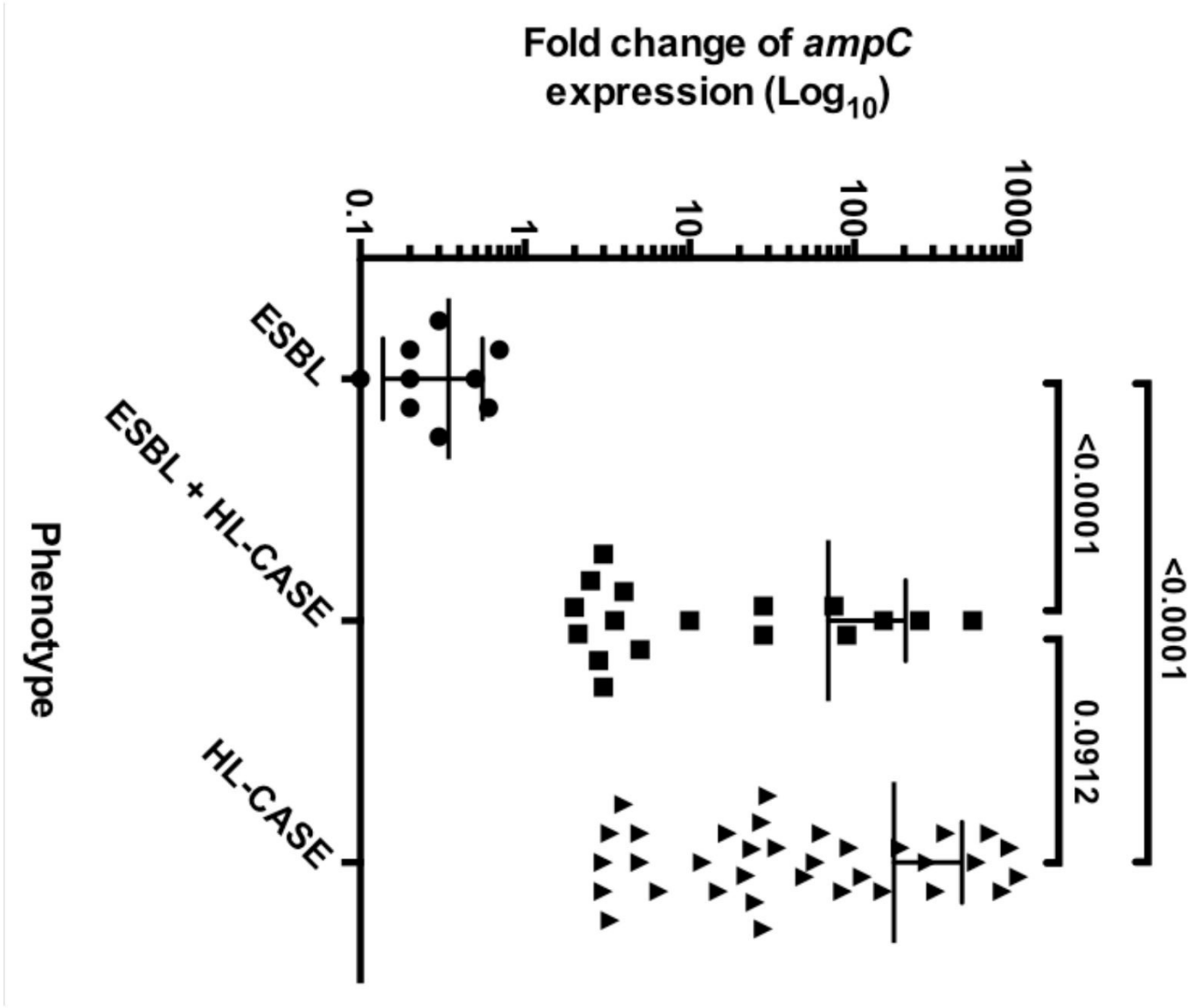
223 **Legend of the figure**

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.


Fold change of ampC expression (Log10)
0.1
1
10
100
1000
ESBL
ESBL + HL-CASE
HL-CASE
Phenotype
<0.0001
0.0912
<0.0001