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1 **Expression of CTX-M-15 limits the efficacy of ceftolozane/tazobactam against**
2 *Escherichia coli* in a high inoculum murine peritonitis model

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19 Short title: Ceftolozane/tazobactam and *Escherichia coli*

1 ABSTRACT

2 Objectives: Among carbapenem-sparing therapies, ceftolozane/tazobactam has been proposed
3 for the treatment of infections due to CTX-M-15 producing *Escherichia coli*. However, few
4 data exist on its *in vivo* activity in infections associated with a high bacterial inoculum.

5 Methods: We analyzed ceftolozane/tazobactam activity against susceptible *E. coli* CFT073-
6 RR and its CTX-M-15-producing transconjugant *E. coli* CFT073-RR Tc *bla*_{CTX-M-15}, *in vitro*
7 at low and high inoculum, and in a high-inoculum murine model of peritonitis.

8 Results: Against *E. coli* CFT073-RR Tc *bla*_{CTX-M-15}, ceftolozane/tazobactam bactericidal
9 effect was impaired *in vitro* with only a minor inoculum effect; this translated into a reduced
10 activity as compared with imipenem in the mouse peritonitis model.

11 Conclusions: Combination of ESBL expression and high inoculum size may be a clinical
12 situation at risk of reduced bactericidal activity of ceftolozane/tazobactam.

13

1 INTRODUCTION

2 Since carbapenems may select for carbapenem resistance [1], efforts have been made to
3 evaluate the activity of beta-lactam/beta-lactamase inhibitors (BLBLI), such as
4 piperacillin/tazobactam (PTZ/TZ), for the treatment of extended spectrum beta-lactamase
5 (ESBL)-producing *Escherichia coli* infections, with conflicting clinical results [2,3]. One
6 explanation for therapeutic failures observed with PTZ/TZ may be the inoculum effect, that
7 was shown to impact its efficacy [4]. Ceftolozane/tazobactam (CTZ/TZ), a novel BLBLI,
8 showed *in vitro* and *in vivo* potency against most ESBL-positive *E. coli* [5–7]. We therefore
9 analyzed the *in vitro* impact of the inoculum size on the activity of CTZ/TZ, and compared its
10 activity to that of imipenem in a high-inoculum murine model of peritonitis due to *E. coli*
11 producing or not a CTX-M-15-type ESBL.

12

13 METHODS

14 *Bacterial strains and in vitro antibiotic activities.*

15 Wild-type *E. coli* CFT073-RR, a reference clinical uropathogenic strain already sequenced,
16 and its transconjugant *E. coli* CFT073-RR Tc *bla*_{CTX-M-15}, harboring a plasmid carrying
17 *bla*_{CTX-M-15}, *bla*_{OXA-1} and *aac*(6′)-1b, obtained by conjugation of CFT073-RR with a clinical
18 isolate of community-acquired urinary tract infection [8,9], were used. Minimal inhibitory
19 concentrations (MICs) of ceftolozane (CTZ), tazobactam (TZ), CTZ/TZ (with a fixed TZ
20 concentration of 4 mg/L according to the reference method, and with a 2:1 ratio as in the
21 manufactured formulation for therapeutic use), and imipenem, were determined in triplicate
22 by microdilution at standard or high inoculum (10^5 and 10^7 CFU/mL, respectively).

1 For time-kill curves, exponentially growing bacteria were diluted in 10 mL glass tubes
2 containing Tryptic-Soy (TS) broth with CTZ at 1, 4 or 8 fold the MIC of the combination (i.e.
3 0.25, 1 and 2 mg/L) and TZ 4 mg/L, or with CTZ/TZ in a 2:1 ratio (manufactured
4 formulation). Viable counts were enumerated after 0, 1, 3, 6 and 24 hours of incubation at
5 37°C. A bactericidal effect was defined as a decrease of $\geq 3 \log_{10}$ CFU/mL between the initial
6 inoculum and the bacterial count at 24 h.

7

8 *Murine model of peritonitis.*

9 The experimental murine peritonitis model [10] was approved by the Departmental Direction
10 of Veterinary Services from Paris, France (CE121 #4949). Female ICR/Swiss mice (5-6
11 week-old, 22-30g, Janvier, St-Berthevin, France) were inoculated intraperitoneally with 0.25
12 ml NaCl containing approximately 2.5×10^7 CFU of *E. coli* and 10% autoclaved porcine mucin
13 (Sigma-Aldrich). Beginning 2h after inoculation, mice (n=15 mice for each strain) were
14 treated for 24 h with CTZ/TZ, or imipenem 100 mg/kg every 2h (imipenem regimen best
15 reproducing the pharmacokinetic/pharmacodynamics obtained with 1g TID in humans,
16 according to our previous experiments [11]). A group of 15 “start-of-treatment” controls was
17 sacrificed just before treatment, to determine the bacterial load before treatment. Treated mice
18 were sacrificed 4h after the last injection. Immediately after sacrifice, a peritoneal wash was
19 performed by intraperitoneal injection of 2 mL of sterile saline solution followed by collection
20 of 1 mL of fluid.. Spleen was removed and homogenized in 1 mL of sterile saline solution.
21 Aliquots of peritoneal fluid or spleen were then diluted and plated onto agar to enumerate
22 surviving bacteria, and onto agar containing CTZ/TZ (1 and 4 mg/L, respectively) to search
23 for the in vivo selection of resistant mutants. Results were expressed as median [range] for
24 continuous variables; \log_{10} CFU/g in spleen and \log_{10} CFU/mL in peritoneal fluids were

1 compared by the Mann–Whitney U-test or the Kruskal–Wallis test when appropriate. Non
2 parametric tests were preferred to parametric tests because they are more appropriate
3 considering the small numbers of mice per groups and the fact that the data do not follow a
4 normal distribution. A $P < 0.05$ was considered significant.

5

6 *CTZ/TZ pharmacokinetics in mice.*

7 To determine the therapeutic regimen that best reproduced the peak serum concentrations
8 obtained with the recommended dose of CTZ/TZ 1g /500mg TID in humans, groups of 4 mice
9 received different doses of CTZ/TZ subcutaneously and were sacrificed 30 or 60 minutes after
10 injection by intraperitoneal injection of 200 mL of sodium thiopental. CTZ and TZ serum
11 concentrations were measured by chromatographic analysis with a TLX-1 chromatographic
12 system with an online turbulent flow SPE TurboFlow® MCX-2 column (60 μ m, 0.5x50 mm,
13 Thermo Scientific). The detection was performed with a High Resolution Mass Spectrometric
14 (HRMS) analyzer (Exactive Plus, Thermo Scientific) equipped with a heated electrospray
15 ionization source (h-ESI-II).

16

17

18 RESULTS

19 *In vitro results.*

20 As shown in Table 1, CFT073-RR was fully susceptible to CTZ, CTZ/TZ and imipenem. *E.*
21 *coli* CFT073-RR Tc *bla*_{CTX-M-15} was highly resistant to CTZ but adjunction of TZ 4 mg/L fully
22 restored the CTZ activity. Using the 4 mg/L TZ concentration, the increase of the inoculum

1 size had no impact on CTZ/TZ activity but increased by two dilutions the MICs of imipenem
2 for both strains. Using the 2:1 CTZ/TZ ratio, MICs of CTZ/TZ against CFT073-RR and its
3 transconjugant increased by 3 dilutions (from 0.25 to 2 mg/L) with a standard inoculum, and
4 by 4 dilutions (from 0.25 to 4 mg/L) with a high inoculum, indicating that TZ concentrations
5 were insufficient to fully restore the CTZ/TZ bacteriostatic activity, with a limited additional
6 impact of the inoculum size.

7 As shown in Fig.1A, a bactericidal effect was observed for *E. coli* CFT073-RR with
8 concentrations of CTZ 1 mg/L (i.e. 4 fold the MIC) or more, and TZ 4 mg/L, at standard and
9 high inoculum. For *E. coli* CFT073-RR Tc *bla*_{CTX-M-15}, no bactericidal effect of the
10 combination was observed for concentrations up to 8 fold the MIC of CTZ (i.e. 4 mg/L) and
11 TZ 4 mg/L, indicating that bactericidal effect of CTZ/TZ was impaired by acquired ESBL
12 resistance, even with a standard inoculum of 10⁵ CFU/mL. Increase of inoculum size had only
13 a mild effect on CTZ/TZ activity. To get as close as possible to the situation observed in
14 human therapeutics, we also studied the in vitro bactericidal activity of CFZ/TZ in a 2:1 ratio
15 (manufactured formulation) (Fig. 1B). For these experiments, CFZ/TZ MICs taken into
16 account were those obtained with the 2:1 ratio (i.e. 0.25 for *E. coli* CFT073-RR, 2 and 4 at
17 standard and high inoculum, respectively, for *E. coli* CFT073-RR Tc *bla*_{CTX-M-15}). For *E. coli*
18 CFT073-RR, a bactericidal effect was observed for concentrations of 8 fold the MIC (CTZ 2
19 mg/L and TZ 1 mg/L) at standard and high inoculum. For *E. coli* CFT073-RR Tc *bla*_{CTX-M-15},
20 concentrations of 4 fold the MIC (i.e. CTZ 8 mg/L and TZ 4 mg/L) at standard inoculum and
21 8 fold the MIC (CTZ 32 mg/L and TZ 16 mg/L) at high inoculum were necessary to observe a
22 bactericidal effect. Our results confirmed that the activity of the combination was impaired by
23 acquired resistance since higher antibiotic concentrations were necessary to obtain a
24 bactericidal effect against the ESBL-producing strain. In addition, increasing the inoculum
25 size required to reach high antibiotic concentrations (CTZ 32 mg/L and TZ 16 mg/L) to obtain

1 a bactericidal effect against the ESBL-producing strain when mimicking the CTZ/TZ ratio
2 obtained in human therapeutics.

3

4 *CTZ/TZ pharmacokinetics in mice.*

5 The unitary dose that best reproduced the peak concentration obtained in humans with
6 CTZ/TZ 1g /500mg (median concentrations in humans [range], 91.5 [65.7-110.7] and 17.5
7 [15.4-27.3] mg/L, respectively [12]) was 100/50 mg/kg, which led to median [range] CTZ and
8 TZ peak concentrations at 30 minutes of 115.6 [95.9-201.2] and 13.85 [8.6-18.2] mg/L,
9 respectively. The rate of injections every 2 hours was chosen according to the elimination
10 half-lives of CTZ and TZ that were approximately 15 minutes for each component.

11

12 *Murine model of peritonitis.*

13 By comparison with “start of treatment” controls, CTZ/TZ significantly reduced the bacterial
14 counts in the spleen and peritoneal fluid of animals infected with *E. coli* CFT073-RR and *E.*
15 *coli* CFT073-RR Tc *bla*_{CTX-M-15} (Table 2). For mice infected with susceptible *E. coli* CFT073-
16 RR, the efficacies of CTZ/TZ and imipenem were similar. By contrast, CTZ/TZ led to a lower
17 decrease in bacterial counts in the spleens and peritoneal fluids of animals infected with *E.*
18 *coli* CFT073-RR Tc *bla*_{CTX-M-15} than imipenem (P< 0.05). In addition, in animals treated with
19 CTZ/TZ, bacterial counts in the spleens and peritoneal fluid of animals infected with *E. coli*
20 CFT073-RR Tc *bla*_{CTX-M-15} were higher than those of animals infected with CFT073-RR (P<
21 0.05). No resistant mutants to CTZ/TZ were detected.

22

23 DISCUSSION

1 Overall, our results indicate an impact of acquired CTX-M-15-type ESBL resistance in *E. coli*
2 on the activity of CTZ/TZ. The presence of the OXA-1 enzyme, coproduced by CFT073-RR
3 Tc *bla*_{CTX-M-15} strain, like a large proportion of CTX-M-15-producing *E. coli* clinical strains
4 [13–15], should not have any implication in the lower CTZ/TZ activity we observed as
5 cephalosporins are stable to OXA-1 enzymes [15]. A limited but significant inoculum effect
6 was observed since high antibiotic concentrations were necessary to obtain a bactericidal
7 effect in vitro when the CTZ/TZ ratio corresponded to that used for human therapeutics.
8 Although CTZ/TZ remained active in vivo, the inoculum effect may have contributed to the
9 lower reduction in bacterial counts observed in vivo with CTZ/TZ, as compared to imipenem,
10 when animals were infected with the CTX-M-15-producing strain. However, using a dosing
11 regimen of CTZ/TZ simulating human pharmacokinetics, bacterial killing may be sufficient to
12 cure infection, despite a decrease in CTZ/TZ activity in the presence of ESBL in high
13 inoculum conditions. One limitation of our work is that, to limit the number of sacrificed
14 animals, we did not compare the activity of CTZ/TZ to that of PTZ/TZ that was shown to be
15 impacted by the inoculum effect in vivo [4]. Another point of debate is the choice of non
16 parametric tests rather than Student's test adjusted for unequal variances; however, the
17 differences of distribution were sufficiently large to justify our choice. Overall, our results
18 indicate that ombination of ESBL expression and high inoculum size may be a clinical
19 situation at risk of reduced bactericidal activity of CTZ/TZ at the recommended dose (1g/0.5g
20 thrice a day) against severe high inoculum infections due to ESBL-producing *E. coli*. Whether
21 higher doses of CTZ/TZ (2g/1g thrice a day), recently evaluated for the treatment of
22 nosocomial pneumonia [16] enables to restore a full activity, remains to be demonstrated.

23

24

25

1

2 CONFLICTS OF INTEREST DISCLOSURE

3 The authors do not declare any conflicts of interest. No external funding was received for this
4 study.

5

6 AUTHOR'S CONTRIBUTIONS

7

8 Conceptualization, methodology and validation: AL, BF

9 Formal analysis: AL, JC

10 Investigation: JC, FC, GP, ALM

11 Writing – Original draft: AL, JC

12 Writing – Review and editing: AL, BF

13

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- 23

1 Table 1. MICs of CTZ, TZ, imipenem and CTZ/TZ according to the reference method (fixed
 2 TZ concentration of 4 mg/L) and using a 2:1 CTZ/TZ ratio, using a standard (10^5 CFU/mL) or
 3 a high (10^7 CFU/mL) inoculum.

4

	MIC (mg/L) / reference method					MIC (mg/L) / manufactured solution
	Inoculum	CTZ	TZ	CTZ + TZ 4 mg/L	Imipenem	CTZ/TZ Ratio 2:1
<i>E. coli</i>	Standard	0.25	256	0.25	0.25	0.25
CFT073-RR	High	0.25	256	0.25	1	0.25
<i>E. coli</i>	Standard	64	256	0.25	0.25	2
CFT073-RR Tc <i>bla</i> _{CTX-M-15}	High	>1024	256	0.25	1	4

5

6

7

1 Table 2. Effects of antibiotics on viable organisms in the spleen and peritoneal fluid of mice
 2 infected with *E. coli* CFT073-RR or *E. coli* CFT073-RR Tc *bla*_{CTX-M-15} and treated with
 3 CTZ/TZ or imipenem during 24h.

4

	<i>E. coli</i> CFT073-RR		<i>E. coli</i> CFT073-RR Tc <i>bla</i> _{CTX-M-15}	
	Median [range] log ₁₀ CFU/g of spleen	Median [range] log ₁₀ CFU/mL of peritoneal fluid	Median [range] log ₁₀ CFU/g of spleen	Median [range] log ₁₀ CFU/mL of peritoneal fluid
“Start of treatment” controls	7.47 [6.1-7.91]	7.41 [5.6-7.95]	7.49 [7.22-7.91]	7.30 [7.01-7.70]
CTZ/TZ 100/50 mg/kg q2h	3.51* [#] [2.94- 4.62]	1.78* [#] [1.3-3.19]	4.38* [3.32- 4.99]	2.30* [1.3-2.89]
Imipenem 100 mg/kg q2h	3.72* [3.28- 4.81]	1.78* [1.3-2.6]	3.98* ^{\$} [2.6- 4.52]	1.95* ^{\$} [1.3-5.64]

5 **P*<0.05 versus controls; [#]*P*<0.05 versus *E. coli* CFT073-RR Tc*bla*_{CTX-M-15}; ^{\$}*P*<0.05 versus
 6 CTZ/TZ.

7

8

9

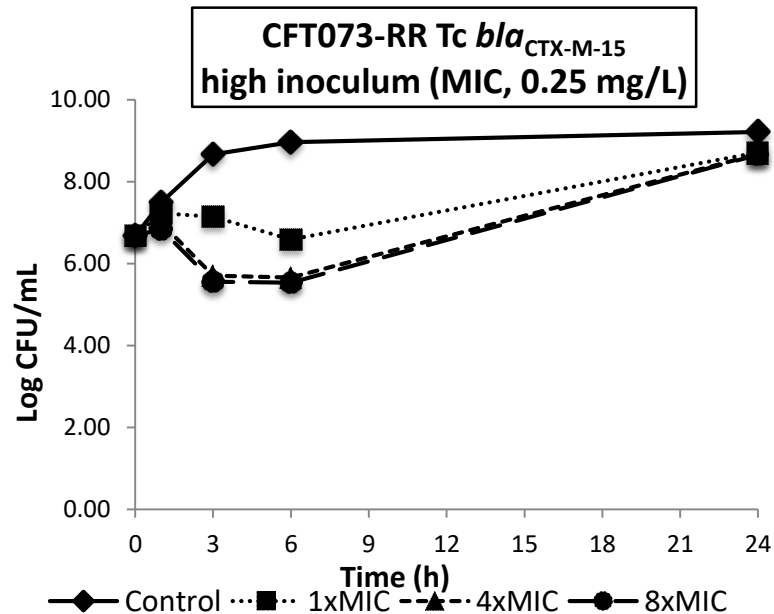
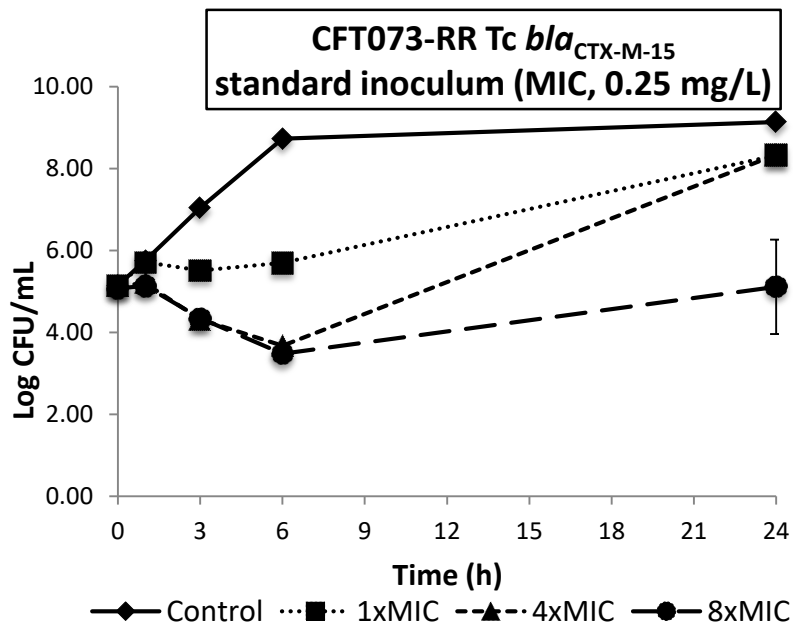
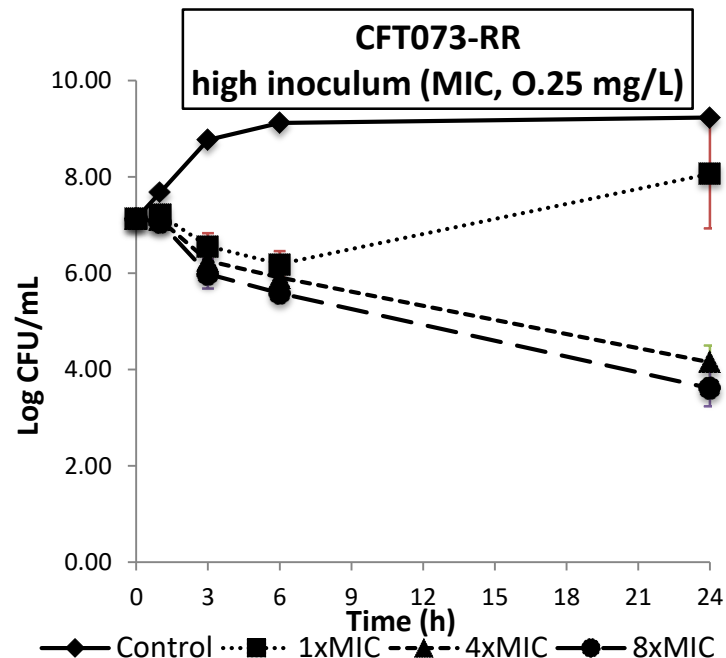
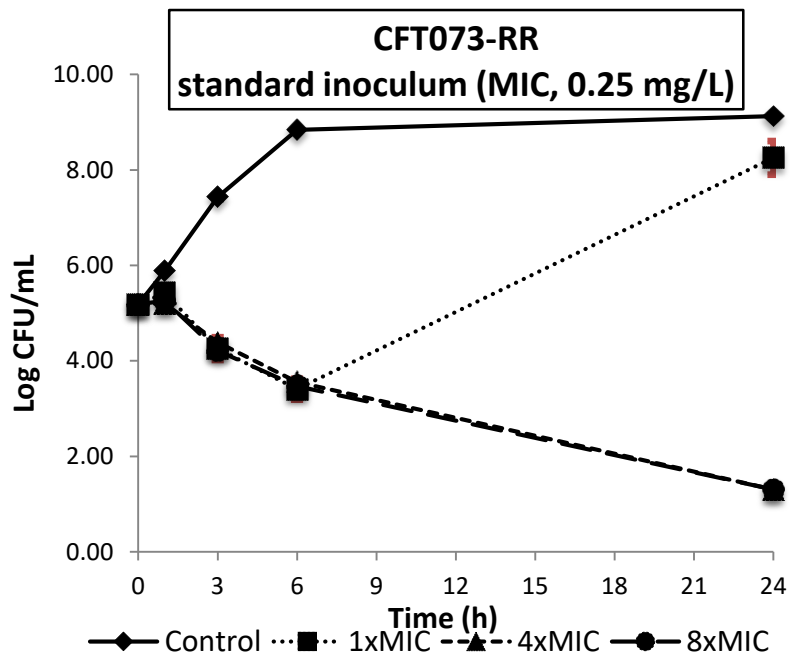
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1 FIG 1. Time-kill curves of CTZ at various concentrations and TZ at a fixed 4 mg/L
2 concentration (A) or in a 2:1 ratio (B) against *E. coli* CFT073-RR and *E. coli* CFT073-RR Tc
3 *bla*_{CTX-M-15}, at standard and high inoculum

4

A. CTZ + TZ 4 mg/L



B. CTZ + TZ ratio 2:1

