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Short Communication

Evaluation of ceftolozane-tazobactam susceptibility on a French nationwide collection of *Enterobacterales*

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ABSTRACT

Objectives: Ceftolozane-tazobactam (C/T) proved its efficacy for the treatment of infections caused by non-carbapenemase producing *Pseudomonas aeruginosa* and *Enterobacterales*. Here, we aimed to provide susceptibility data on large series of *Enterobacterales* since the revision of EUCAST categorization breakpoints in 2020.

Methods: First, C/T susceptibility was determined on characterized *Enterobacterales* resistant to 3rd generation cephalosporins (3GC) (ESBL production or different levels of AmpC overexpression) (n=213) and carbapenem resistant *Enterobacterales* (CRE) (n=259) including 170 carbapenemase producers (CPE). Then, 1,632 consecutive clinical *Enterobacterales* responsible for infection were prospectively collected in 23 French hospitals. C/T susceptibility was determined by Etest® (biomérieux) and broth microdilution (BMD) (Sensititre™, Thermo Scientific) to perform method comparison.

Results: Within the collection isolates, 88% of 3GC resistant strains were susceptible to C/T, with important variation depending on the resistance mechanism: 93% *vs* 13% susceptibility for CTX-M and SHV-ESBL producers, respectively. Only 20% of the CRE were susceptible to C/T. Among CPE, 80 % of OXA-48-like producers were susceptible to C/T, whereas all metallo-β-lactamase producers were resistant. The prospective study revealed that 95.6% of clinical isolates were susceptible to C/T. Method comparison performed on these 1,632 clinical isolates demonstrated 99 % of categorization agreement between MIC to C/T determined by Etest® compared to BMD (reference) and only 74% of essential agreement.

Conclusion: Overall, C/T showed good activity against wild-type *Enterobacterales*, AmpC producers and ESBL-producing *E. coli* but is less active against ESBL-producing *K. pneumoniae* and CRE. Etest® led to an underestimation of the MICs in comparison to BMD.

KEYWORDS

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Ceftolozane-tazobactam, ESBL, carbapenemase, *Enterobacterales*, epidemiology, France, MIC.

HIGHLIGHTS

- 88% of *Enterobacterales* resistant to 3rd generation cephalosporins (3GC) remained susceptible to ceftolozane-tazobactam
- Among ESBL producers, susceptibility to ceftolozane-tazobactam varies from 93% to only 13% for CTX-M and SHV-ESBL producers, respectively
- In France, 95.6% of enterobacterial isolates responsible for infections (except urinary tract infections) collected prospectively were susceptible to ceftolozane-tazobactam
- Etest® led to an underestimation of the ceftolozane-tazobactam MIC compared to a commercial broth microdilution method used as reference but the categorization agreement remained high (99%).

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1. INTRODUCTION

The dissemination of multidrug resistant (MDR) bacteria represents a public health concern and only few last-line antimicrobials such as colistin, tigecycline and the new combination (e.g. ceftazidime-avibactam) may be effective to treat infections caused by these pathogens. Novel molecules are required to fight against MDR bacteria. Ceftolozane-tazobactam (C/T) is a combination of a new oxyimino-cephalosporin and a classical inhibitor of β-lactamase. Ceftolozane was proved to be more stable than classical 3GC against the overexpression of the natural AmpC of *Pseudomonas aeruginosa.* [1] The activity of this combination has been similarly encouraging against 3GC susceptible *Enterobacterales* [2]. However, tazobactam activity is dependent on the acquired β-lactamases and on the species itself, especially in *Enterobacterales* that naturally produce a cephalosporinase [3,4]. EUCAST categorization breakpoint for susceptibility has been revised in 2020 for *Enterobacterales*. It has been changed from ≤ 1 mg/L to ≤ 2 mg/L. Since this change, evaluation of C/T susceptibility on large series of *Enterobacterales* with multiple resistance phenotypes (wild-type to carbapenemase-producing bacteria) remain scarce in Europe.

The purpose of the study was to evaluate the *in vitro* activity of C/T on two large series of *Enterobacterales*. First, we reviewed a collection of 472 well-characterized multidrug resistant isolates with numerous β-lactamase variants. Secondly, in a prospective clinical study, we considered 1632 consecutive clinical isolates responsible for infections and collected in 23 hospitals distributed all over the French territory during 2019. An additional objective was to compare two methods of MIC determination for C/T: Etest® strips *vs* a commercial broth microdilution method (used as reference).

2. MATERIAL AND METHODS

2.1. Bacterial isolates

Collection isolates:

A total of 472 characterized *Enterobacterales* were tested for C/T susceptibility. This collection included (i) 213 strains resistant to 3GC due to ESBL production or AmpC overexpression but remaining susceptible to carbapenems and (ii) 259 enterobacterial strains that were resistant to carbapenems due to carbapenemase production (n=170) or to combined mechanisms such as ESBL +/- AmpC overexpression +/- permeability defect (n=89). Among these 259 carbapenem-resistant *Enterobacterales* (CRE), 100 consecutive *Enterobacterales* sent to the French NRC for expertise were included, and the remaining 159 isolates were provided by the laboratory collection to ensure a wide diversity of carbapenemase variants.

Prospective study:

Besides, a prospective study was conducted during 2019 in 23 hospitals distributed on the French territory (Figure S1). Each center aimed to collect 70 consecutive *Enterobacterales* isolates cultured from clinical samples. Bacterial isolates responsible for urinary tract infection or from screening samples were excluded (Figure S1). This prospective study has been approved by an ethic committee (Research Project number: 18.08.01.47952 RIPH 3).

2.2. Antimicrobial susceptibility testing and characterization of β-lactamase content

Antimicrobial susceptibility testing was performed on each isolate using disk diffusion method on Muller-Hinton agar (Biorad®, Marnes-la-Coquette, France) as recommended by EUCAST guidelines. If resistance to 3GC was observed, synergy with clavulanate was investigated to look for ESBL production. In that case, ESBL related genes were sought using

home-made simplex PCRs targeting *bla*$_{CTX-M}$, *bla*$_{SHV}$, *bla*$_{TEM,}$ *bla*$_{VEB}$ as previously described [8].

If the susceptibility to carbapenems or broad-spectrum cephalosporins was fully restored on Muller-Hinton agar supplemented with cloxacillin (bioMérieux), AmpC overexpression (or acquisition) was confirmed.

On all isolates, MICs for C/T, ceftazidime and imipenem were measured using Etest® strip (biomérieux, La Balmes les Grottes, France). For the prospective clinical study, strains identification was performed using MALDI-TOF (Burker Daltonics) and MICs were also determined by broth microdilution (BMD) method using customed plate manufactured by Sensititre™ (Thermo Scientific, Les Ulis, France). This plate enabled to test the activity of C/T, ceftazidime, ceftazidime-avibactam and imipenem in particular. All MIC results were interpreted using EUCAST recommendations revised in 2020 [5].

On each CRE, carbapenemase production was investigated using Carba NP test as previously described [6] coupled with the Lateral flow immunoassay NG-Test CARBA 5 assay (NG Biotech, Guipry, France) for the detection of OXA-48-like, KPC, NDM, IMP and VIM enzymes [7]. If one of these tests were positive, carbapenemase encoding genes were amplified by PCR for the detection of *bla*$_{VIM}$, *bla*$_{IMP}$, *bla*$_{NDM}$, *bla*$_{KPC}$ and *bla*$_{OXA-48}$ as previously described [9]. PCR products were directly sequenced by the Sanger method with an ABI 3130 Applied Biosystems. The nucleotide sequences were analyzed with the BLDB BLAST program [10].

2.3. Methods comparison

MIC determination of C/T by Etest® strip (bioMérieux) was compared to broth microdilution (BMD) (Sensititre™, Thermo Scientific) used as reference method. Categorical agreements

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[(CAs) results within the same category], essential agreements [(EAs) MIC results differing by a maximum of 1 $\log_2$ concentration], very major errors [(VMEs) false susceptibility] and of major errors [(MEs) false resistance] were calculated in reference to EUCAST breakpoints of 2020 [11].

3. RESULTS & DISCUSSION

3.1. Evaluation of C/T susceptibility on characterized isolates

3.1.1. 3GC resistant Enterobacterales

A collection of 213 *Enterobacterales* strains resistant to 3GC due to ESBL and/or AmpC overexpression was tested (Table S1). This collection was mostly composed of CTX-M- (n=155), SHV- (n=15), and TEM-producing strains (n=14) reflecting the global epidemiology of ESBL nowadays. Coproduction of different ESBLs was present in 18 isolates. Four different variants of TEM and nine of SHV, known to confer an ESBL phenotype, were included as well as 18 variants of CTX-M (Table S2). Rare ESBL such as VEB (n=6) and GES (n=1) and acquired AmpC (n=4) were also present in this collection. Overall, distribution of the MICs showed that 88% of the strains were susceptible to C/T. Of note, this proportion rose to 93% with CTX-M producers (Table 1). At the opposite, production of SHV-ESBL was associated with higher resistance rate to C/T (87%, n=13/15 of isolates). TEM-ESBL producers showed 79% of susceptibility (11/14) and VEB producers harbored 83% of susceptibility to C/T (5/6). All strains co-producing CTX-M + SHV (n=16) or TEM + SHV (n=2) were resistant to C/T (Table 1). Most studies focused on studying C/T susceptibility on ESBL-producing strains regardless the enzyme and the variant. Here, we provide susceptibility data on isolates with fully characterized β-lactamase contents

demonstrating that SHV-ESBL producers were more prone to be resistant to C/T as opposed to CTX-M producers.

3.1.2. Carbapenem-resistant Enterobacterales

Susceptibility to C/T was also determined on a collection of 259 CRE either due to carbapenemase production (n=170) or to the acquisition of an ESBL and/or AmpC overexpression combined with a supposed permeability defect (n=89) (Table 1). Among them, 100 consecutive *Enterobacterales* sent to the French NRC for expertise were included, and the rest was provided by the laboratory collection to ensure a wide diversity of carbapenemase variants (Table S3).

Overall, only 20% of the carbapenemase-producing *Enterobacterales* (CPE) were susceptible to C/T (n=35/170). The lowest MIC_{50} and MIC_{90} were observed in OXA-48-like producers alone (without ESBL or AmpC associated) displaying 80% (n=24) of susceptibility (Table 1). Indeed, OXA-48-like enzymes barely hydrolyze cephalosporins, so the activity of ceftolozane is preserved. Regarding Ambler class A carbapenemase producers, KPC-producing isolates (n=25) were all resistant to C/T confirming the ceftolozane hydrolyzing activity of KPC enzymes and the low inhibitory capacity of tazobactam towards these enzymes. At the opposite, several rarer Ambler class A carbapenemases with poor cephalosporinase activity, such as IMI/Nmc-A producers (n=5) and SME producers (n=2) remained fully susceptible to C/T. Finally, as expected all metallo-β-lactamase (MBL) producing strains (n=55) were resistant to C/T with MIC_{50} > 256 mg/L. To conclude, the rare C/T activity on CPE is not due the inhibiting capacity of tazobactam but to the small proportion of CPE that remains susceptible to 3GC, that means OXA-48-like producers that do not co-produce an ESBL or an AmpC.

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Interestingly, non carbapenemase-producing CRE showed the same level of susceptibility to C/T as CPE with only 22% of isolates (n=20/89) with MICs below the breakpoint. These isolates are resistant to 3GC due to ESBL production or AmpC overexpression. These enzymes have very low hydrolysis activity towards carbapenems but their association to membrane permeability defect due to porins loss by example can lead to carbapenem resistance. Among the CRE, comparison of C/T MIC distributions revealed higher values in strains for which carbapenem resistance was due to impaired permeability (Figure S2), suggesting that permeability defect impacts the susceptibility to C/T.

Overall, given the high rate of C/T resistance within CRE, regardless of the mechanism of resistance, C/T should not be considered as part of the therapeutic arsenal for the treatment of infections caused by such resistant pathogens.

3.2. Clinical prospective study

In 2019, 1,632 *Enterobacterales* collected by 23 microbiology laboratories from clinical samples were analyzed (Figure S1). Most of them were isolated from blood culture (n=1028). The most representative species were *Escherichia coli* (n=825), *Klebsiella pneumoniae* (n=261), *Enterobacter cloacae* complex (ECC) (n=156), *Proteus mirabilis* (n=74), *Klebsiella oxytoca* (n=58) and *Serratia marcescens* (n=50) (Figure S1). The objective was to evaluate C/T susceptibility on a large collection of strains involved in patient's infections (except urinary tract infections) with various β-lactamase contents (from wild-type phenotype, i.e. no acquired β-lactamase encoding genes or no chromosomal mutations implicated in resistance to β-lactams, to acquired carbapenemase) (Table S1).

3.2.1. Overall susceptibility

As determined by BMD, ceftazidime and imipenem susceptibilities were of 82.4% and 98.8% respectively (Figure S3). Of note, 17% (285/1632) of *Enterobacterales* isolates were resistant to 3GC due to the acquisition of an ESBL (11%, n=177), or the overexpression of an AmpC (intrinsic or acquired), referred as HL-CASE for high-level production of cephalosporinase (6%, n= 95). These results are in agreement with data collected by the EARS-net European surveillance program that indicates a mean of 13% of resistance to 3GC in *E. coli* and *K. pneumoniae* species combined responsible for invasive infections in a total 30 participating countries [12].

In ESBL-producing strains, CTX-M was identified in 92% (171/185) of isolates whereas SHV-ESBL, VEB and TEM-ESBL producers represented 5.9% (11/185), 1.0% (2/185) and 0.5% (1/185) of ESBL producers, respectively (Table S4).

Ten CPE (0.6%) were collected during this prospective study. The identified carbapenemases were OXA-48 (n=3), NDM-1 (n=4) and OXA-181 (n=2). One *K. pneumoniae* coproduced OXA-48 and NDM-5 enzymes. Of note, 9 of the 10 CPE also produced an ESBL of CTX-M type.

Overall, in this collection made up of 1632 clinical isolates the susceptibility for C/T regardless the phenotype was of 95.6% (Figure S3). Similar data have been published in Europe with some disparities between countries, with susceptibility rates ranging from 79.4% in Eastern Europe to 95% in Western Europe [2,13,14].

In ESBL-, AmpC- and carbapenemase-producing groups, C/T susceptibility rate fell to 80%, 74% and 0% respectively (Table 2). C/T was very active towards non-ESBL producers (99.7%) and ESBL-producing *E. coli* (96%). However, C/T remained less active on ESBL-producing *K. pneumoniae* with only 66% of susceptibility. Similar patterns were reported in the SMART Asia-Pacific surveillance program where 93% of ESBL-producing *E. coli* and

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66% of ESBL-producing *K. pneumoniae* isolates were found to be susceptible to C/T [15]. This lower C/T susceptibility in ESBL-producing *K. pneumoniae* compared to ESBL-producing *E. coli* was also observed in USA [16] and in Spain [17] and could be the result of membrane permeability defects that preferentially affect *K. pneumoniae* [18].

Among all tested molecules, ceftazidime-avibactam and imipenem possessed the highest susceptibility rate (99.8% and 99.7%, respectively) on the clinical collection strains (Figure S3).

3.2.2. Natural producers of AmpC β-lactamase

We focused on studying *Enterobacterales* that naturally produce a cephalosporinase, from wild-type phenotype to AmpC being overexpressed (ESBL- and carbapenemase-producing strains were excluded). The objective was to evaluate the activity of C/T on several AmpC subtypes and different levels of expression (from wild-type phenotype to HL-CASE). The read-out of the AmpC expression was the ceftazidime MIC. Over the 297 isolates, 92% were susceptible to C/T. Tazobactam is known to be a poor inhibitor of most cephalosporinases. But when we looked deeper into the MICs distributions for each species, we found that all *Morganella morganii* (n=40) remained susceptible to C/T as it has been previously suggested on a small series by Livermore *et al.* (Table S4) [3]. Over the 40 tested isolates, 10 were HL-CASE. This observation suggests that *Morganella*'s AmpC (=DHA-type) has less activity towards ceftolozane or that tazobactam keeps its inhibitory capacity against these AmpC as previously reported [19].

The highest MIC_{90} (8 mg/L) was observed for isolates of the *Enterobacter cloacae* complex (ECC) (n=124). Within this complex, 100% of susceptibility was observed in the wild-type phenotype subgroup (n=93), and this rate dropped to 50% in the AmpC overexpressed subgroup (n=31). Similar findings have been reported by Robin *et al*. who demonstrated that

only 24% of ECC isolates with high-level of cephalosporinase production were susceptible to C/T versus 100% of wild-type isolates [4]. Accordingly for all AmpC producers combined, the increase of MICs to CAZ (*e.g.* the level of AmpC expression) correlates with the increase of MICs to C/T (Figure S4).

4. Comparison of methods for C/T MIC determination

On this large collection of clinical strains, MIC for C/T was also determined by Etest® and compared with values obtained by a BMD technique used as reference. Overall, Etest® led to an underestimation of the MICs with a 73.6% (1201/1632) of essential agreement (EA) (Figure 1). However, categorical agreement (CA) occurred for 99% (1608/1632) of the isolates. A total of 24 very major error (VME) were identified corresponding to 1.5% of the total number of tested isolates (n=1632) and 33.3% of the resistant isolates (n=72) (Figure 1). By excluding the MICs that were in the essential agreement zone, the number of VME dropped to 15 isolates, 21% of the resistant population and 0.9% of total isolates. No major error was identified using Etest®. Of note, underestimation of the MICs values to C/T determined by Etest® has been previously observed on a large collection of 200 *P. aeruginosa* with a VME rate of 25% of the resistant isolates [20]. For *Enterobacterales*, EUCAST categorization breakpoint for susceptibility has been revised in 2020 and changed from ≥ 1 mg/L to ≥2 mg/L. To our knowledge, method comparison has not been evaluated since the implementation of this new cutoff. Based on our results, the Etest® might not fulfil the CLSI requirements (CA and EA ≥ 90%, MEs <3% and VMEs <3%) to assess the *in vitro* susceptibility of *Enterobacterales* to C/T using the EUCAST breakpoints of 2020.

CONCLUSION

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Evaluation of C/T susceptibility on 472 fully characterized isolates provided useful information on rare β-lactamases. Comparison of C/T activity on 3GC-resistant strains susceptible to carbapenems versus those resistant to carbapenems revealed the importance of membrane permeability on C/T susceptibility. This has been confirmed in the clinical study where difference in susceptibly rates was observed between ESBL-producing *K. pneumoniae* and *E. coli*. Overall, if C/T showed good activity against wild-type *Enterobacterales*, natural AmpC producers and ESBL-producing *E. coli*, it lacked useful activity against ESBL-producing *K. pneumoniae* and CRE. Of note, study limitation resides in the fact that two different methodologies were employed for C/T susceptibility testing on characterized strains (Etest) and on the clinical study (Etest + broth microdilution).

Despite this limit, the high percentage of susceptibility of C/T on 3GC-susceptible *Enterobacterales* as well as on ESBL-producing *E. coli* enables to consider C/T as part of the empirical antimicrobial therapy of polymicrobial infections, including those caused by a combination of *Enterobacterales* and *Pseudomonas aeruginosa*.

Regarding C/T susceptibility testing, microbiologists should be aware that Etest® might lead to an underestimation of the MICs compare to commercial broth microdilution methods that are commonly considered as reference method.

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TRANSPARENCY DECLARATION

Declaration of competing interest: None to declare

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Ethical Approval: not required

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Table 1. Ceftolozane-tazobactam MICs distribution of the 213 collection isolates resistant to 3rd generation cephalosporins (3GC) and the 259 isolates resistant to carbapenems.

		MICs distribution (mg/L)																							EUCAST categorization	
		0.19	0.25	0.38	0.5	0.75	1	1.5	2	3	4	6	8	12	16	24	32	48	64	96	128	192	>256	Total	MIC_{50}	MIC_{90}	S (%)	R (%)
Strains 3GC resistant - Carbapenem S (n=213)																												
Mechanisms of resistance to 3GC (n=213)	CTX-M	1	9	26	37	30	20	10	11	3	4	0	1	1		1							1	155	0.75	2	93%	7%
	TEM	1	2	1	1	1	3	1	1		2												1	14	1	4	79%	21%
	SHV				1			1					2		1		1		1	2	1	1	4	15	64	>256	13%	87%
	CTX-M + SHV									1	0	0	2	1	0	2	3	1	1	2	1		2	16	3	>256	0%	100%
	VEB				1	3			1				1											6	0,75	8	83%	17%
	TEM+SHV														1			1						2	NA	NA	NA	NA
	GES														1									1	NA	NA	NA	NA
	ACC												2											2	NA	NA	NA	NA
	DHA		1			1																		2	NA	NA	NA	NA
Strains Carbapenem R (n=259)																												
Without carbapenemase (n=89)	AmpC				1		1		4		2	1	1	9	2	6	3	2		1			1	34	12	48	18%	82%
	ESBL						1	3	2	3	2	2	3	1	3	1	1						12	34	12	>256	18%	82%
	AmpC + ESBL					1			2		1	2		2		2		1						11	6	24	27%	73%
	OXA-163																						2	2	NA	NA	NA	NA
	OXA-405																						1	1	NA	NA	NA	NA
	other	1		1	1		1	1		1													1	7	1	>256	71%	29%
With carbapenemase (n=170)	Ambler class A		1	2	1	2	1		1	1	2	1		4	1	1	2	5	4			1	4	34	16	>256	24%	76%
	Ambler class B																						55	55	>256	>256	0%	100%
	Ambler Class D		1		6	8	2	3	4	1	2	1			1								1	30	0.75	4	80%	20%
	Ambler Class D + ESBL							1	2		4	3	2	3	3	5	4	1	3		1	1	7	40	24	>256	8%	93%
	Ambler Class D + AmpC											1		2				1	1					5	12	64	0%	100%
	Ambler class D + class B																						6	6	>256	>256	0%	100%

NA not applicable: analysis of MIC_{50}, MIC_{90}, susceptibility (S)/resistance (R) rates were not performed due to low number of isolates

Table 2. Ceftolozane-tazobactam MICs distribution (determined by broth microdilution) of the 1632 isolates collected during the clinical study

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	MICs distribution (mg/L)											EUCAST category	
	≤0.25	0.5	1	2	4	8	16	32	>32	Total	MIC50	MIC90	Susceptible (%)	Resistant (%)
ESBL	33	55	32	21	10	4	6	8	8	177	1	16	80%	20%
HL-CASE[a]	8	25	14	23	8	7	7	2	1	95	2	16	74%	26%
Carbapenemase					1			1	8	10	>32	>32	0%	100%
OXA-48-like + ESBL					1			1	3					
NDM									4					
OXA-48-like + NDM									1					
other	888	360	82	17	1	2				1350	≤0.25	0.5	99.8%	0.2%

S = susceptible; R = Resistant
ESBL, Extended spectrum β-lactamase
HL-Case, high level production of AmpC (intrinsic or acquired)

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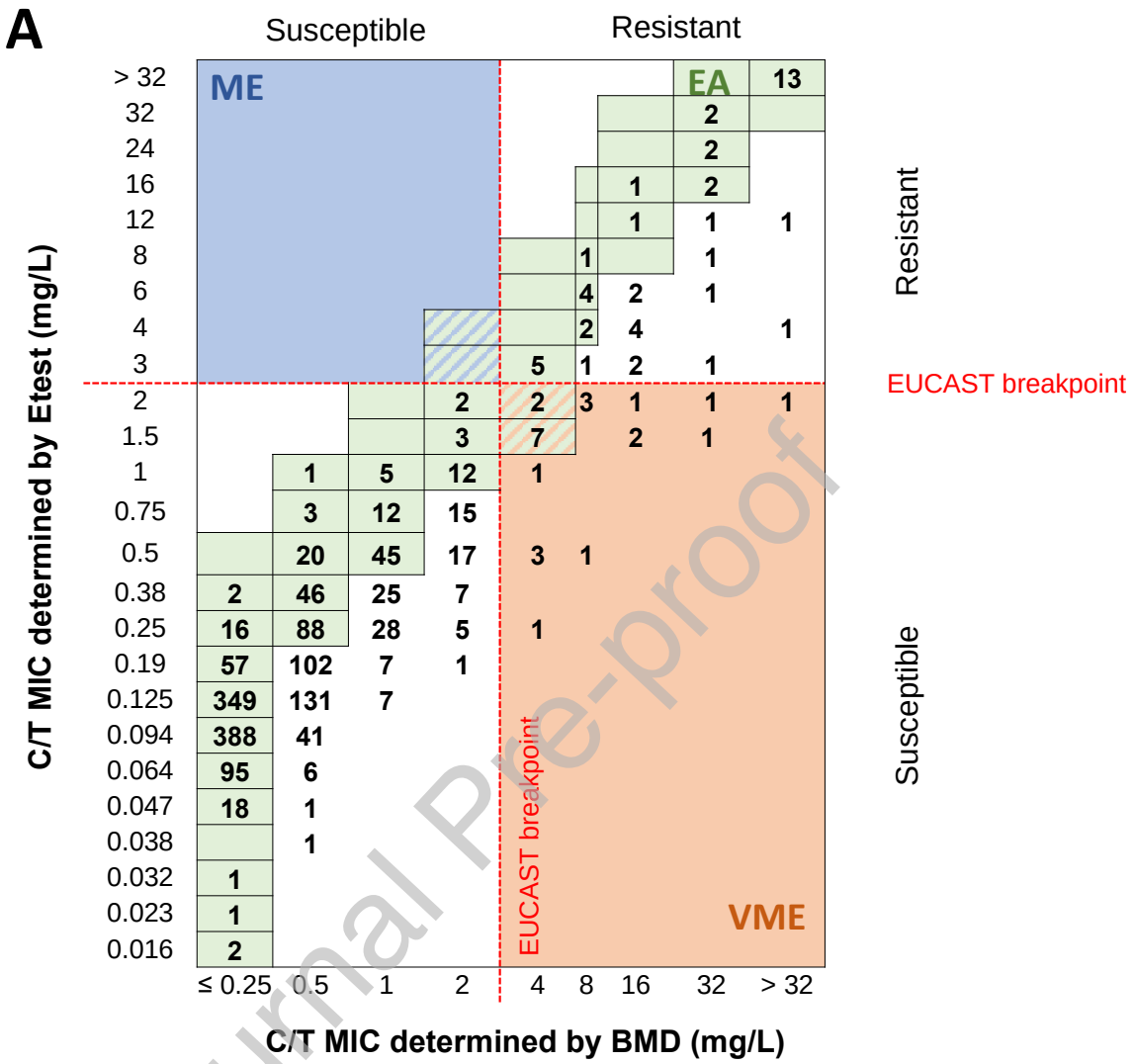
Figure 1.



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LEGEND OF THE FIGURE

Figure 1. Comparison of Etest® and broth microdilution (reference method) for the determination of MIC to ceftolozane-tazobactam. EA = essential agreement is colored in green, ME major errors are colored in blue, VME = very major errors are colored in orange.