Occurrence and Detection of Extended-Spectrum β-Lactamases in Members of the Family *Enterobacteriaceae* at a Veterans Medical Center: Seek and You May Find

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A total of 907 consecutive isolates of members of the family *Enterobacteriaceae* recovered during a 20-week period were tested for production of extended-spectrum β-lactamases (ESBLs) by the double-disk (DD) potentiation method. Of 84 DD-positive isolates, 83 (9.2%) produced ESBLs based on isoelectric focusing. SHV-derived ESBLs and several TEM-derived ESBLs were present in nine species, including the first isolate of *Citrobacter koserii* and *Morganella morganii* known to harbor an SHV-derived ESBL. Results of testing 58 nonrepeat isolates for ESBL production by several recommended methods were as follows (percent detected in parentheses): DD method with aztreonam (95), ceftazidime (79), ceftriaxone (88), or cefpodoxime (90); broth microdilution method with ceftazidime (86) or cefotaxime (91) alone or in combination with clavulanate; and the standard disk diffusion method with new breakpoints and standard concentrations of aztreonam (78), ceftazidime (79), ceftriaxone (83), or cefpodoxime (98) or a novel concentration (5 μg) of ceftazidime (88). In three instances during an extended part of the study, an ESBL-producing isolate and a non-ESBL-producing isolate of the same species were recovered from a single blood culture bottle. These data indicate that ESBLs occur in several species of *Enterobacteriaceae* and at a relatively high incidence at our institution and that the standard disk diffusion method with cefpodoxime and the DD method with several β-lactams are practical and cost-effective methods for detecting ESBL-producing isolates of *Enterobacteriaceae*.

In recent years, bacterial resistance to β-lactam antibiotics has risen dramatically (15). Contributing to this increase has been the spread of extended-spectrum β-lactamases (ESBLs), enzymes that hydrolyze the expanded-spectrum cephalosporins, like ceftazidime and cefotaxime, and/or the monobactam aztreonam (3). The first isolate resistant to expanded-spectrum cephalosporins was found in Germany in 1983, and it produced an SHV-type β-lactamase (12). Subsequently, many ESBLs, predominantly SHV and TEM variants, have been reported in clinical isolates (3, 5–9, 15, 27). The first reported outbreak of ESBL-producing organisms occurred in France in 1985 (27). More recently, outbreaks have occurred worldwide, including several cities in the United States (2, 16, 19, 22, 23). These reports underscore the importance of early detection of ESBL-producing organisms.

Unfortunately, the resistance to expanded-spectrum cephalosporins of many of these strains is not detected by routine susceptibility testing methods that follow current National Committee for Clinical Laboratory Standards (NCCLS) breakpoints (11). Standard disk diffusion testing of cefpodoxime appears to be a promising method for screening *Klebsiella pneumoniae* and *Escherichia coli* (32), but this drug is not routinely tested in many laboratories. A 5-μg ceftazidime disk has recently been proposed for use to discriminate between ESBL-producing and non-ESBL-producing strains of *E. coli* and *K. pneumoniae* (8). In addition, a clavulanate double-disk (DD) potentiation procedure has improved detection (9).

Few studies have compared the abilities of different laboratory methods to detect ESBL-producing organisms among members of the family *Enterobacteriaceae*. Few have examined the frequency with which these organisms are recovered from specimens received in hospital laboratories within the United States. Most studies conducted to date have been done in Europe or involved only *K. pneumoniae* and *E. coli*, the two species in which ESBLs are most common (4, 5, 7, 8, 13, 28, 30, 33). Therefore, a study was designed to determine the occurrence of ESBLs among all genera of the family *Enterobacteriaceae* isolated at the McGuire Veterans Affairs Medical Center (VAMC), Richmond, Va., during a 20-week period. Determination of true prevalence would have required testing all consecutive isolates with labor-intensive procedures including isoelectric focusing (IEF). Therefore, isolates were screened for ESBLs with the DD method. Isolates that were DD positive were examined further for ESBL production with definitive procedures. The abilities of different methods to detect ESBLs in these organisms were also compared.

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MATERIALS AND METHODS

Tests for ESBL-producing members of the family *Enterobacteriaceae*. A total of 907 consecutive isolates of *Enterobacteriaceae* recovered at the VAMC during a 20-week period (November 1995 to March 1996) were tested by the DD method (9). Mueller-Hinton agar (Remel, Lenexa, Kans.) was inoculated from a blood agar plate culture grown overnight, as recommended for the standard disk diffusion test (18). Disks (BBL, Cockeysville, Md.) containing the standard 30 μg of aztreonam, ceftazidime, and ceftriaxone were placed 15 mm (edge to edge) from an amoxicillin-clavulanic acid disk (20 and 10 μg, respectively). Disk placement was expedited by melting holes in the lid of a petri plate and using the lid as a template to mark the bottom of the agar plate for proper disk location. A total of 321 isolates were also tested by the DD method with cefpodoxime (10 μg; Difco Laboratories, Detroit, Mich.). Inoculated media were incubated overnight at 35°C. An enhanced zone of inhibition between any one of the β-lactam disks and the disk containing clavulanic acid was interpreted as presumptive evidence for the presence of an ESBL. Isolates with this pattern were recorded as DD

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positive. In addition to recording enhanced zones of inhibition from the DD plate, standard zone diameters were determined by measuring the zones directly, or if necessary, by measuring the radius and multiplying by two.

The 15-mm distance between disks was based on results of a preliminary study at our institution involving 155 isolates of members of the family *Enterobacteriaceae* collected from intensive care units. For these isolates, β-lactam disks were placed at distances of both 15 and 20 mm from the clavulanic acid disk (edge to edge). Although results from disks placed at both distances were similar, enhanced inhibition zones with disks 15 mm apart were frequently more pronounced and easier to read than were zones of disks placed 20 mm apart.

MICs of ceftazidime and cefotaxime with and without 2 μg clavulanic acid per ml (fixed concentration) (31) were determined by the standard microdilution broth method (17). Control strains included *E. coli* ATCC 25922, *E. coli* ATCC 35218, and *Pseudomonas aeruginosa* ATCC 27853. Some isolates were also tested by the standard disk diffusion method with disks containing 5 μg of ceftazidime (CAZ-5), which were prepared by adding ceftazidime (Sigma Chemical Co., St. Louis, Mo.) to blank disks (BBL). Zone diameters were recorded.

β-Lactamases. The presence of ESBLs in DD-positive isolates was confirmed by IEF of cell extracts. IEF was performed as described previously (14) with an LKB Multiphor unit (LKB Instruments, Inc., Rockville, Md.) or a Bio-Rad Mini IEF cell (Bio-Rad Laboratories, Hercules, Calif.). Cells were grown in tryptic soy broth for 4 h, and crude preparations of enzymes were obtained by sonication of cell pellets. Enzyme standards included TEM-1, TEM-2, TEM-3, SHV-2, SHV-3, SHV-4, and SHV-5 and were obtained from strains containing well-characterized β-lactamases. To detect enzyme activity, the surfaces of focused gels were overlaid with molten agar containing 50 μg of nitrocephin per ml (BBL) (26). In addition, enzyme inhibition was shown for all non-*K. pneumoniae* and non-*E. coli* isolates by briefly applying a filter paper strip moistened with 1 mM potassium clavulanate to the focused gel surface prior to adding the molten agar (26). Last, for six of the isolates, a second focused gel was overlaid with agar containing 0.094 μg of cefotaxime per ml (1). The agar was inoculated with *E. coli* ATCC 25922 by a standard procedure (18), and growth was recorded after overnight incubation at 35°C. Because *E. coli* ATCC 25922 is susceptible to 0.094 μg of cefotaxime per ml, growth indicated hydrolysis of the extended-spectrum β-lactam in the agar.

One or more isolates from each species that were DD positive were also tested for transfer of resistance to recipient strains. Resistance was transferred by filter mating to an *E. coli* strain, CGSC 1867 or HB101 (1:20 ratio of donor and recipient). Selective media contained 400 μg of sodium azide (Sigma Chemical Co.) per ml and 4 or 8 μg of aztreonam (Bristol-Myers Squibb Co., Princeton, N.J.) per ml, or 1,750 μg of streptomycin per ml and 32 μg of ampicillin (Sigma) per ml. When appropriate, colonies were initially screened for transconjugants by a spot indole test. Transfer of resistance was confirmed for all transconjugants by DD testing and in some cases by IEF. DD patterns of transconjugants mimicked patterns of donors.

RESULTS

Occurrence of ESBL-producing organisms. Of the 907 isolates of members of the family *Enterobacteriaceae* tested by the DD method, enhanced zones of inhibition were observed with 84 isolates. For one of these isolates, a *Klebsiella oxytoca* isolate, the interpretation of enzyme inhibition with clavulanic acid was unclear. Therefore, test results for this isolate were not included in data analyses.

IEF was performed on extracts prepared from the remaining 83 DD-positive isolates or their transconjugants (see below). IEF profiles for β-lactamases recovered from 77 isolates indicated the presence of at least one ESBL, which is most likely a derivative of an SHV β-lactamase due to the range of pIs encountered (7.0 to 8.2) and their comigration with known SHV-derived ESBLs. IEF profiles of β-lactamases recovered from the remaining six isolates, all of which were *E. coli*, showed an enzyme with a pI of 5.4 that was inhibited by clavulanic acid and capable of hydrolyzing cefotaxime. This suggested the presence of an ESBL, probably derived from a TEM β-lactamase. Therefore, of 906 consecutive isolates, 83 (9.2%) were ESBL-producing organisms. The number of isolates of each species that produced ESBLs is listed in Table 1.

Of 32 ESBL-producing organisms that were tested, resistance was transferred from 23 isolates, including each of the following species (the number of isolates given in parentheses): *K. pneumoniae* (4), *Enterobacter aerogenes* (6), *E. coli* (1), *Serratia marcescens* (1), *K. oxytoca* (3), *Citrobacter freundii* (1),

TABLE 1. Numbers of isolates of members of the family *Enterobacteriaceae* that produced ESBLs

Species	No. of isolates tested	ESBL-producing isolates			
		No.	%(ESBL)[a]	%(Spp.)[b]	No. of repeats[c]
K. pneumoniae	164	31	37	19	8
E. aerogenes	68	17	21	25	6
E. coli	316	13	16	4	3
S. marcescens	48	8	10	17	3
M. morganii	24	7	8	29	4
K. oxytoca	27	3	4	11	1
C. freundii	23	2	2	9	0
C. koserii	19	1	1	5	0
E. cloacae	62	1	1	2	0
Other species	155	0	0	0	
Total	906	83	100		25

[a] Percentage of all isolates that produced ESBLs.
[b] Percentage of isolates within each species that produced ESBLs.
[c] Number of ESBL-producing isolates within each species that were repeat isolates (see text).

Citrobacter koserii (1), *Enterobacter cloacae* (1), and *Morganella morganii* (5).

The 83 ESBL-producing isolates were recovered from 47 patients and from the following specimens (number of specimens in parentheses): urine (65), blood (3), respiratory fluid (2), wound (2), and knee fluid (1). For seven patients, two different species that produced ESBLs were recovered from the same specimen. A repeat isolate was defined as an isolate that was the same species as an isolate recovered from another specimen of the same patient earlier during the 20-week study period, and both isolates produced ESBLs. Twenty-five (30%) of the 83 isolates were repeat isolates and, in general were proportionately distributed among the different species (Table 1).

Since ESBL production is usually plasmid mediated, it was possible for one specimen to contain both ESBL-producing and non-ESBL-producing cells of the same species. To address this issue, the following additional tests were performed with isolates recovered from blood specimens during an extended part of the study (12 months). Samples from blood culture bottles containing gram-negative bacilli were inoculated onto blood agar and streaked for isolation. After overnight incubation, several colonies were suspended in saline and this suspension was used to perform the DD test. Of 88 blood culture bottles (88 patients) containing members of the family *Enterobacteriaceae*, seven (8%) had ESBL-producing organisms and 3 of these (3.4%) had both ESBL-producing and non-ESBL-producing organisms of the same species (one *K. pneumoniae* and two *E. aerogenes* strains). Mixed populations of ESBL-producing and non-ESBL-producing cells of the same species were also detected in urine specimens, but the additional measures outlined above were not performed with these specimens.

Detection of ESBL-producing organisms. The abilities of various tests to detect the presence of ESBLs among clinical isolates of *Enterobacteriaceae* were assessed. With the DD method, enhanced zones indicative of ESBL production were readily apparent for most isolates (Fig. 1A). Enhancement was less apparent with some isolates, as shown in Fig. 1B and C with the aztreonam and ceftriaxone disks, respectively. Resistance was transferred for all isolates shown in Fig. 1. Of the 58 nonrepeat ESBL-producing isolates tested by the DD method,
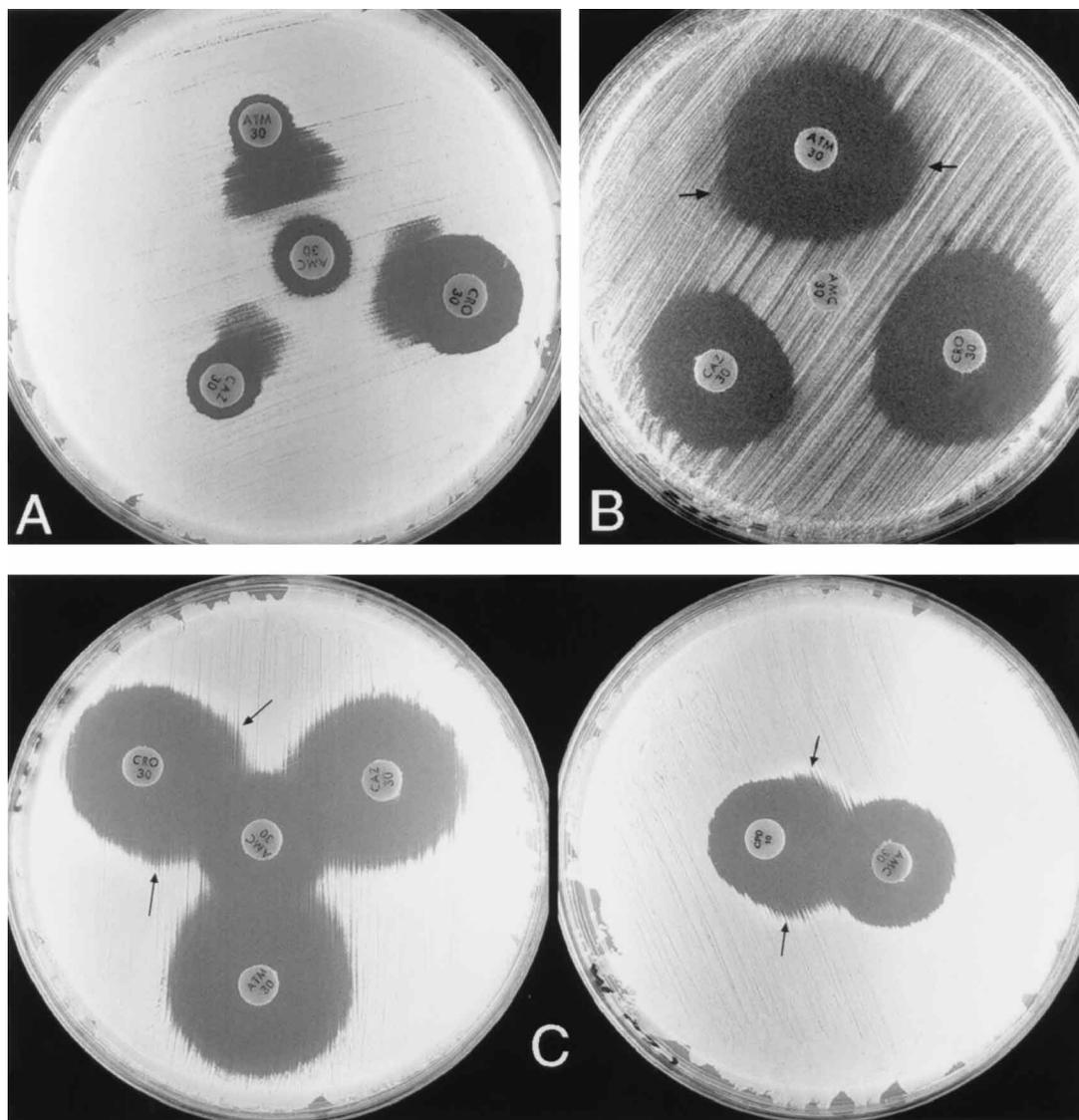
FIG. 1. DD potentiation patterns for three isolates of members of the family *Enterobacteriaceae*. (A) *E. aerogenes*. Enhanced zones of inhibition are readily apparent between disks containing clavulanic acid and β-lactams. (B) *M. morganii*. An area of slightly enhanced inhibition is visible by the ATM disk (arrows). (C) *E. coli*. Areas of slightly and moderately enhanced inhibition are apparent by the CRO and CPD disks (arrows), respectively. Drug abbreviations on disks: ATM, aztreonam; CAZ, ceftazidime; CRO, ceftriaxone; CPD, cefpodoxime; AMC, amoxicillin-clavulanic acid. The numbers on the disks are the amounts of drug (in micrograms).

the following numbers of isolates gave clavulanate enhanced zones with the β-lactam disk indicated: aztreonam, 55 (95%); ceftazidime, 46 (79%); ceftriaxone, 51 (88%); and cefpodoxime, 52 (90%) (Table 2). ESBLs were indicated in tests with all isolates with two disks, aztreonam and ceftriaxone or aztreonam and cefpodoxime.

Some investigators have used a fourfold difference in MICs between a β-lactam tested alone and in combination with an inhibitor to classify organisms as putative ESBL-producing isolates (10). Therefore, the MICs of ceftazidime and cefotaxime alone and in combination with 2 μg of clavulanate per ml were determined for the 58 nonrepeat ESBL-producing isolates. With the exception of two *E. aerogenes* isolates and three *M. morganii* isolates, all MICs for one or both of the β-lactams with clavulanate were at least fourfold less than the MICs of the same agent without clavulanate (Table 2). Of the isolates with MIC differences of less than fourfold, resistance was

transferred for four of the five organisms. New NCCLS guidelines for interpreting results of MICs for β-lactams alone suggest that *Klebsiella* and *E. coli* strains with ceftazidime or cefotaxime MICs of >1 μg/ml may be ESBL-producing strains (17). By these criteria, only 48 (83%) and 42 (72%) of the 58 isolates would have been suspected as being ESBL-producing isolates from MICs determined for ceftazidime and cefotaxime, respectively.

New guidelines for disk susceptibility tests state that some ESBLs may confer high-level resistance to certain β-lactams and may be detected by disk diffusion testing but that other isolates may display a lower level of resistance (18). The latter isolates may not reach standard breakpoints for resistance, and therefore, the following breakpoints were recommended for detecting ESBL-producing *E. coli* and *Klebsiella* species: aztreonam, <28 mm; ceftazidime, <23 mm; ceftriaxone, <26 mm; and cefpodoxime, <23 mm (18). With the standard break-

TABLE 2. Numbers of nonrepeat ESBL-producing isolates that were detected by different methods

| Species (no. of isolates) | No. of nonrepeat ESBL-producing isolates detected by: | | | | | | | | | | | |
| | DD[a] | | | | MD[b] | | SDD[c] | | | | CAZ-5[d] | |
	ATM	CAZ	CRO	CPD	CAZ	CTX	ATM	CAZ	CRO	CPD	<18	<21
K. pneumoniae (23)	22	19	23	22	21	23	20	20	22	22	19	20
E. aerogenes (11)	11	9	10	9	9	9	10	11	11	11	11	11
E. coli (10)	8	8	6	10	9	10	3	6	4	10	7	7
S. marcescens (5)	5	4	5	5	5	5	5	4	5	5	5	5
M. morganii (3)	3	1	2	1	0	0	2	1	1	3	2	2
K. oxytoca (2)	2	2	2	2	2	2	2	1	2	2	2	2
C. freundii (2)	2	1	2	2	2	2	1	1	1	2	1	2
C. koserii (1)	1	1	1	1	1	1	1	1	1	1	1	1
E. cloacae (1)	1	1	0	0	1	1	1	1	1	1	1	1
Total (58)	55	46	51	52	50	53	45	46	48	57	49	51

[a] Double-disk (DD) potentiation method. Disks contained standard concentrations of aztreonam (ATM), ceftazidime (CAZ), ceftriaxone (CRO), and cefpodoxime (CPD).

[b] Microdilution method (MD). Wells contained ceftazidime (CAZ) and cefotaxime (CTX) with and without clavulanate. Differences between MICs with and without inhibitor were at least fourfold.

[c] Standard disk diffusion (SDD) method. Disks contained standard concentrations of β-lactams, and zone diameters were interpreted with new breakpoints for detecting ESBL-producing isolates.

[d] Disk diffusion method. Each disk contained 5 μg of ceftazidime (CAZ-5). Inhibition zone diameters of <18 and <21 mm were measured.

points, the following numbers of the 58 nonrepeat ESBL-producing isolates were intermediate or resistant to each β-lactam listed: aztreonam, 35 (60%); ceftazidime, 28 (48%); ceftriaxone, 27 (47%); and cefpodoxime, 54 (93%). At least 66% of the *E. coli* and *M. morganii* isolates appeared susceptible to the first three agents. With the new breakpoints (18), however, the numbers of the 58 ESBL-producing isolates identified were as follows: aztreonam, 45 (78%); ceftazidime, 46 (79%); ceftriaxone, 48 (83%); and cefpodoxime, 57 (98%) (Table 2). At least 40% of the *E. coli* isolates still appeared susceptible to the first three agents with these new interpretive criteria.

Jacoby and Han (8) have proposed that a CAZ-5 disk be used to detect ESBL-producing members of the family *Enterobacteriaceae*. The numbers of 58 ESBL-producing isolates with zones of inhibition of <18 and <21 mm were 49 (85%) and 51 (88%), respectively.

DISCUSSION

A number of studies have assessed the occurrence of ESBLs among members of the family *Enterobacteriaceae* (4–9, 13, 21, 24, 28, 30, 33). Most, however, have focused primarily on *E. coli* and *K. pneumoniae* (8, 9, 13, 21, 24, 30, 33), and many have involved hospitals outside the United States (4–7, 9, 13, 28, 30, 33). One French study that used the DD test to examine the occurrence of ESBLs among 9,382 consecutive isolates from 12 hospitals in 1990 found that 139 (1.5%) of the isolates appeared to produce ESBLs (28). In this study, disks were placed 30 mm apart (center to center) and results were not confirmed by more-definitive testing. The detection of 83 ESBL-producing isolates from 906 consecutive isolates at our institution represents an occurrence around six times higher than that reported in this earlier French study. Clearly, the relatively high occurrence of ESBL-producing organisms underscores the importance of testing all *Enterobacteriaceae* for this characteristic in the clinical laboratory.

Several features of the current study were worthy of note. First, the occurrence of an SHV-derived ESBL in *C. koserii* and *M. morganii* has not been reported previously. Second, the finding of both ESBL-producing and non-ESBL-producing organisms of the same species from the *Enterobacteriaceae* in a single specimen suggests that for optimal detection, several

colonies must be tested from a primary culture plate. Due to cost constraints, we performed DD testing of several colonies by combining cells from several colonies into one suspension rather than testing each colony separately, and we limited multiple-colony testing to isolates recovered from blood samples.

A number of methods for the detection of ESBL production among *Enterobacteriaceae* are currently under development (8, 11, 17, 18, 20, 25, 32). The reliability of these methods and the DD test has not been thoroughly evaluated to date. Although the current study did not assess the ability of a variety of tests to detect ESBL production among all 906 *Enterobacteriaceae* studied by the DD test, results obtained with DD-positive isolates did reveal differences and limitations.

In this and previous studies, the inability of standard interpretive criteria applied to results of disk diffusion tests to detect ESBL production among *Enterobacteriaceae* has been amply demonstrated (8, 28). Although the percentages of isolates detected with the new interpretive criteria were higher, only tests with cefpodoxime were acceptably high (98%). These results agree with those reported previously by Thomson, who showed that cefpodoxime was the most reliable for detecting ESBL production among *Enterobacteriaceae* (32). Jacoby and Han (8) tested *K. pneumoniae* and *E. coli* strains with the CAZ-5 disk and reported that a zone of inhibition of <18 or <21 mm could be used to detect ESBL production in these two species. In the current study, 6 of the 7 ESBL-producing isolates that had zones of inhibition of >20 mm by testing with the CAZ-5 disk were *K. pneumoniae* or *E. coli*.

The ability of other tests to detect ESBL production among *Enterobacteriaceae* varied with the species involved. Although the inclusion of 2 μg of clavulanate per ml in MIC tests with cefotaxime indicated the presence of ESBLs in all *K. pneumoniae* and *E. coli* isolates, it was not as reliable in tests with ESBL-producing *Enterobacter* or *Morganella* isolates. Ceftazidime was not as reliable an indicator of ESBL production in these tests as cefotaxime. Overall, the DD test was the most practical and least expensive test to perform in this study. Unlike other methods, the DD test utilizes more than one test agent, and this combination approach allows maximal detec-

tion of ESBL production among all species of *Enterobacteriaceae* (29).

In summary, during a 20-week period, 9.2% of all members of the family *Enterobacteriaceae* isolated at the McGuire VAMC produced ESBLs. Most of these appeared to be SHV-derived ESBLs, although several TEM-derived ESBLs were also involved. This finding is significant because many of these isolates did not appear to be resistant to expanded-spectrum cephalosporins in routine susceptibility tests. In addition, it was noted that some specimens may harbor both ESBL-producing and non-ESBL-producing isolates of the same species in the *Enterobacteriaceae*. Thus, multiple colonies should be tested for ESBL production for maximal detection. The optimal laboratory test for detection of ESBL production has not been identified to date. However, results of this study indicate that the DD test can be very useful, and disk diffusion tests with cefpodoxime warrant further study.

ACKNOWLEDGMENTS

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