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Use of Microdilution Panels with and without β-Lactamase Inhibitors as a Phenotypic Test for β-Lactamase Production among *Escherichia coli*, *Klebsiella* spp., *Enterobacter* spp., *Citrobacter freundii*, and *Serratia marcescens*

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Over the past decade, a number of new β-lactamases have appeared in clinical isolates of *Enterobacteriaceae* that, unlike their predecessors, do not confer β-lactam resistance that is readily detected in routine antibiotic susceptibility tests. Because optimal methodologies are needed to detect these important new β-lactamases, a study was designed to evaluate the ability of a panel of various β-lactam antibiotics tested alone and in combination with β-lactamase inhibitors to discriminate between the production of extended-spectrum β-lactamases, AmpC β-lactamases, high levels of K1 β-lactamase, and other β-lactamases in 141 isolates of *Escherichia coli*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Enterobacter cloacae*, *Enterobacter aerogenes*, *Citrobacter freundii*, and *Serratia marcescens* possessing well-characterized β-lactamases. The microdilution panels studied contained aztreonam, cefpodoxime, ceftazidime, cefotaxime, and ceftriaxone, with and without 1, 2, and 4 μg of clavulanate per ml or 8 μg of sulbactam per ml and cefoxitin and cefotetan with and without 8 μg of sulbactam per ml. The results indicated that a minimum panel of five tests would provide maximum separation of extended-spectrum β-lactamase high AmpC, high K1, and other β-lactamase production in *Enterobacteriaceae*. These included cefpodoxime, cefpodoxime plus 4 μg of clavulanate per ml, ceftazidime, ceftriaxone, and ceftriaxone plus 8 μg of sulbactam per ml. Ceftriaxone plus 2 μg of clavulanate per ml could be substituted for cefpodoxime plus 4 μg of clavulanate per ml without altering the accuracy of the tests. This study indicated that tests with key β-lactam drugs, alone and in combination with β-lactamase inhibitors, could provide a convenient approach to the detection of a variety of β-lactamases in members of the family *Enterobacteriaceae*.

Over the past decade, there have appeared a number of new β-lactamases in clinical isolates of members of the family *Enterobacteriaceae* (1–12, 14–17, 20, 25–28, 34–40, 42, 46, 50, 51, 54, 55). Unlike their predecessors, many of these enzymes do not confer β-lactam resistance that is readily detected in routine antibiotic susceptibility tests (12, 18, 22–24, 46, 51, 53, 56). Nevertheless, it is apparent that at least some of these enzymes confer resistance to expanded-spectrum cephalosporins and aztreonam. For example, there are an increasing number of reports of clinical failure of these drugs to adequately treat infections caused by *Enterobacteriaceae* producing extended-spectrum β-lactamases (ESBLs) (10, 46, 51). High-level production of AmpC β-lactamase by *Escherichia coli* and production of plasmid-mediated AmpC β-lactamases by various members of the family *Enterobacteriaceae* may also cause similar diagnostic problems, but published therapeutic data are currently lacking. Although the detection of resistance produced by each of these β-lactamases can be problematic for the clinical laboratory, the majority of recent interest has focused on the accurate detection of ESBLs.

ESBL detection is a problem confronting clinical laboratories worldwide. Mutated from older β-lactamases of gram-negative bacteria, such as TEM-1, TEM-2, and SHV-1, ESBLs have extended substrate profiles that include drugs such as aztreonam and the newer cephalosporins as well as penicillins and the older cephalosporins (19, 28, 37). Currently, there are two main approaches to enhancing the detection of ESBL-producing *Enterobacteriaceae*. The first involves the use of modified susceptibility criteria for certain indicator drugs to screen for the presence of ESBL-producing *Enterobacteriaceae* (30, 31, 51, 53). This approach was developed because, although the results for some ESBL-producing strains of *E. coli* and *Klebsiella* spp. fall in the susceptible range for cefotaxime, ceftriaxone, ceftazidime, and aztreonam, they are different from those obtained with strains that produce older β-lactamases, such as TEM-1, TEM-2, or SHV-1 (30–32, 51, 53). This approach enhances the detection of ESBLs, but lacks sensitivity and specificity for some strains, and appears relevant for only limited species of *Enterobacteriaceae*, like *E. coli*, *Klebsiella pneumoniae*, and *Proteus mirabilis* (12, 30, 32, 34, 51, 53). In a study using the current National Committee for Clinical Laboratory Standards (NCCLS) ESBL disk screening criteria, Coudron et al. reported that only 3, 4, and 6 out of 10 ESBL-producing strains of *E. coli* were correctly identified in tests with aztreonam, ceftriaxone, and ceftazidime, respectively (12). Using the criterion of ≥2 μg/ml (30, 51) for a positive ESBL screen in a recent study of dilution tests, Thomson and Sanders detected only 43, 57, 93, and 79% of ESBL-producing *E. coli* and *K. pneumoniae* strains in tests with cefotaxime, ceftriaxone, ceftazidime, and aztreonam respectively (53). Furthermore, specificity problems arise in some populations, because strains of *E. coli* and *K. pneumoniae* that produce plasmid-mediated AmpC β-lactamases and strains of *E. coli* that hyperproduce their chromosomally mediated AmpC β-lactamase have sus-

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ceptibility patterns for the indicator drugs similar to those of ESBL-producing strains (1, 4, 14, 16, 21, 25, 35, 36, 39, 54).

These problems of sensitivity and specificity were recently highlighted in a study of the use of commercially available MicroScan microdilution panels as a screen for β-lactamase production in *E. coli* and *Klebsiella* spp. (48). In this study, a cefpodoxime MIC of ≥2 μg/ml was the most accurate indicator of ESBL or AmpC production in isolates of *E. coli* or *K. pneumoniae*. However, no single drug was able to completely discriminate between these two types of β-lactamases. More importantly, the results showed that the currently recommended screening criteria were inadequate for isolates of *Klebsiella oxytoca*, because only ceftazidime or cefotaxime correctly identified ESBL producers among this species (48).

The second approach to the identification of ESBL-producing *E. coli* and *Klebsiella* utilizes special tests to demonstrate enzymatic activity against the indicator drugs (8, 20, 23, 24, 44, 46, 51, 52, 56). The majority of these tests are based on the principle of the double disk test (8, 20), in which a β-lactamase inhibitor is used to potentiate the activity of an indicator drug against an ESBL-producing strain (8, 20, 23, 24, 32, 44, 46, 51). This approach can be used either as a screen or as a confirmatory test for ESBL production.

ESBLs are not the only new β-lactamases that clinical laboratories may be encountering. Although epidemiological data are scanty, the occurrence of new plasmid-mediated derivatives of AmpC β-lactamases (1, 3, 5, 6, 10, 17, 21, 25, 27, 39, 41, 51) and inhibitor-resistant TEM and SHV β-lactamases (7, 10, 15, 26, 40, 49–51, 55), and also of these enzymes in new host species, suggests that these enzymes may be increasing in occurrence. Of these, the plasmid-mediated AmpC β-lactamases present the greatest threat clinically because they most seriously limit therapeutic choices, even more so than ESBLs (39). Inhibitor-resistant TEM and SHV β-lactamases do not appear to cause significant clinical problems at present, especially in the United States.

At present, clinical laboratories may use NCCLS-recommended screening and confirmatory tests to detect ESBL production by *E. coli* and *Klebsiella* spp. (32). There is now a need for screening and confirmatory tests for ESBL detection in organisms other than *E. coli* and *Klebsiella* spp. and also for the detection of plasmid-mediated AmpC β-lactamases. There have been some attempts to address these issues. Thomson and Sanders (52) reported that positive three-dimensional tests with cefoxitin discriminated between strains of *E. coli* and *K. pneumoniae* that produced a plasmid-mediated AmpC β-lactamase and those that produced an ESBL. Smith Moland et al. (47) showed that this test also discriminated between strains that produced a plasmid-mediated AmpC β-lactamase and strains that produced an ESBL and/or possessed a porin mutation that conferred cefoxitin resistance.

Because optimal methodologies are needed to detect these important new β-lactamases, a feasibility study was performed to determine if an isogenic panel of *E. coli* strains containing many different β-lactamases could be used to identify which drugs alone and in combination with β-lactamase inhibitors might be most useful in developing a definitive test for β-lactamases in *Enterobacteriaceae* (13). The results of this feasibility study suggested that cefpodoxime, ceftazidime, and ceftriaxone, with and without clavulanate and the cephamycins, would have the greatest utility. Therefore, a second study was undertaken to assess the ability of a panel of various β-lactam antibiotics tested alone and in combination with β-lactamase inhibitors to discriminate between the production of a variety of different β-lactamases among isolates of *Enterobacteriaceae*. In this study, discrimination between ESBLs, AmpC β-lactamases, high levels of K1 β-lactamase, and other β-lactamases was assessed in isolates of *E. coli*, *K. pneumoniae*, *K. oxytoca*, *Enterobacter cloacae*, *Enterobacter aerogenes*, *C. freundii*, and *S. marcescens* possessing well-characterized β-lactamases. Some of these strains had been used in a previous study of commercially available microdilution panels (48).

TABLE 1. β-Lactamase groupings of study strains

Organism (no. of strains)	No. of strains in β-lactamase group:			
	ESBL[a]	High AmpC[b]	High K1[c]	OBL[d]
E. coli (32)	16 (3)[e]	5	0	11 (1)[f]
K. pneumoniae (36)	20 (17)[g]	5 (1)[h]	0	11 (2)[i]
K. oxytoca (13)	3	0	4	6
Enterobacter spp. and *C. freundii* (48)	16	17	0	15
S. marcescens (12)	4	2	0	6
Total	59	29	4	49

[a] ESBLs (Bush group 2be [10]) included TEM-3, TEM-4, TEM-5, TEM-6, TEM-7, TEM-8, TEM-9, TEM-10, TEM-12, SHV-2, SHV-3, SHV-4, SHV-5, and four TEM- or SHV-derived ESBLs.

[b] AmpC β-lactamases (Bush group 1) among *E. coli* and *Klebsiella* spp. included strains that produced the plasmid-mediated enzymes LAT-1, LAT-2, MIR-1, MOX-1, and FOX-1 and *E. coli* strains hyperproducing their chromosomal AmpC β-lactamase. Derepressed mutants of *Enterobacter* spp., *C. freundii*, and *S. marcescens* were also included in this group.

[c] Hyperproducers of the chromosomally-encoded K1 β-lactamase (Bush group 2be) of *K. oxytoca*.

[d] OBLs were basal-level K1 (Bush group 2be) in wild-type strains of *K. oxytoca*; Bush group 2 enzymes, TEM-1 (low-level and hyperproduction), TEM-2, SHV-1 (low-level and hyperproduction), and PSE-1; TRC-1 (Bush group 2br) in *E. coli* and *Klebsiella* spp.; NMC-A and Sme-1 (Bush group 2f) in *S. marcescens*; and inducible AmpC (Bush group 1) in wild-type strains of *Enterobacter* spp., *C. freundii*, and *S. marcescens*.

[e] Multienzyme production: two strains produced an ESBL plus an OBL; one strain produced two ESBLs.

[f] Multienzyme production: TEM-1 plus TRC-1.

[g] Multienzyme production in 17 strains: 3 strains produced two ESBLs plus an OBL, 7 strains produced two ESBLs, and 7 strains produced an ESBL plus an OBL.

[h] Multienzyme production: AmpC plus two ESBLs plus three OBLs (TEM-1 plus SHV-1 plus OXA-9).

[i] Multienzyme production in two strains: SHV-1 plus TEM-1 and SHV-1 plus PSE-1.

MATERIALS AND METHODS

Strains. Tests were performed with 141 isolates, including *E. coli* ($n = 32$), *K. pneumoniae* ($n = 36$), *K. oxytoca* ($n = 13$), *E. cloacae* ($n = 17$), *E. aerogenes* ($n = 15$), *C. freundii* ($n = 16$), and *S. marcescens* ($n = 12$), chosen to provide a wide variety of β-lactamase types. These strains were collected from multiple centers across the United States, Europe, Southeast Asia, and Mexico. All isolates were from clinical sources, except for 13 laboratory strains of *E. coli*. The laboratory strains produced the enzymes TEM-3, TEM-4, TEM-5, TEM-7, TEM-9, TEM-10, TEM-12, SHV-1, SHV-2, SHV-5, TRC-1, LAT-1, and LAT-2. For the purposes of this study, the isolates were divided into groups according to the type of β-lactamase produced. These groups included strains that produced (i) ESBLs, (ii) high levels of AmpC, (iii) high levels of K1, and (iv) other β-lactamases (OBLs) (Table 1). Within the high-AmpC group, there were *E. coli* strains that hyperproduced chromosomal AmpC β-lactamase, *E. coli* and *K. pneumoniae* strains that produced a plasmid-mediated AmpC β-lactamase, and derepressed *Enterobacter*, *C. freundii*, and *S. marcescens* mutants producing elevated levels of chromosomal AmpC β-lactamase. Within the OBL group, there were strains producing older-spectrum β-lactamases or carbapenemases, *K. oxytoca* strains producing low levels of chromosomal K1 β-lactamase, and wild-type *Enterobacter*, *C. freundii*, and *S. marcescens* strains producing low basal levels of chromosomal AmpC β-lactamase (Table 1). A number of organisms produced several different β-lactamases, and these were assigned to the group representing the broadest-spectrum enzyme that contributed to resistance because this enzyme was the phenotypic determinant. Hyperproduction of β-lactamase was defined as hydrolysis by a sonic extract of greater than 400 nmol of nitrocefin per min per mg of protein in spectrophotometric hydrolysis assays at pH 7.0 and 37°C. All β-lactamase identifications were confirmed in our laboratory by the appropriate biochemical or molecular procedures, such as isoelectric focusing

TABLE 2. In vitro activity of study drugs against organisms separated by β-lactamase group

Organism	β-Lactamase group (no. of strains)[a]	Drug[b]	Cumulative no. of strains inhibited by concn (μg/ml): ≤0.5	1	2	4	8	≥16
E. coli or *K. pneumoniae*	ESBL (36)	CAZ	3	3	4	6	7	36
	AmpC (10)				1	2	3	10
	OBL (22)		18	20	20	22		
	ESBL (36)	CTX	4	6	7	10	29	36
	AmpC (10)			1	2	3	4	10
	OBL (22)		22					
	ESBL (36)	CTR	4	5	6	7	9	36
	AmpC (10)		1	2	4	4	4	10
	OBL (22)		22					
	ESBL (36)	AZT	3	6	8	10	13	36
	AmpC (10)		1	1	1	3	3	10
	OBL (22)		22					
	ESBL (36)	POD			1	1	4	36
	AmpC (10)							10
	OBL (22)		17	22				
	ESBL (36)	CX		2	13	25	32	36
	AmpC (10)							10
	OBL (22)			1	10	18	22	
	ESBL (36)	CTT	29	29	35	36		
	AmpC (10)					1	3	10
	OBL (22)		22					
K. oxytoca	ESBL (3)	CAZ						3
	Hi K1 (4)		3	4				
	OBL (6)		6					
	ESBL (3)	CTX				1	1	3
	Hi K1 (4)		2	2	3	4		
	OBL (6)		6					
	ESBL (3)	CTR						3
	Hi K1 (4)							4
	OBL (6)		6					
	ESBL (3)	AZT						3
	Hi K1 (4)							4
	OBL (6)		4	6				
	ESBL (3)	POD						3
	Hi K1 (4)			2	2	2	3	4
	OBL (6)		6					
Enterobacter spp. or *C. freundii*	ESBL (16)	CAZ		1	3	4	5	16
	AmpC (17)							17
	OBL (15)		11	11	13	15		
	ESBL (16)	CTX					1	16
	AmpC (17)							17
	OBL (15)		11	12	12	14		15
	ESBL (16)	CTR						16
	AmpC (17)						1	17
	OBL (15)		11	12	12	14	15	
	ESBL (16)	AZT	2	3	3	6	7	16
	AmpC (17)						1	17
	OBL (15)		11	12	14	14	15	
	ESBL (16)	POD						16
	AmpC (17)							17
	OBL (15)		2	3	6	10	10	15
S. marcescens	ESBL (4)	CAZ				1	2	4
	AmpC (2)				2			
	OBL (6)		5	5	6			
	ESBL (4)	CTX						4
	AmpC (2)							2
	OBL (6)		3	4	4	6		
	ESBL (4)	CTR						4
	AmpC (2)							2
	OBL (6)		2	3	6			
	ESBL (4)	AZT				1	2	4
	AmpC (2)				1	1	2	
	OBL (6)		3	4	4	5	6	
	ESBL (4)	POD						4[c]
	AmpC (2)							2[c]
	OBL (6)				2	3	4	6

[a] Hi K1, strains of *K. oxytoca* producing high levels of chromosomal K1 β-lactamase. See text for complete explanation.
[b] CAZ, ceftazidime; CTX, cefotaxime; CTR, ceftriaxone; AZT, aztreonam; POD, cefpodoxime; CX, cefoxitin; CTT, cefotetan.
[c] MICs for all ESBL and AmpC producers were >16 μg/ml, while those for OBL producers were ≤16 μg/ml.

(29, 45), substrate profile (2, 33), inhibitor profile (45), plasmid isolation, recombinant DNA techniques, and transformations (43).

The quality control strains were *E. coli* ATCC 25922 and *E. coli* PAB-C14. The latter is a laboratory strain that produces the SHV-2 β-lactamase.

Susceptibility tests. Antibiotic susceptibilities were determined according to the manufacturer's recommendations by an overnight microdilution method using dehydrated investigational panels provided by Dade MicroScan, Inc. (Sacramento, Calif.). Results were interpreted according to current NCCLS breakpoints and recommendations (32). The panels contained doubling dilutions of cefpodoxime, ceftriaxone, cefotaxime, ceftazidime, and aztreonam, alone and in combination with clavulanate (at fixed concentrations of 1, 2, and 4 μg/ml) or sulbactam (8 μg/ml) and cefoxitin and cefotetan alone and in combination with sulbactam (8 μg/ml).

RESULTS

In vitro susceptibility. The in vitro susceptibility of the 141 test strains to the study drugs is shown in Table 2. Results have been combined for *E. coli* and *K. pneumoniae* and for *E. cloacae*, *E. aerogenes*, and *C. freundii* due to their similarities within the same β-lactamase group. For *E. coli* and *K. pneumoniae*, strains possessing ESBLs or AmpC β-lactamases were more resistant to the study drugs than strains possessing OBLs. However, ranges of MICs overlapped between the three β-lactamase groups, making it impossible to distinguish between the three groups on the basis of MIC alone. For example, for ceftazidime, 3 ESBL-producing strains were as susceptible as 18 of 22 OBL-producing strains. The three ESBL-producing strains were clinical isolates of *K. pneumoniae* ($n = 1$) and *E. coli* ($n = 2$), the ceftazidime MIC for each of which was 0.5 μg/ml, and each produced the ceftaxime-preferring ESBL SHV-3, either alone or in combination with another SHV β-lactamase with a pI of 7.6. Two OBL-producing strains were as resistant to ceftazidime as some ESBL-producing strains. Both of these OBL-producing strains were clinical isolates of *K. pneumoniae* that produced high levels of SHV-1. Thus, if the screening criteria for indicating ESBL production currently recommended by the NCCLS (MIC, ≥2 μg/ml) (32) were applied to these strains, there would be three false negatives (8%) among ESBL producers and two false positives (9%) among OBL producers. These findings add support to the NCCLS recommendation that the sensitivity of ESBL screening is increased by testing more than one agent (32). All of the

TABLE 3. Ability of β-lactamase inhibitors to reduce MICs of study drugs ≥8-fold in tests with *E. coli* and *K. pneumoniae*

Drug[a]	Inhibitor[b]	No. of strains with reduced MIC requirement in β-lactamase group (no. of strains tested)[c]:		
		ESBL (36)	AmpC (10)	OBL (22)
CAZ	C1	30	0	1[k]
	C2	31	0	3 (2)[l]
	C4	33	0	3 (2)[l]
	S8	25	1 (1)[d,f]	0
CTX	C1	33	0	1[m]
	C2	33	0	1[n]
	C4	32	1 (1)[g]	0
	S8	28	4 (3)[h]	0
CTR	C1	35[e]	0	0
	C2	35[e]	0	0
	C4	32[e]	2 (1)[i]	0
	S8	28	3 (2)[j]	0
AZT	C1	35	0	1[k]
	C2	34	0	1[k]
	C4	34	0	0
	S8	28	1 (1)[f]	0
POD	C1	35	0	2[o]
	C2	35	0	2[o]
	C4	36	0	2[p]
	S8	29	0	0

[a] See footnote *b* of Table 2.
[b] C1, C2, and C4, 1, 2, and 4 μg of clavulanate per ml, respectively; S8, 8 μg of sulbactam per ml.
[c] See footnote *a* of Table 2.
[d] The number in parentheses indicates the number of strains for which the MIC of the test drug alone was ≥2 μg/ml, thus fulfilling current NCCLS screening criteria for ESBL production. These strains would therefore be false positives for ESBL production in the inhibitor-drug combination tests.
[e] The ceftriaxone MIC for one strain of *E. coli*, which produced a low level of TEM-12, was ≤0.06 μg/ml. This value was too low to permit detection of an eightfold decrease in MIC when ceftriaxone was tested in combination with a β-lactamase inhibitor.
[f] *E. coli* (LAT-1).
[g] *K. pneumoniae* (ESBL plus AmpC).
[h] *E. coli* (LAT-1 [one strain]), *K. pneumoniae* (ESBL plus AmpC [one strain]), and *E. coli* (high-level chromosomal cephalosporinase [two strains]).
[i] *E. coli* (LAT-1 [one strain]) and *E. coli* (high-level chromosomal cephalosporinase [one strain]).
[j] *E. coli* (LAT-1 [one strain]), *K. pneumoniae* (ESBL plus AmpC [one strain]), and *E. coli* (high-level chromosomal cephalosporinase [one strain]).
[k] *K. pneumoniae* (PSE-1 plus SHV-1).
[l] *K. pneumoniae* (PSE-1 plus SHV-1 [one strain]) and *K. pneumoniae* (high SHV-1 [two strains]).
[m] *E. coli* (SHV-1).
[n] *K. pneumoniae* (high SHV-1).
[o] *K. pneumoniae* (high SHV-1 [two strains]).
[p] *K. pneumoniae* (high SHV-1 [one strain]) and *E. coli* (high TEM-1 [one strain]).

high-AmpC producers would be considered ESBL producers by these criteria, but none were confirmed as such by the recent NCCLS ESBL confirmatory tests (32).

Among the study drugs, the only one that discriminated between OBL producers and ESBL or high-AmpC producers was cefpodoxime, with only the former inhibited by concentrations of ≤1 μg/ml. Cefpodoxime did not discriminate between ESBL or high-AmpC producers. It was possible that one of the cephamycins would discriminate between ESBL and high-AmpC producers, since only the latter enzyme confers resistance to this drug class. However, this did not occur, and MIC ranges for these two groups overlapped (Table 2). MICs of cefoxitin were >16 μg/ml for 2 of 36 ESBL producers (*K. pneumoniae* strains producing SHV-3 and SHV-5) and 10 of 10 high-AmpC producers. MICs of cefotetan were ≤4 μg/ml for 1 of 10 high-AmpC producers (*E. coli*, presumed chromosomally mediated AmpC) and 36 of 36 ESBL producers.

With the exception of a TEM-12-producing laboratory strain of *E. coli* which was more susceptible than most isolates to several of the drugs tested (see footnote *e* of Table 3), the grouping together of laboratory and clinical strains of *E. coli* did not influence the trends of the data. In general, irrespective of the type of β-lactamase produced (OBL, ESBL, or AmpC), the MIC patterns for the clinical strains were more extreme than those of the laboratory strains. That is, the laboratory strains were less sensitive than the most sensitive clinical strains and less resistant than the most resistant clinical strains.

For *K. oxytoca*, several of the study drugs did not have overlapping MIC ranges for all three types of enzyme production. This allowed discrimination between the three enzyme groups (Table 2). Only ESBL producers were resistant to ceftazidime. Strains producing high levels of the K1 β-lactamase could be differentiated from those producing low levels of the enzyme with ceftriaxone or aztreonam.

For tests with *E. cloacae*, *E. aerogenes*, and *C. freundii*, OBL producers tended to be more susceptible to the study drugs than ESBL or high-level AmpC producers (Table 2). However, overlapping MIC ranges for each of the three β-lactamase-producing groups prevented complete discrimination between the groups. For *S. marcescens*, OBL producers could be discriminated from ESBL or high-AmpC producers with cefo-

TABLE 4. Ability of β-lactamase inhibitors to reduce MICs of study drugs ≥8-fold in tests with *E. cloacae*, *E. aerogenes* and *C. freundii*

Drug[a]	Inhibitor[b]	No. of isolates with reduced MIC requirement in β-lactamase group (no. of strains tested)[c]:		
		ESBL (16)	AmpC (17)	OBL (15)
CAZ	C1	8	0	0
	C2	9	0	0
	C4	9	0	0
	S8	10	2 (2)[d]	0
CTX	C1	10	0	0
	C2	10	0	0
	C4	11	0	0
	S8	14	3 (3)	1 (1)
CTR	C1	11	0	1
	C2	10	0	1
	C4	12	0	0
	S8	15	2 (2)	0
AZT	C1	10	0	0
	C2	9	0	1
	C4	9	0	0
	S8	9	1 (1)	1
POD	C1	10	0	0
	C2	9	0	0
	C4	8	0	0
	S8	9	1 (1)	6 (6)

[a] See footnote *b* of Table 2.
[b] C1, C2, and C4, 1, 2, and 4 μg of clavulanate per ml, respectively; S8, 8 μg of sulbactam per ml.
[c] See footnote *a* of Table 2.
[d] The number in parentheses indicates the number of strains for which the MIC of the test drug alone was ≥2 μg/ml, thus fulfilling current NCCLS screening criteria for ESBL production by *E. coli* and *Klebsiella* spp. If the criteria were extended to these organisms, these strains would therefore be false positives for ESBL production in the inhibitor-drug combination tests.

TABLE 5. Induction of AmpC β-lactamase by clavulanate reflected by elevated MICs in inhibitor-drug combinations

Strain	β-Lactamase(s)	MIC (μg/ml)[a]				
		CAZ	CAZ + C1	CAZ + C2	CAZ + C4	CAZ + 8S
E. cloacae 84	AmpC (wild type)	2	>8	8	16	2
Vega 104	AmpC (wild type) + SHV-2 + TEM-1	4	>8	>8	16	2
Vega 484	AmpC (wild type) + TEM-1	4	8	>8	16	8
C. freundii GB51	AmpC (wild type)	0.5	1	0.5	2	0.25
S. marcescens M1	AmpC (mutant)	2	4	8	8	2

[a] CAZ, ceftazidime; C1, C2, and C4, 1, 2, and 4 μg of clavulanate per ml, respectively; 8S, 8 μg of sulbactam per ml.

taxime, ceftriaxone, or cefpodoxime. Ceftazidime discriminated between ESBL and high-AmpC producers (Table 2).

Inhibitor-drug combinations. Since single drugs alone were not capable of accurately discriminating between the ESBL and high-AmpC β-lactamase groups in certain species tested, β-lactamase inhibitor–β-lactam drug combinations were evaluated. If MICs of a given drug were reduced by at least eightfold (three twofold dilutions) in the presence of an inhibitor, the test strain was considered to have an inhibitor-susceptible ESBL. In tests with *E. coli* and *K. pneumoniae*, sulbactam added to each study drug gave the largest number of false positives with high-AmpC-producing strains (Table 3). Clavulanate added to either ceftazidime or cefpodoxime gave the largest number of false positives with OBL producers. However, if NCCLS screening criteria for ESBL production (MIC, ≥2 μg/ml for test drug) (30) were applied to the strains before the results of combination testing were considered, false positives among OBL producers occurred only with two strains (*K. pneumoniae* producing high levels of SHV-1) in tests with clavulanate-ceftazidime (Table 3). False negatives among ESBL producers were highest with ceftazidime and lowest with cefpodoxime. Addition of sulbactam to cefoxitin or cefotetan did not reliably reduce the MIC of either drug in tests with ESBL or high-AmpC producers (data not shown).

In tests with *E. cloacae*, *E. aerogenes*, and *C. freundii*, addition of clavulanate to the study drugs failed to lower MICs at least eightfold in tests with 25 to 50% of ESBL producers (Table 4). This poor performance was due in large part to the ability of clavulanate to induce the AmpC β-lactamase in these strains, which often resulted in MICs of the combinations higher than that of the drug alone (Table 5). This phenomenon was not observed in combinations containing sulbactam. Sulbactam-ceftriaxone correctly identified 15 of 16 ESBL producers; however, there were two false positives (12%) among high-AmpC producers (Table 4).

In tests with *S. marcescens*, ESBL producers were correctly separated from high-AmpC and OBL producers with ceftazidime plus 8 μg of sulbactam per ml, cefotaxime plus 1 μg of clavulanate per ml, or cefotaxime plus 2 μg of clavulanate per ml (Table 6).

DISCUSSION

This study addressed issues surrounding the detection of clinically important β-lactamases among strains of *Enterobacteriaceae* possessing well-characterized enzymes. Results obtained with the study drugs when tested alone indicated that it was not possible to select interpretive criteria that would accurately identify ESBL- or high-AmpC-producing strains specifically among isolates of *E. coli*, *K. pneumoniae*, *E. cloacae*, *E. aerogenes*, or *C. freundii*. It was possible by using a breakpoint of ≥2 μg of cefpodoxime per ml to identify producers of either ESBLs or high-AmpC β-lactamases among isolates of *E. coli* or *K. pneumoniae*, but discrimination between these two β-lactamase groups was not possible when any drug was used alone. Cefotetan was almost suitable for this purpose, inhibiting all producers of ESBLs and only one high-AmpC producer at a concentration of 4 μg/ml. For *K. oxytoca*, it was possible to distinguish between ESBL, high K1, and OBL producers by using ceftazidime and either ceftriaxone or aztreonam. For *S. marcescens*, it was possible to distinguish between ESBL, high-AmpC, and OBL producers by using ceftazidime and ceftriaxone or cefotaxime.

For *E. coli* and *K. pneumoniae*, it was necessary to use an inhibitor-drug combination to accurately separate ESBL from high-AmpC producers. The best combinations for achieving this were cefpodoxime plus 4 μg of clavulanate per ml or ceftriaxone plus 1 or 2 μg of clavulanate per ml. One of the recently recommended NCCLS ESBL confirmatory tests, with ceftazidime tested alone and in combination with 4 μg of clavulanate per ml (32), yielded false positives with three OBL-producing strains of *K. pneumoniae*. Two of these strains hyperproduced SHV-1 and were ESBL screen positive in tests with ceftazidime alone (MICs, 2 and 4 μg/ml), but not with

TABLE 6. Ability of β-lactamase inhibitors to reduce MICs of study drugs ≥8-fold in tests with *S. marcescens*

Drug[a]	Inhibitor[b]	No. of isolates with reduced MIC requirement in β-lactamase group (no. of strains tested)[c]:		
		ESBL (4)	AmpC (2)	OBL (6)
CAZ	C1	4	0	1 (1)[d]
	C2	3	0	1 (1)
	C4	4	0	1 (1)
	S8	4	0	0
CTX	C1	4	0	0
	C2	4	0	0
	C4	3	0	0
	S8	4	0	1 (1)
CTR	C1	3	0	2 (1)
	C2	3	0	1 (1)
	C4	3	0	2 (1)
	S8	4	0	2 (1)
AZT	C1	4	0	1 (1)
	C2	4	0	2 (2)
	C4	3	0	2 (2)
	S8	4	1 (1)	1 (1)
POD	C1	2	0	0
	C2	2	0	0
	C4	2	0	1 (1)
	S8	2	0	2 (2)

[a] See footnote *b* of Table 2.
[b] C1, C2, and C4, clavulanate at 1, 2, and 4 μg/ml, respectively; S8, sulbactam at 8 μg/ml.
[c] See footnote *a* of Table 2.
[d] The number in parentheses indicates the number of strains for which MIC of the test drug alone was ≥2 μg/ml, thus fulfilling current NCCLS screening criteria for ESBL production by *E. coli* and *Klebsiella* spp. If the criteria were extended to these organisms, these strains would therefore be false positives for ESBL production in the inhibitor-drug combination tests.

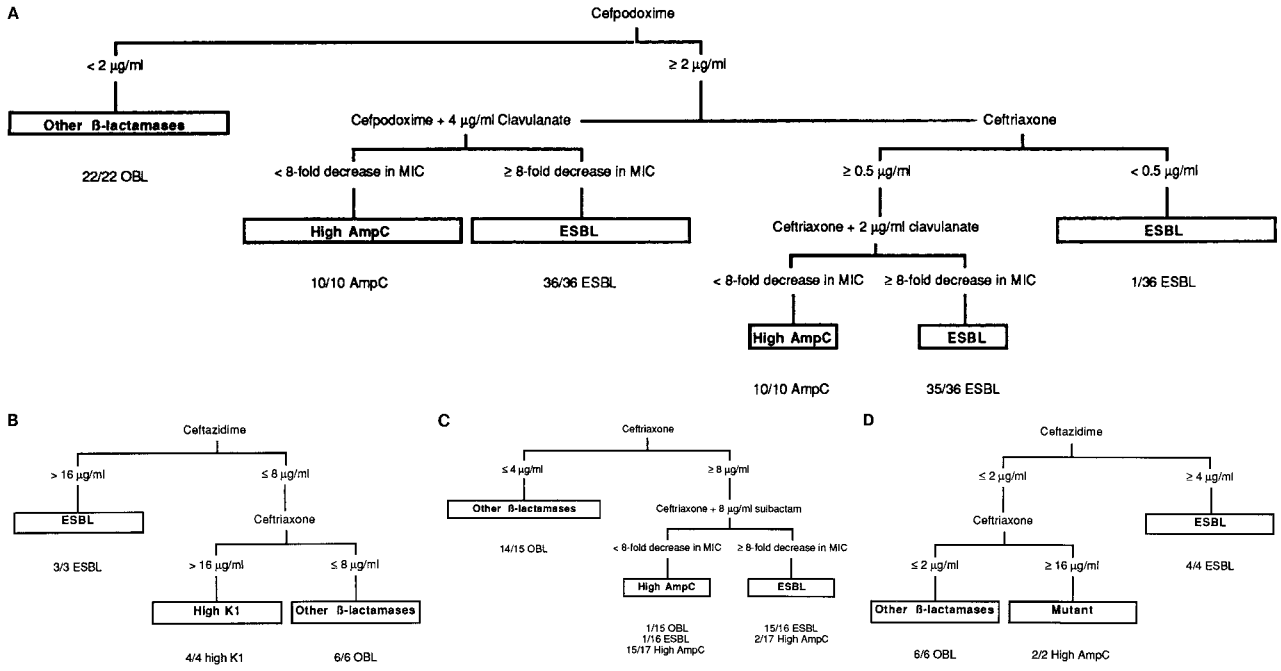

FIG. 1. Flow chart for identification of β-lactamase groups in isolates of *E. coli* and *K. pneumoniae* (A), *K. oxytoca* (B), *Enterobacter* spp. and *C. freundii* (C), and *S. marcescens* (D).

other NCCLS-recommended screening drugs. The other strain produced SHV-1 and PSE-1 but was ESBL screen negative in tests with all screening drugs (i.e., MIC, <2 μg/ml). These findings suggest that positive ESBL screens arising from the use of ceftazidime as a screening drug should not be evaluated with the ceftazidime-clavulanate confirmatory test and that this inhibitor-based test should not be used on its own as a single test for ESBL detection. For *E. cloacae*, *E. aerogenes*, and *C. freundii*, the best combination for separating ESBL from high-AmpC producers was ceftriaxone plus 8 μg of sulbactam per ml.

From these results, a minimum panel of five tests could be identified that would provide maximum separation of ESBL, high AmpC, high K1, and OBL production in *Enterobacteriaceae* (Fig. 1). These included cefpodoxime, cefpodoxime plus 4 μg of clavulanate per ml, ceftazidime, ceftriaxone, and ceftriaxone plus 8 μg of sulbactam per ml. Ceftriaxone plus 2 μg of clavulanate per ml could be substituted for cefpodoxime plus 4 μg of clavulanate per ml without altering the accuracy of the tests (Fig. 1A). Such a panel would correctly separate ESBL, high-AmpC, high-K1, and OBL producers among all species tested, except *E. cloacae*, *E. aerogenes*, and *S. marcescens*. For these species, one OBL producer and one ESBL producer would be incorrectly identified as high-AmpC producers, and two high-AmpC producers would be incorrectly identified as ESBL producers. None of these errors involved identification of an ESBL or high-AmpC producer as a OBL producer—an error that could lead to serious therapeutic hazard.

The approach outlined in Fig. 1 could be useful for clinical laboratories, provided its limitations are recognized. One limitation is that in strains producing multiple β-lactamases, the presence of a broader-spectrum β-lactamase may mask the presence of narrower-spectrum β-lactamases. Another limitation involves the small number of strains of species other than *E. coli* and *K. pneumoniae* included in this study. Until additional strains of these species have been tested, the proposed schemes for *K. oxytoca*, *Enterobacter* spp., *C. freundii*, and *S. marcescens* should be considered provisional. Nevertheless, even with this limitation, the current study has been useful for identifying approaches which do not work well with these organisms. These include the use of clavulanate-based tests with organisms that produce inducible β-lactamases and the use of the same interpretive criteria for *K. oxytoca* as for *K. pneumoniae* (32). The presence of the K1 β-lactamase in the former species dictates the use of separate criteria for interpreting tests with these two species.

Overall, this study demonstrated that the application of MIC tests which include the use of appropriate β-lactamase inhibitors can provide a simple, convenient, and inexpensive approach for routine clinical laboratories to recognize the types of β-lactamases produced by *E. coli*, *Klebsiella*, *Enterobacter*, *C. freundii*, and *S. marcescens*. With further studies, additional guidelines can be identified to recognize other β-lactamase types that are produced by these and other species of bacteria.

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