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In Vitro Activity of Ceftolozane-Tazobactam as Determined by Broth Dilution and Agar Diffusion Assays against Recent U.S. *Escherichia coli* Isolates from 2010 to 2011 Carrying CTX-M-Type Extended-Spectrum β-Lactamases

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Ceftolozane MIC_{50}/MIC_{90}s were 4/8 μg/ml when tested against 26 CTX-M-14-type-producing isolates and 64/>64 μg/ml against 219 CTX-M-15-type-producing isolates. The addition of 4 μg/ml tazobactam lowered the ceftolozane MIC_{50}/MIC_{90}s to ≤0.25/0.5 μg/ml by broth microdilution and Etest. The zone diameters for the ceftolozane-tazobactam disks were 23 to 29 mm for 92.2% of the isolates.

Ceftolozane, a novel cephalosporin with potent activity, particularly against *Pseudomonas aeruginosa* (1), has intrinsic microbiological activity against *Enterobacteriaceae*. However, ceftolozane is somewhat compromised when hydrolyzed by selected β-lactamases, particularly the extended-spectrum β-lactamases (ESBLs), but not the pseudomonal AmpC cephalosporinase (2–4). Thus, the addition of tazobactam, a well-established inactivator of many class A β-lactamases (3, 5–7), to ceftolozane has expanded its utility for the treatment of many pathogens producing common ESBLs (2, 3, 8–10), particularly CTX-M-14 and CTX-M-15, the most prevalent ESBLs globally (11–16). Phase 3 trials with ceftolozane-tazobactam have been completed for the treatment of complicated urinary tract infections (17) and complicated intra-abdominal infections (18), and the agent is being studied for the treatment of ventilator-associated nosocomial pneumonia. In this study, three testing methods for ceftolozane-tazobactam were evaluated against a set of 245 recently collected CTX-M-producing *Escherichia coli* isolates (13) to determine the correspondence of the data among the different assays.

TEM-1 was purchased from Invitrogen (PV3575; Carlsbad, CA). CTX-M-15 was purified by Evotec Ltd. (Abingdon, United Kingdom) after cloning into pET26-b(+) and being expressed in *E. coli*. CTX-M-14 was expressed and purified at GenScript (Piscataway, NJ). Ceftolozane and tazobactam were supplied by Cubist Pharmaceuticals (Lexington, MA). Clavulanic acid was from Fluka/Sigma-Aldrich (St. Louis, MO). Piperacillin, ceftazidime, levofloxacin, and tobramycin were from Sigma-Aldrich. Sulbactam, cefepime, and meropenem were from the U.S. Pharmacopeial Convention (Rockville, MD). Caftolozane-tazobactam Etest strips were from bioMérieux (lot no. 1001256360; Marcy-l'Étoile, France). Ceftolozane-tazobactam disks (30 μg of ceftolozane and 10 μg of tazobactam) were manufactured by BD (Franklin Lakes, NJ).

β-Lactamase assays were performed in phosphate buffer saline containing 137 mM sodium chloride, 2.7 mM potassium chloride, and 10 mM phosphate buffer (pH 7.4), with 0.1 mg/ml bovine serum albumin (BSA), in 96-well half-area plates (50 μl). The 50% inhibitory concentrations (IC_{50}s) were determined by incubating inhibitor for 5 min at 25°C with 0.125 nM TEM-1, 0.1 nM CTX-M-15, or 0.015 nM CTX-M-14 (final concentrations). Enzymatic activity was measured spectrophotometrically at 486 nm after the addition of 0.1 mM nitrocefin. IC_{50}s were calculated using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA).

TABLE 1 Inhibitory activity of tazobactam against CTX-M-15 and CTX-M-14 in comparison with that of clavulanic acid or sulbactam

Inhibitor	IC_{50} (nM)[a]		
	TEM-1	CTX-M-14	CTX-M-15
Clavulanic acid	143 ± 15	120 ± 10	36.7 ± 1.9
Sulbactam	223 ± 17	438 ± 77	335 ± 63
Tazobactam	2.3 ± 0.1	3.6 ± 0.1	2.7 ± 0.2

[a] IC_{50}, concentration of inhibitor required to reduce enzymatic activity by 50%. Each data point in an 11-point dose-response curve was measured in duplicate; the results represent the averages ± standard deviations from three separate experiments.

The *E. coli* isolates obtained from 2010 to 2011 were from a large medical center representing 8 hospitals in Detroit and southeast Michigan (13). Isolates that were phenotypically positive for ESBL production were screened by PCR in a previous study for $bla_{\text{CTX-M-14-type}}$, $bla_{\text{CTX-M-15-type}}$, $bla_{\text{SHV-type}}$, and $bla_{\text{TEM-type}}$ genes, and single enzymes were confirmed by isoelectric focusing in representative strains (13). Isolates that were positive for only $bla_{\text{CTX-M-14-type}}$ ($n = 26$) or $bla_{\text{CTX-M-15-type}}$ ($n = 219$) genes and that were reproducibly culturable were tested. Each β-lactamase gene was not sequenced, nor was clonality assessed. For the purposes of this study, the enzymes encoded are called CTX-M-14 and CTX-M-15.

Susceptibilities were determined in cation-adjusted Mueller-

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TABLE 2 Susceptibilities of 245 *E. coli* isolates with *bla* genes encoding either CTX-M-14-type or CTX-M-15-type ESBLs, as determined by broth microdilution testing

Enzyme type (*n*)	Antibiotic	MIC data (μg/ml)			% Susceptible	% Resistant
		Range	MIC_{50}	MIC_{90}		
CTX-M-14 (26)						
	Ceftolozane	≤1 to 32	4	8	NA[a]	NA
	Ceftolozane-tazobactam[b]	≤0.25 to 1	≤0.25	0.5	NA	NA
	Piperacillin	64 to >256	>256	>256	0.0	92.3
	Piperacillin-tazobactam[b]	0.5 to 8	2	4	100.0	0.0
	Ceftazidime	≤1 to 16	4	8	53.8	7.7
	Cefepime	≤1 to >64	8	32	7.7	53.8
	Meropenem	≤0.06 to 0.5	≤0.06	0.12	100.0	0.0
	Levofloxacin	≤0.25 to >16	8	>16	11.5	80.5
	Tobramycin	≤1 to 64	≤1	32	76.9	19.2
CTX-M-15 (219)						
	Ceftolozane	≤1 to >64	64	>64	NA	NA
	Ceftolozane-tazobactam[b]	≤0.25 to 1	≤0.25	0.5	NA	NA
	Piperacillin	32 to >256	>256	>256	0.0	97.3
	Piperacillin-tazobactam[b]	≤0.25 to 16	2	8	100.0	0.0
	Ceftazidime	≤1 to >64	16	64	10.0	78.1
	Cefepime	≤1 to >64	16	64	8.2	63.9
	Meropenem	≤0.06 to 1	≤0.06	≤0.06	100.0	0.0
	Levofloxacin	≤0.25 to >16	8	16	2.7	91.0
	Tobramycin	≤1 to >64	16	64	27.9	70.0

[a] NA, not applicable. No breakpoints have been assigned.
[b] Tazobactam was tested at a fixed concentration of 4 μg/ml.

Hinton II (Sigma-Aldrich, St. Louis, MO) by broth microdilution (BMD) and disk diffusion (DD), according to Clinical and Laboratory Standards Institute (CLSI) methodology (19), and by Etest, according to the instructions from the manufacturer (bioMérieux). In BMD testing, tazobactam was used at a fixed concentration of 4 μg/ml. MICs from the Etest assays were rounded up to the next doubling dilution associated with the BMD concentrations. All MIC values and zone sizes were determined from at least two assays. If duplicate values were not identical, a third assay was conducted, and the median value was used.

In isolated enzyme studies with purified TEM-1, CTX-M-14, and CTX-M-15, tazobactam had the greatest inhibitory activity compared with that of clavulanic acid and sulbactam (Table 1); clavulanic acid and sulbactam exhibited IC_{50}s ≥10-fold greater than those of tazobactam. The clavulanic acid and sulbactam IC_{50}s for TEM-1 and CTX-M-15 were within 3-fold of published values, with the exception of TEM-1 with sulbactam, for which our value of 223 nM was as much as 7-fold lower than previously reported IC_{50} data (20–22). Compared to previous reports, this tazobactam IC_{50} tended to be similar for CTX-M-15 but lower for TEM-1 (20–22). The discrepancies among the studies may be due to the preincubation times (either 0 min or 5 min), the addition of BSA and salt to the reaction mixtures, or an incubation temperature of 25°C compared with 37°C, with the higher temperature facilitating more complete hydrolysis of inactivator before inhibition was measured.

In vitro susceptibility testing by BMD was conducted for ceftolozane with and without tazobactam and compared with other antipseudomonal agents (Table 2). By BMD, the ceftolozane MICs were ≤4 μg/ml for 17/26 (65%) of the CTX-M-14-positive isolates but only 5/219 (2.3%) of the CTX-M-15-positive isolates (Table 3). Ceftolozane had lower MICs when tested against CTX-M-14-producing isolates, with 92% of the strains inhibited at 8 μg/ml compared with only 5% of the CTX-M-15-producing strains. The ceftolozane MIC_{50}/MIC_{90}s were 4/8 μg/ml for CTX-M-14-producing strains and 64/>64 μg/ml for CTX-M-15-producing strains.

TABLE 3 Distribution of MICs for ceftolozane-tazobactam against *E. coli* isolates with $bla_{CTX\text{-}M}$ genes

Antibiotic used	Enzyme type (*n*)	Testing method	No. (cumulative %) of isolates with MIC (μg/ml) of:										MIC (μg/ml)	
			≤0.25	0.5	1 or ≤1	2	4	8	16	32	64	>64	MIC_{50}	MIC_{90}
Ceftolozane[a]	CTX-M-14 (26)	BMD			1 (4)	3 (15)	13 (65)	7 (92)	1 (96)	1 (100)			4	8
Ceftolozane-tazobactam[b]	CTX-M-14	BMD	20 (77)	4 (92)	2 (100)								≤0.25	0.5
Ceftolozane-tazobactam	CTX-M-14	Etest	22 (85)	3 (96)	1 (100)								≤0.25	0.5
Ceftolozane	CTX-M-15 (219)	BMD			2 (1)	1 (1)	2 (2)	5 (5)	31 (19)	67 (49)	52 (73)	59 (100)	64	>64
Ceftolozane-tazobactam	CTX-M-15	BMD	161 (74)	45 (94)	13 (100)								≤0.25	0.5
Ceftolozane-tazobactam	CTX-M-15	Etest	170 (78)	37 (95)	11 (99.5)	1 (100)[c]							≤0.25	0.5

[a] Ceftolozane was tested at ≥1 μg/ml and was tested for MICs by BMD only.
[b] Tazobactam was tested at a concentration of 4 μg/ml.
[c] MIC was recorded as 1.5 μg/ml in duplicate readings.

TABLE 4 Scattergrams of ceftolozane-tazobactam comparing susceptibilities of 245 CTX-M-producing *E. coli* strains across testing methods

Ceftolozane-tazobactam MIC (μg/ml) by BMD/Etest	Ceftolozane-tazobactam zone diam (mm)														
	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
BMD[a]															
2			1			1	1	2	4	4	1	1			
1					1	1	4	11	11	7	5	4	3	1	
0.5		1			3	3	4	15	28	45	45	21	10	6	1
≤0.25															
Etest[b]															
2								1							
1			1			4	1	3	2	1					
0.5		1			1	1	4	5	10	9	6	1	2		
≤0.25					3		4	19	31	46	45	25	11	7	1

[a] Ceftolozane MICs in μg/ml as determined by broth microdilution in the presence of 4 μg/ml tazobactam, compared with zone diameters in mm.
[b] Ceftolozane-tazobactam MICs in μg/ml as determined by Etest, compared with zone diameters in mm.

MICs for ceftolozane were decreased as much as 128-fold when tazobactam was added (Tables 2 and 3). Ceftolozane with 4 μg/ml tazobactam (in BMD) had MIC_{50}/MIC_{90}s of ≤0.25/0.5 μg/ml for all strains by both BMD and Etest, regardless of the enzyme (Table 3). These results are similar to those of a recent study with CTX-M-14- and CTX-M-15-producing *E. coli* and *Klebsiella pneumoniae* isolates, in which concentrations of 4 or 8 μg/ml tazobactam lowered ceftolozane MICs to ≤1 μg/ml for 96% of the isolates (10).

Among the comparator agents, meropenem had the lowest MICs, with MIC_{50}/MIC_{90}s of ≤0.06/0.12 μg/ml for the CTX-M-14-producing strains and ≤0.06/≤0.06 μg/ml for the CTX-M-15-producing strains. While no isolates were susceptible to piperacillin (MICs ≥ 32 μg/ml), the addition of 4 μg/ml tazobactam restored piperacillin susceptibility in all isolates (MICs ≤ 16 μg/ml). Ceftazidime maintained >50% susceptibility among the CTX-M-14-producing isolates, while only 10% of the CTX-M-15-producing isolates were susceptible. As in previous studies, CTX-M-14-producing strains were generally more susceptible to expanded-spectrum cephalosporins than strains producing CTX-M-15 ESBLs (23, 24). The cefepime MICs mirrored those for ceftazidime against the CTX-M-15-producing strains, with 8.2% susceptibility reported, similar to the low susceptibility (7.7%) seen for cefepime against CTX-M-14-producing strains. Tobramycin resistance was more strongly related with CTX-M-15 production (70% resistant) than to a CTX-M-14 ESBL (19% resistant). Fluoroquinolone resistance was prevalent (90% levofloxacin resistance), similar to that seen in the larger population of *E. coli* isolates from which this set of strains was selected (13). In the Hayakawa et al. study (13), ciprofloxacin resistance was 94.7% among the 319 CTX-M-producing *E. coli* strains that tested positive for ESBLs, in contrast to 60.7% of the strains that produced a non-CTX-M ESBL. These data suggest that CTX-M production is linked to fluoroquinolone resistance, possibly due to the prevalence of a CTX-M-producing *E. coli* sequence type 131 (ST131) clone with chromosomal mutations in *gyrA*, often with mutations in *parC* and *parE* (25, 26). Other clonal relationships may also be present in this selected population of isolates.

In these isolates, the highest ceftolozane-tazobactam MIC was 1.5 μg/ml against a CTX-M-15-producing isolate tested by Etest (rounded up to 2 μg/ml), corresponding to an MIC of 1 μg/ml by BMD. When the Etest and BMD data were aligned by strain, only nine (3.7%) of the ceftolozane-tazobactam results differed by more than one doubling dilution; six exhibited 4-fold lower MICs and three had 4-fold higher MICs by Etest. The zone diameters for a 30-μg–10-μg ceftolozane-tazobactam disk ranged from 18 to 31 mm for all isolates, with 83.3% (204/245) of the zones in the range of 24 to 28 mm and 92.2% (226/245) between 23 and 29 mm (Table 4).

In this study, ceftolozane-tazobactam exhibited consistent inhibitory activity against recent *E. coli* clinical isolates carrying the widespread CTX-M-14 and CTX-M-15 ESBLs, and it may provide a novel therapeutic option in the future for the treatment of infections caused by these organisms.

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