

## Effects of Inoculum and $\beta$ -Lactamase Activity in AmpC- and Extended-Spectrum $\beta$ -Lactamase (ESBL)-Producing *Escherichia coli* and *Klebsiella pneumoniae* Clinical Isolates Tested by Using NCCLS ESBL Methodology

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*Escherichia coli* and *Klebsiella pneumoniae* isolates with extended-spectrum  $\beta$ -lactamases (ESBLs) or AmpC cephalosporinases generally respond as predicted to NCCLS tests for ESBL production. However, inoculum size may affect MICs. The effect of inoculum level in clinical isolates expressing  $\beta$ -lactamases were studied at inocula within 0.5 log unit of the standard inoculum, using broth microdilution methodology with ceftazidime, cefotaxime, cefepime, cefpodoxime, and aztreonam. Strains with TEM-1 or no  $\beta$ -lactamases gave consistent MIC results with inocula of  $10^5$  and  $10^6$  CFU/ml. When the bacteria were screened for ESBL production and the lower inoculum was used, several strains with ESBLs, including CTX-M-10, TEM-3, TEM-10, TEM-12, TEM-6, SHV-18, and K1, gave false-negative results for one or more antimicrobial agents (MICs below the NCCLS screening concentration for detecting suspected ESBLs). When the higher inoculum was used, MICs of at least one antimicrobial agent increased at least fourfold in strains producing TEM-3, TEM-10, TEM-28, TEM-43, SHV-5, SHV-18, and K1. All antimicrobial agents showed an inoculum effect with at least one ESBL producer. Confirmatory clavulanate effects were seen for both inocula for all ESBL-producing strains with all antimicrobial agents tested, except for the CTX-M-10-producing *E. coli* with ceftazidime and the SHV-18-producing *K. pneumoniae* with cefotaxime. In kinetic studies, cefpodoxime and cefepime were hydrolyzed by ESBLs in a manner similar to that of cefotaxime. When total  $\beta$ -lactamase activity and hydrolysis parameters were evaluated, however, no single factor was predictive of inoculum effects. These results indicate that the NCCLS screening and confirmation tests are generally predictive of ESBL production, but false-negative results can arise when a lower inoculum is used in testing.

Extended-spectrum  $\beta$ -lactamases (ESBLs) are considered one of the most important resistance mechanisms for penicillins and cephalosporins when these enzymes are produced in *Escherichia coli* and *Klebsiella* spp. (7). The genes encoding these enzymes are most often carried by multidrug-resistant plasmids and are capable of being readily transferred among different species of the family *Enterobacteriaceae* (2). Infections caused by ESBL-producing pathogens may not be responsive to treatment by most penicillins and cephalosporins (32). Hence, their appearance in a hospital setting creates a situation in which ESBL-producing organisms should be identified quickly so that appropriate antibiotic usage and containment measures can be implemented (21).

Clinical microbiologists have devised a number of testing strategies based on phenotypic testing to identify putative ESBL-producing organisms. One testing strategy is the recently adopted set of NCCLS guidelines for *E. coli*, *Klebsiella pneumoniae*, and *Klebsiella oxytoca* isolates with elevated cephalosporin MICs (16). In the protocol for MIC testing, strains with cefotaxime, ceftazidime, ceftriaxone, or aztreonam MICs of  $\geq 2$   $\mu$ g/ml or cefpodoxime MICs of  $\geq 8$   $\mu$ g/ml are suspected

of producing an ESBL; confirmation tests are then performed with ceftazidime and cefotaxime with and without the  $\beta$ -lactamase inhibitor clavulanic acid. ESBL production is confirmed when a  $\geq 3$  twofold concentration decrease in MIC for either cephalosporin is observed in the comparative tests in the absence and presence of clavulanate. Similar testing is recommended for disk diffusion determinations (15). When ESBL production is confirmed, susceptibility results for aztreonam and for all penicillins and cephalosporins, excluding cephamycins, are to be reported as resistant.

Many questions have arisen as a result of the NCCLS recommendations. Why must both cefotaxime and ceftazidime be tested? Should cefepime be treated the same as the other extended-spectrum cephalosporins? Why does cefpodoxime not behave like the other reporter cephalosporins? How does inoculum affect the MICs obtained? Although some of these questions have been addressed with phenotypic approaches (19, 30), there has been no comparative study of the microbiological properties of these cephalosporins and the biochemical properties of recently identified ESBLs and AmpC  $\beta$ -lactamases that may appear in *E. coli* and *Klebsiella* spp. In many cases, the ESBLs found in clinical isolates efficiently hydrolyze cefotaxime and/or ceftazidime, and these  $\beta$ -lactams are often used in hydrolytic profile studies. However, for cephalosporins of more recent interest, such as cefepime and cefpodoxime, minimal hydrolysis data are available to characterize the action

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TABLE 1. Bacterial strains used in this study

Strain designation	$\beta$ -Lactamase(s)	Functional group(s) <sup>a</sup>	$\beta$ -Lactamase sp act <sup>b</sup>	Reference	Original strain designation
<i>E. coli</i>					
ATCC 25922	None	None	16		
ATCC 35218	TEM-1	2b	700		
OC4075	TEM-1	2b	7,500	10	TEM
OC4229	TEM-1, SHV-12	2b, 2be	3,800	32	EC 2859 case 12
OC6028	TEM-1, CTX-M-10	2b, 2be	2,500	18	97/38582
OC4087	TEM-3	2be	900	27	SC15011
					CF 102
OC6042	TEM-10	2be	370	33	166
OC4227	TEM-12	2be	1,700	32	EC 1924 case 10
OC6043	TEM-28	2be	2,500	3	2300
OC6044	TEM-43	2be	4,100	33	156
OC4138	AmpC	1	700	32	EC 3102 case 3
OC4136	AmpC	1	1,300	32	EC 1201 case 1
OC4249	ACT-1 <sup>c</sup>	1	12,000	This work	Transformant
<i>K. pneumoniae</i>					
ATCC 13883	None	None	0.35		
ATCC 700603	SHV-18	2be	240	25	K6
OC4244	TEM-1, SHV-5	2b, 2be	690	32	KP 3160 case 20
OC4239	TEM-6, SHV-1	2be, 2b	750	32	KP 2679 case 17
OC4110	TEM-10, SHV-1	2be, 2b	850	26	2351
OC4105	TEM-26, SHV-1	2be, 2b	970	14	SC 15923
OC4074	TEM-1, MIR-1 <sup>c</sup>	2b, 1	12,000	20	96D
OC4250	ACT-1, 2 TEM type, 2 SHV type	1, 2b, 2be, unknown	17,000	4	MCQ-95
OC5064	FOX-5 <sup>c</sup> , TEM type, SHV-11	1, 2b	3,200	24	OC5064
<i>K. oxytoca</i> OC4076	K-1	2be	14,000	23	SC10436

<sup>a</sup> Functional group according to reference 8.

<sup>b</sup> Specific activity reported in nanomoles of nitrocefin hydrolyzed per minute per milligram of protein.

<sup>c</sup> Plasmid-encoded AmpC  $\beta$ -lactamase.

of ESBLs on these  $\beta$ -lactams. Also, the effect of hydrolysis on small variations in inoculum has not been fully characterized.

In the set of studies described herein, a limited set of clinical isolates of *E. coli*, *K. pneumoniae*, and *K. oxytoca* was selected for their production of some of the most commonly identified ESBL types in the United States and Europe. These strains were characterized for ESBL production using NCCLS methodology with inocula within 0.5 log unit of the NCCLS-recommended  $5 \times 10^5$  CFU/ml standard. Hydrolysis data for purified enzymes against cefotaxime, ceftazidime, cefpodoxime, cefepime, and aztreonam were determined. Although NCCLS methodology includes ceftioxone among the cephalosporins that may be used for ESBL testing, this cephalosporin was not studied. Previous work has shown that the biochemical and microbiological profiles of ceftioxone were similar to those of cefotaxime in ESBL producers (22). Instead, cefepime was selected for this study because we wished to compare the microbiological and enzymatic characteristics of five distinguishable  $\beta$ -lactams against the same ESBL-producing organisms. In addition, several AmpC, functional group 1 (8), cephalosporinase-producing isolates were included to show the effects of these antimicrobial agents on strains with non-ESBL-producing, cephalosporin-hydrolyzing enzymes. Note that for many strains, multiple enzymes were present, especially in *K. pneumoniae*, as these represent a frequently increasing set of ESBL-producing isolates that are being observed in many hospitals.

#### MATERIALS AND METHODS

**Strains.** The clinical isolates of *E. coli*, *K. pneumoniae*, and *K. oxytoca* used in this study are listed in Table 1. Original strain numbers and sources are listed

where known. For strains that contained multiple  $\beta$ -lactamases or clinical strains with low  $\beta$ -lactamase expression, the ESBL was purified from the following *E. coli* cloning strains in which the ESBL gene is on a plasmid: OC4249 for ACT-1, OC5032 for SHV-18, and OC4107 for TEM-26.

**Antimicrobial agents.** The following  $\beta$ -lactams were used for both susceptibility testing and biochemical assays. Extinction coefficients used in the kinetic assays are given in parentheses for each of the  $\beta$ -lactamase substrates. Ceftazidime ( $\Delta\epsilon_{260} = -8,660 \text{ M}^{-1} \text{ cm}^{-1}$ ), cefotaxime ( $\Delta\epsilon_{267} = -6,690 \text{ M}^{-1} \text{ cm}^{-1}$ ), and clavulanic acid were obtained from U.S. Pharmacopeia (Rockville, Md.). Cefepime ( $\Delta\epsilon_{265} = -5,160 \text{ M}^{-1} \text{ cm}^{-1}$ ) and aztreonam ( $\Delta\epsilon_{318} = -660 \text{ M}^{-1} \text{ cm}^{-1}$ ) were gifts from Bristol-Myers Squibb (Princeton, N.J.). Cefpodoxime ( $\Delta\epsilon_{280} = -2,710 \text{ M}^{-1} \text{ cm}^{-1}$ ) was a gift from Pharmacia Corporation (Kalamazoo, Mich.), and tazobactam was a gift from Wyeth (Pearl River, N.Y.). Cephaloridine ( $\Delta\epsilon_{295} = -889 \text{ M}^{-1} \text{ cm}^{-1}$ ) and benzylpenicillin ( $\Delta\epsilon_{240} = -546 \text{ M}^{-1} \text{ cm}^{-1}$ ) were purchased from Sigma (St. Louis, Mo.). Nitrocefin ( $\Delta\epsilon_{495} = -14,060 \text{ M}^{-1} \text{ cm}^{-1}$ ) was obtained from Becton-Dickinson (Sparks, Md.).

**Susceptibility testing.** MICs were determined by the NCCLS broth microdilution method in cation-adjusted Mueller-Hinton broth (Becton Dickinson) using inocula of  $10^5$ ,  $10^6$ , and  $5 \times 10^7$  CFU/ml and drug concentrations from 0.12 to 128  $\mu\text{g/ml}$  (16). For some strains, a small degree of haze on the bottom of the wells was observed for all drug concentrations at the higher inocula. This haze appeared different from turbid growth and was assumed to be settling due to the high inoculum. Haze was ignored when reading MICs. Plate counts were used to verify inocula.

**$\beta$ -Lactamase purification.** All  $\beta$ -lactamases were purified from 2- to 4-liter cultures grown overnight at 37°C in tryptic soy broth (Difco) containing 50  $\mu\text{g}$  of ampicillin per ml. Cells were harvested by centrifugation, washed in 50 mM phosphate buffer (pH 7.0), and resuspended in 5 ml of 0.2 M sodium acetate (pH 5.5). The cells were then subjected to four freeze-thaw cycles (9), followed by centrifugation at 20,000  $\times g$ . The supernatants that were produced were loaded onto a Superdex 75 gel filtration column (Amersham-Pharmacia, New Brunswick, N.J.) and eluted in 50 mM phosphate buffer (pH 7.0). Fractions with nitrocefin-hydrolyzing activity were pooled, and in some cases, further purified by ion-exchange chromatography. The column, buffer, and pH chosen were dependent on the isoelectric point (pI) of the  $\beta$ -lactamase. Details about purification of individual enzymes are available upon request. Purity of  $\beta$ -lactamase prepa-

TABLE 2. *E. coli* MIC values for extended-spectrum cephalosporins and aztreonam

Strain	$\beta$ -lactamase	Inoculum (CFU/ml)	MIC ( $\mu$ g/ml) of antimicrobial agent(s) <sup>a</sup> :									
			CTX	CTX-CA	CAZ	CAZ-CA	ATM	ATM-CA	CPD	CPD-CA	FEP	FEP-CA
ATCC 25922	None	10 <sup>5</sup>	≤0.12	≤0.12	0.5	0.25	≤0.12	≤0.12	0.5	0.5	≤0.12	≤0.12
		10 <sup>6</sup>	≤0.12	≤0.12	0.25	0.25	0.25	≤0.12	0.5	0.5	≤0.12	≤0.12
ATCC 35218	TEM-1	10 <sup>5</sup>	≤0.12	≤0.12	≤0.12	≤0.12	≤0.12	≤0.12	0.25	0.25	≤0.12	≤0.12
		10 <sup>6</sup>	≤0.12	≤0.12	0.25	≤0.12	≤0.12	≤0.12	0.5	0.25	≤0.12	≤0.12
OC4075	TEM-1	10 <sup>5</sup>	≤0.12	≤0.12	0.5	0.25	≤0.12	≤0.12	0.5	0.25	≤0.12	≤0.12
		10 <sup>6</sup>	≤0.12	≤0.12	0.5	0.5	0.25	≤0.12	1	0.5	≤0.12	≤0.12
OC4229	TEM-1, SHV-12	10 <sup>5</sup>	2	≤0.12	16	0.25	16	≤0.12	4	0.5	0.25	≤0.12
		10 <sup>6</sup>	2	≤0.12	32	0.5	16	0.25	16	0.5	0.5	≤0.12
OC6028	TEM-1, CTX-M-10	10 <sup>5</sup>	8	≤0.12	0.5	0.25	1	≤0.12	32	0.5	2	≤0.12
		10 <sup>6</sup>	16	≤0.12	1	0.25	2	≤0.12	64	1	2	0.25
OC4087	TEM-3	10 <sup>5</sup>	4	≤0.12	4	0.25	1	≤0.12	128	0.25	1	≤0.12
		10 <sup>6</sup>	8	≤0.12	32	0.25	4	≤0.12	>128	0.5	2	≤0.12
OC6042	TEM-10	10 <sup>5</sup>	1	≤0.12	128	0.5	32	≤0.12	32	0.5	4	≤0.12
		10 <sup>6</sup>	2	≤0.12	128	0.5	32	≤0.12	128	0.5	4	≤0.12
OC4227	TEM-12	10 <sup>5</sup>	0.25	≤0.12	8	0.5	1	≤0.12	8	1	1	≤0.12
		10 <sup>6</sup>	1	≤0.12	32	1	1	≤0.12	8	1	16	≤0.12
OC6043	TEM-28	10 <sup>5</sup>	2	0.25	>128	1	128	0.25	32	1	8	≤0.12
		10 <sup>6</sup>	16	0.25	>128	2	>128	0.5	>128	2	32	0.25
OC6044	TEM-43	10 <sup>5</sup>	32	0.25	>128	2	>128	0.5	>128	1	16	0.5
		10 <sup>6</sup>	>128	0.25	>128	4	>128	0.5	>128	2	>128	0.5
OC4138	AmpC	10 <sup>5</sup>	1	0.5	2	1	4	2	16	8	≤0.12	≤0.12
		10 <sup>6</sup>	2	2	2	2	4	4	32	16	≤0.12	≤0.12
OC4136	AmpC derepressed	10 <sup>5</sup>	8	4	128	64	16	8	128	64	1	0.5
		10 <sup>6</sup>	8	4	128	64	16	16	>128	64	2	1
OC4249	ACT-1	10 <sup>5</sup>	8	8	16	16	8	8	16	64	≤0.12	≤0.12
		10 <sup>6</sup>	16	16	16	16	64	16	64	128	≤0.12	≤0.12

<sup>a</sup> The antimicrobial agents were each tested alone and with clavulanic acid (CA) at 4  $\mu$ g/ml. Drug abbreviations: CTX, cefotaxime; CAZ, ceftazidime; ATM, aztreonam; CPD, cefpodoxime; FEP, cefepime.

rations was examined on Novex NuPAGE 10% BT gels stained with colloidal blue (Invitrogen, Carlsbad, Calif.). Protein concentrations were measured by the MicroBCA protein assay microwell format (Pierce, Rockford, Ill.).

**Hydrolysis studies.** Initial hydrolysis rates of  $\beta$ -lactam substrates were measured in 50 mM phosphate buffer (pH 7.0) using a Shimadzu 1601-UV spectrophotometer at 25°C.  $\beta$ -Lactam substrates were made fresh as 1-mg/ml stocks. Hydrolysis rates were measured at least twice, with cephaloridine included as a reference each day.  $K_m$  and  $V_{max}$  values were calculated by averaging the results from Hanes, Eadie-Hofstee, and Cornish-Bowden plots and a least-squares fit to the Michaelis-Menten equation. Values for  $k_{cat}$  were calculated if the  $\beta$ -lactamase purity was >75% by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Specific activity was measured in sonicated cell extracts (9) using 100  $\mu$ M nitrocefin as a substrate, and corrected for protein concentration as determined by the Pierce MicroCoomassie blue protein assay.

## RESULTS

**Susceptibility testing at the lower and upper ranges of NCCLS inocula.** The clinical isolates used in this study are shown in Table 1. They include classic historical isolates, such as the original TEM-1 strain described by Datta and Richmond (10) and recently isolated strains that carry ESBLs alone or in combination with TEM-1 or SHV-1. Also included were NCCLS quality control standards, AmpC-producing strains, and the *K. oxytoca* strain producing the K1 group 2be enzyme, originally defined in 1989 as a functional ESBL (6). Both *E. coli* and *Klebsiella* spp. were represented.

The current NCCLS protocol indicates that *E. coli* and *Klebsiella* strains with cefotaxime, ceftazidime, ceftriaxone, or aztreonam MICs of  $\geq 2$   $\mu$ g/ml or with cefpodoxime MICs of  $\geq 8$ , are to be identified as possible ESBL producers and undergo confirmation testing. When inocula within 0.5 log unit of the standard inoculum were used (10<sup>5</sup> and 10<sup>6</sup> CFU/ml), strains with TEM-1 or no  $\beta$ -lactamase had cephalosporin MICs under

2  $\mu$ g/ml that did not differ by more than 1 dilution (Tables 2 and 3). However, several ESBL producers also had  $\beta$ -lactam MICs below 2  $\mu$ g/ml at the lower inoculum, including the NCCLS ESBL reference strain *K. pneumoniae* ATCC 700603, which had a cefotaxime MIC of 1  $\mu$ g/ml. Also in this group were *E. coli* OC4087 (TEM-3) with an aztreonam MIC of 1  $\mu$ g/ml, *K. pneumoniae* OC4239 (TEM-6) with a cefotaxime MIC of 0.25  $\mu$ g/ml, *K. pneumoniae* OC4110 and *E. coli* OC6042 (both with TEM-10) with cefotaxime MICs of 1  $\mu$ g/ml, *K. pneumoniae* OC4227 (TEM-12) with cefotaxime and aztreonam MICs of  $\leq 1$   $\mu$ g/ml, *E. coli* OC6028 (CTX-M-10) with a ceftazidime MIC of 0.5  $\mu$ g/ml, *K. oxytoca* OC4076 (K1) with cefotaxime and ceftazidime MICs of  $\leq 1$   $\mu$ g/ml, and *E. coli* OC4229 (SHV-12) with a cefpodoxime MIC of 4  $\mu$ g/ml. Even at the higher inoculum of 10<sup>6</sup> CFU/ml, MICs were not above 2  $\mu$ g/ml when tested in the following combinations: *E. coli* OC6028 (CTX-M-10) for ceftazidime, *E. coli* OC4227 (TEM-12) for cefotaxime and aztreonam, *K. pneumoniae* OC4239 (TEM-6) for cefotaxime, and *K. oxytoca* OC4076 (K1) for ceftazidime.

In addition to their ability to hydrolyze extended-spectrum cephalosporins, ESBLs are identified in confirmation testing by their inhibition by clavulanic acid in combination with both cefotaxime and ceftazidime. As expected, clavulanic acid reduced MICs for most of the ESBL-containing strains tested with cefotaxime, ceftazidime, cefepime, cefpodoxime, and aztreonam (Tables 2 and 3). There were, however, several cases where the clavulanic acid confirmation failed with ESBL-producing strains, e.g., for cefpodoxime in *K. pneumoniae* ATCC 700603 (SHV-18) at the higher inoculum and for ceftazidime in *E. coli* OC6028 (CTX-M-10) at both inocula.

TABLE 3. *K. pneumoniae* MIC values for extended-spectrum cephalosporins and aztreonam

Strain	$\beta$ -Lactamase	Inoculum (CFU/ml)	MIC ( $\mu$ g/ml) of antimicrobial agent(s) <sup>a</sup> :									
			CTX	CTX-CA	CAZ	CAZ-CA	ATM	ATM-CA	CPD	CPD-CA	FEP	FEP-CA
ATCC 13883	None	10 <sup>5</sup>	$\leq 0.12$	$\leq 0.12$	$\leq 0.12$	$\leq 0.12$	$\leq 0.12$	$\leq 0.12$	$\leq 0.12$	$\leq 0.12$	$\leq 0.12$	$\leq 0.12$
		10 <sup>6</sup>	$\leq 0.12$	$\leq 0.12$	0.25	$\leq 0.12$	$\leq 0.12$	$\leq 0.12$	$\leq 0.12$	$\leq 0.12$	$\leq 0.12$	$\leq 0.12$
ATCC 700603	SHV-18	10 <sup>5</sup>	1	0.5	8	0.5	2	0.25	8	1	0.5	0.5
		10 <sup>6</sup>	4	0.5	8	1	4	0.25	8	2	0.5	0.5
OC4244	SHV-5, TEM-1	10 <sup>5</sup>	>128	0.25	>128	0.5	>128	$\leq 0.12$	>128	0.5	32	$\leq 0.12$
		10 <sup>6</sup>	>128	0.25	>128	1	>128	$\leq 0.12$	>128	0.5	>128	$\leq 0.12$
OC4239	TEM-6, SHV-1	10 <sup>5</sup>	0.25	$\leq 0.12$	64	1	32	$\leq 0.12$	16	0.5	2	$\leq 0.12$
		10 <sup>6</sup>	0.5	$\leq 0.12$	>128	2	64	$\leq 0.12$	64	0.25	2	$\leq 0.12$
OC4110	TEM-10	10 <sup>5</sup>	1	$\leq 0.12$	128	1	64	$\leq 0.12$	32	0.25	2	$\leq 0.12$
		10 <sup>6</sup>	4	$\leq 0.12$	>128	1	128	$\leq 0.12$	>128	0.5	2	$\leq 0.12$
OC4105	TEM-26, SHV-1	10 <sup>5</sup>	2	$\leq 0.12$	>128	4	128	0.25	16	0.25	8	$\leq 0.12$
		10 <sup>6</sup>	2	$\leq 0.12$	>128	4	128	0.25	32	0.5	16	0.25
OC4074	MIR-1, TEM-1	10 <sup>5</sup>	8	16	32	32	16	16	64	128	0.5	0.5
		10 <sup>6</sup>	8	16	32	32	16	32	128	>128	1	1
OC4076 <sup>b</sup>	K-1	10 <sup>5</sup>	1	$\leq 0.12$	0.25	$\leq 0.12$	16	$\leq 0.12$	8	$\leq 0.12$	1	$\leq 0.12$
		10 <sup>6</sup>	2	$\leq 0.12$	0.25	$\leq 0.12$	128	0.25	32	0.25	4	0.25
OC4250	ACT-1, TEM, SHV	10 <sup>5</sup>	16	32	64	32	32	32	64	64	1	0.5
		10 <sup>6</sup>	32	32	128	64	32	32	>128	>128	2	0.5
OC5064	FOX-5, TEM, SHV	10 <sup>5</sup>	16	16	64	64	4	4	128	128	1	0.5
		10 <sup>6</sup>	16	32	64	128	8	8	>128	128	1	1

<sup>a</sup> The antimicrobial agents were each tested alone and with clavulanic acid (CA) at 4  $\mu$ g/ml. Drug abbreviations: CTX, cefotaxime; CAZ, ceftazidime; ATM, aztreonam; CPD, cefpodoxime; FEP, cefepime.

<sup>b</sup> *K. oxytoca*.

The effect of inoculum on the NCCLS ESBL screening test was evaluated using the criterion of an increase in MIC of at least fourfold between the 10<sup>5</sup> and 10<sup>6</sup> inocula. If the MIC at an inoculum of 10<sup>5</sup> CFU was  $\geq 128$   $\mu$ g/ml or  $\leq 0.12$   $\mu$ g/ml, the strain could not be evaluated for an inoculum effect. As shown in Tables 2 and 3, *E. coli* and *K. pneumoniae* strains lacking a  $\beta$ -lactamase or expressing TEM-1 have similar  $\beta$ -lactam MICs that do not appear to be affected by a 10-fold increase in inoculum, although this cannot be assessed for antimicrobial agents with MICs of  $\leq 0.12$   $\mu$ g/ml. However, when the higher inoculum was used with ESBL-producing strains, MIC increases were seen for selected bacterium-drug combinations.

A fourfold or higher increase in MIC was observed for cefotaxime in strains *K. pneumoniae* OC4110 (TEM-10), *E. coli* OC4227 (TEM-12), *E. coli* OC6043 (TEM-28), *E. coli* OC6044 (TEM-43), and *K. pneumoniae* ATCC 700603 (SHV-18) at the two inocula. MICs increased for ceftazidime with *E. coli* OC4087 (TEM-3), *K. pneumoniae* OC4239 (TEM-6), and *E. coli* OC4227 (TEM-12). Cefepime MICs increased with increasing inoculum with *E. coli* OC4227 (TEM-12), *E. coli* OC6043 (TEM-28), *E. coli* OC6044 (TEM-43), *K. pneumoniae* OC4244 (SHV-5), and *K. oxytoca* OC4076 (K1). Cefpodoxime MICs were high for ESBL- and AmpC-producing strains, with inoculum effects observed for *E. coli* OC4229 (SHV-12), *E. coli* OC6042 and *K. pneumoniae* OC4110 (TEM-10), *E. coli* OC6043 (TEM-28), *E. coli* OC4249 and *K. pneumoniae* OC4250 (ACT-1), *K. pneumoniae* OC4239 (TEM-6), and *K. oxytoca* OC4076 (K1). Only two strains had decreased susceptibility when tested at the higher inoculum with aztreonam, *K. oxytoca* OC4076 (K1) and *E. coli* OC4087 (TEM-3).

The addition of clavulanic acid reduced or eliminated the inoculum effect in all of the ESBL-containing strains (Tables 2 and 3). This supports the view that  $\beta$ -lactamase activity contributes to the inoculum effect. As expected, the presence of

clavulanic acid did not reduce the MICs of the AmpC-containing strains, which are not inhibited by this compound.

At an inoculum of  $5 \times 10^7$  CFU/ml, the MICs for all strains (except *E. coli* OC4075) were  $>32$   $\mu$ g/ml (data not shown). This inoculum is 2 orders of magnitude higher than the NCCLS-recommended inoculum but one that can be present in localized infections. Elevated MICs were seen even in the strains without  $\beta$ -lactamases, *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 13883.

**Hydrolysis parameters of the  $\beta$ -lactamases.** In an effort to correlate the kinetic characteristics of the  $\beta$ -lactamases with the MIC testing results, the  $K_m$ ,  $V_{max}$ ,  $k_{cat}$ , and hydrolytic efficiency ( $k_{cat}/K_m$ ) values for the  $\beta$ -lactamases were obtained (Table 4). TEM-1 is known to hydrolyze cephaloridine and benzylpenicillin readily but hydrolyzes cefotaxime and ceftazidime at rates several orders of magnitude lower (31). Similarly, the newer cephalosporins cefepime and cefpodoxime were not hydrolyzed efficiently by TEM-1, with  $k_{cat}$  values less than 1.2 s<sup>-1</sup>. In contrast, the ESBLs of the TEM and SHV types generally demonstrated measurable hydrolysis for the extended-spectrum cephalosporins. In some cases, no hydrolysis was measured for some of the  $\beta$ -lactamases that demonstrated clearly defined substrate specificity. For example, CTX-M-10 hydrolyzed cefotaxime, cefepime, and cefpodoxime at similar rates but did not hydrolyze ceftazidime or aztreonam efficiently. The oxyimino-cephalosporin hydrolysis profile of enzyme K1 was similar to that of CTX-M-10, but K1 also hydrolyzed aztreonam. Cefepime and cefpodoxime were hydrolyzed by both of the enzymes that exhibited substrate preferences. Generally, cefepime and cefpodoxime were hydrolyzed at rates within the same order of magnitude as those for cefotaxime.

The enzymes of group 1 AmpC type, FOX-5 and ACT-1, demonstrated very low hydrolysis rates for all of the extended-spectrum cephalosporins and aztreonam. Of these compounds,



negative results. This group of strains included the NCCLS ESBL control strain ATCC 700603, which had a cefotaxime MIC of 1  $\mu\text{g/ml}$  in the screening test.

It has been observed that an increase in inoculum size can be correlated with an increase in  $\beta$ -lactam MICs and a decrease in efficacy in vivo (11, 12, 29, 30). Factors that could contribute to these effects are the number, type, and amount of  $\beta$ -lactamases; outer membrane permeability; efflux; number and susceptibility of penicillin-binding protein targets; and phase of growth. Strain dependence has also been observed (1). In this work, we sought to correlate the NCCLS ESBL testing protocols and inoculum effects with levels and hydrolysis properties of ESBL and AmpC  $\beta$ -lactamases.

Our data indicate that an ESBL has the potential to increase cephalosporin MICs when organisms are tested at a slightly elevated standard inoculum. First, if there were no  $\beta$ -lactamases present or if there was no hydrolysis of  $\beta$ -lactams, no inoculum effect was observed (ATCC 25922, ATCC 13883, and the TEM-1-producing strains). Second, ESBLs were associated with the inoculum effect when extended-spectrum  $\beta$ -lactams were tested. Thomson and Moland proposed that the size of the inoculum effect depended on the amount of hydrolysis (30). In our experiments, however, the inoculum effect did not directly correlate with  $k_{\text{cat}}$  (or relative  $V_{\text{max}}$ ) for the  $\beta$ -lactamase expressed by the strain, but catalytic efficiency seemed to provide better predictability. Additionally, the amount of  $\beta$ -lactamase activity did not always correlate with the inoculum effect observed. These results indicate that a number of factors contribute to the inoculum effect and that the presence and activity of a  $\beta$ -lactamase are only two of the factors involved.

At the highest inoculum of  $5 \times 10^7$  CFU/ml, MICs of  $>32$   $\mu\text{g/ml}$  for all drugs were observed for most strains, regardless of  $\beta$ -lactamase status. Inoculum effects of more than 4 doubling dilutions have been previously reported for the NCCLS quality control strain *E. coli* ATCC 25922 (28). Additionally, at this high inoculum, clavulanate was ineffective in reducing the MICs of ESBL-producing strains. The resistance at this inoculum, even in non- $\beta$ -lactamase-producing strains, is most likely due to the excess number of target penicillin-binding proteins in the bacterial population compared to the fixed drug concentration in the testing medium.

Hydrolysis of extended-spectrum cephalosporins was observed for the ESBLs but not the AmpC enzymes, with the exception of cefpodoxime and FOX-5. In addition to the common cephalosporins cefotaxime and ceftazidime that are included in most  $\beta$ -lactamase characterizations (8), cefepime and cefpodoxime were hydrolyzed by ESBLs of TEM, SHV, and CTX-M types. In general, cefepime had lower MICs than ceftazidime, cefotaxime, and cefpodoxime, but similar  $k_{\text{cat}}$  values were observed for the extended-spectrum  $\beta$ -lactams. This may be due to increased permeability of cefepime into members of the family *Enterobacteriaceae* (17). However, inoculum effects in ESBL-producing *Klebsiella* spp. have been reported for cefepime both in vitro and in vivo (12, 29), and they could be due, at least in part, to the hydrolysis of this drug by ESBLs.

There was slow hydrolysis of the extended-spectrum cephalosporins ( $k_{\text{cat}}$  values of  $<1.5 \text{ s}^{-1}$ ) by the group 1  $\beta$ -lactamases ACT-1 and FOX-5, close relatives of the chromosomal AmpC  $\beta$ -lactamases from *Pseudomonas*, *Enterobacter*, and other bacterial species (4, 24). High levels of AmpC  $\beta$ -lactamase activity

with low hydrolysis rates have been associated with cefepime resistance in *Pseudomonas aeruginosa* (11). Therefore, hydrolysis rates alone are not sufficient to explain the high MICs in the AmpC-producing strains, but both rate and amount of enzyme must be taken into account. The high specific activities found in our *E. coli* and *Klebsiella* strains may contribute to the high MICs in multiple ways. (i) The low rate of hydrolysis by a large amount of excess  $\beta$ -lactamase is sufficient to inactivate a high proportion of the  $\beta$ -lactam. (ii) The low  $K_m$  values may contribute to high catalytic efficiencies for some substrates, such as cefpodoxime. (iii) The  $\beta$ -lactamases bind enough  $\beta$ -lactam molecules to lower the effective drug concentration. This is supported by the observation that strains with very high levels of non-ESBLs, such as SHV-1, and with low cephalosporin hydrolysis rates can also have elevated MICs (13).

Cefpodoxime was hydrolyzed well by both types of substrate-specific ESBLs, CTX-M-10 and K1 enzymes. Although cefpodoxime is not a good substrate for AmpC enzymes, cefpodoxime MICs were above 8  $\mu\text{g/ml}$  for both *E. coli* and *K. pneumoniae* AmpC-producing strains. There was a recent proposal for the use of cefpodoxime as an antimicrobial agent for ESBL testing (5). However, false-positive results have been reported with cefpodoxime, due to at least five different phenotypic characterizations (19), including its ability to be hydrolyzed by a variety of  $\beta$ -lactamases in organisms with porin channels.

As expected, hydrolytic specificity for cefotaxime was demonstrated by the CTX-M, TEM-3, and K1 enzymes, while the TEM-6, TEM-10, and TEM-26 enzymes showed preferential hydrolysis of ceftazidime. This is the basis for the NCCLS recommendation for ESBL confirmation to be done with both drugs. Our results give further support to this NCCLS recommendation, because several strains would be falsely considered negative in the screening test if only one of these cephalosporins were tested. However, if all strains were initially screened with both cefotaxime and ceftazidime, using an inoculum at the higher end of NCCLS recommendations, all ESBLs and AmpC producers would have been identified as potential resistant organisms. These data suggest that  $\beta$ -lactamase-positive, resistant organisms with either ESBL or AmpC  $\beta$ -lactamases could be identified if cephalosporin MICs of  $\geq 2$   $\mu\text{g/ml}$  were observed by initial screening with both cefotaxime and ceftazidime. For this screening to provide valid results, however, low inoculum levels should be avoided.

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