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Detection of *Klebsiella pneumoniae* and *Escherichia coli* Strains Producing Extended-Spectrum β-Lactamases

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Plasmids encoding extended-spectrum β-lactamases of the TEM, SHV, and AmpC families were introduced into common *Escherichia coli* and *Klebsiella pneumoniae* hosts to create a homogeneous panel for evaluating the abilities of five test systems to detect resistance to eight β-lactam antibiotics. Although MICs, as determined by agar dilution or E test strips, were increased and disk diffusion zone diameters were diminished, breakpoints for resistance were often not reached, and neither approach was sensitive in detecting resistance to oxyimino-β-lactams. The MicroScan 18-h microdilution or Vitek rapid automated procedures were similarly insensitive. Ceftazidime was the best single test antibiotic for detecting extended-spectrum β-lactamase production. β-Lactamases TEM-7 and TEM-12 were particularly difficult to detect. Because of such difficulties, the prevalence of extended-spectrum β-lactamases is likely to be greater than is currently appreciated.

Strains of *Klebsiella pneumoniae*, *Escherichia coli*, and other gram-negative bacilli that make extended-spectrum β-lactamases have been described from many parts of the world (12, 19, 20). They are probably even more prevalent than is currently recognized because of difficulties in their detection by the clinical laboratory. Some extended-spectrum β-lactamases provide such low levels of resistance to aztreonam, cefotaxime, ceftriaxone, or ceftazidime that they are easily overlooked. In the study described here, we compared several test β-lactam antibiotics and five susceptibility testing procedures to evaluate the abilities of the procedures to detect strong and weak extended-spectrum enzymes introduced on plasmids into common *K. pneumoniae* or *E. coli* hosts.

MATERIALS AND METHODS

Antimicrobial agents. Antibiotics were obtained from the following sources: Glaxo Pharmaceuticals, Research Triangle Park, N.C. (cefuroxime), Hoechst-Roussel Pharmaceuticals Inc., Somerville, N.J. (cefotaxime), Eli Lilly & Co., Indianapolis, Ind. (ceftazidime and cephalothin), Merck & Co., Inc., West Point, Pa. (cefoxitin), Roche Laboratories, Nutley, N.J. (ceftriaxone), E. R. Squibb & Sons, Inc., Princeton, N.J. (aztreonam), and Stuart Pharmaceuticals, Wilmington, Del. (cefotetan). Antibiotic disks for susceptibility testing were obtained from Becton Dickinson Microbiology Systems, Cockeysville, Md. E test strips were provided by Anne Bolmström of AB Biodisk, Solna, Sweden.

Bacterial strains. Susceptible *E. coli* and *K. pneumoniae* recipients were chosen from clinical material. Donor strains were *E. coli* J53-2 (*met pro* Rif^r) (5) derivatives carrying plasmids encoding the extended-spectrum β-lactamase indicated in parentheses: pCFF04 (TEM-3) (4), pIF100 (TEM-7) (9), pMG224 (TEM-12; earlier YOU-2) (22, 23), pMG225 (TEM-26; earlier YOU-1) (22, 23), pMG229 (SHV-2) (13), pUD21 (SHV-4) (2), and pMG233 (MIR-1) (18). Additional properties of these plasmids have been described previously (13).

Strain construction. Plasmids were transferred to the prototrophic *E. coli* and *K. pneumoniae* recipients by conjugation in L broth and selection on plates containing minimal medium A (6), 0.5% glucose, and 2% agar without the methionine and proline required by strain J53-2. An antibiotic other than a β-lactam was used to select for plasmid transfer to avoid inadvertent host mutations that would enhance β-lactam resistance. Transconjugants were screened to confirm their full resistance phenotype prior to susceptibility testing.

Susceptibility tests. Agar dilution and disk susceptibility tests were performed and interpreted by following standard procedures of the National Committee for Clinical Laboratory Standards (16, 17). E test strips were inoculated, incubated, and interpreted according to the manufacturer's recommendations. Unsupplemented Mueller-Hinton agar was used for agar dilution, E test, and disk test procedures. An inoculum of 10^4 CFU per spot was used for agar dilution testing, with an end point of less than 10 surviving colonies. MicroScan Neg/Urine MIC Type 6 panels (18 h) (Baxter Diagnostics, Deerfield, Ill.) and Vitek GNS-DE, GNS-DF, and GNS-F4 susceptibility cards (4 h) (Vitek Systems, Hazelwood, Mo.) were inoculated and incubated according to the manufacturer's recommendations. MicroScan panels were read manually, and Vitek cards were interpreted by the Vitek AutoMicrobic System by using software version R06.4.

RESULTS

Test organisms. Clinical isolates of *E. coli* and *K. pneumoniae* that were susceptible to β-lactam and other antibiotics and that would accept plasmids carrying a variety of extended-spectrum β-lactamases were selected. The extended-spectrum β-lactamases TEM-7 and TEM-12 were chosen as representatives of enzymes that provide low-level resistance to oxyimino-β-lactams, whereas TEM-3, TEM-26, SHV-2, and SHV-4 were selected for their higher-level resistances. β-Lactamase MIR-1 was chosen as a plasmid-mediated AmpC-type enzyme that

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TABLE 1. Detection of extended-spectrum β-lactamases in *E. coli*[a]

β-Lactam and β-lactamase	MIC (μg/ml)				Disk diffusion zone diam (mm)
	Agar dilution	E test strip	MicroScan microdilution	Vitek automated	
Aztreonam					
R[−]	0.06	0.032	≤8.0	≤8.0	33
TEM-3	4.0	6.0	>16.0 (R)	≤8.0	22
TEM-7	0.5	0.38	≤8.0	≤8.0	30
TEM-12	0.12	0.125	≤8.0	≤8.0	32
TEM-26	8.0	6.0	>16.0 (R)	≤8.0	20 (I)
SHV-2	2.0	1.0	≤8.0	≤8.0	30
SHV-4	8.0	3.0	16.0 (I)	≤8.0	25
MIR-1	1.0	1.0	≤8.0	≤8.0	27
Cefotaxime					
R[−]	0.03	0.064	≤4.0	≤4.0	30
TEM-3	8.0	24.0 (I)	>32.0 (R)	6.0	14 (R)
TEM-7	0.12	0.094	≤4.0	≤4.0	31
TEM-12	0.06	0.094	≤4.0	≤4.0	34
TEM-26	0.25	0.50	≤4.0	≤4.0	30
SHV-2	8.0	4.0	32.0 (I)	≤4.0	17 (I)
SHV-4	0.5	0.75	≤4.0	≤4.0	29
MIR-1	1.0	1.0	≤4.0	≤4.0	26
Cefotetan					
R	0.125		≤4.0	≤16.0	30
TEM-3	0.5		≤4.0	≤16.0	29
TEM-7	0.25		≤4.0	≤16.0	30
TEM-12	0.125		≤4.0	≤16.0	32
TEM-26	0.25		≤4.0	≤16.0	33
SHV-2	0.25		≤4.0	≤16.0	32
SHV-4	0.25		≤4.0	≤16.0	32
MIR-1	4.0		≤4.0	≤16.0	22
Cefoxitin					
R[−]	8.0	3.0	8.0		
TEM-3	8.0	4.0	≤2.0		
TEM-7	8.0	2.0	8.0		
TEM-12	8.0	4.0	4.0		
TEM-26	8.0	6.0	8.0		
SHV-2	8.0	3.0	4.0		
SHV-4	8.0	3.0	4.0		
MIR-1	32.0 (R)	32.0 (R)	>16.0 (R)		
Ceftazidime					
R[−]	0.25	0.125	≤2.0	≤8.0	30
TEM-3	16.0 (I)	24.0 (I)	>16.0 (R)	≥32.0 (R)	16 (I)
TEM-7	8.0	12.0 (I)	>16.0 (R)	≥32.0 (R)	19
TEM-12	2.0	3.0	8.0	≤8.0	24
TEM-26	128.0 (R)	256.0 (R)	>16.0 (R)	≥32.0 (R)	11 (R)
SHV-2	4.0	2.0	4.0	≤8.0	26
SHV-4	4.0	4.0	8.0	≤8.0	24
MIR-1	1.0	1.0	≤2.0	≤8.0	28
Ceftriaxone					
R[−]	0.03	0.047	≤4.0	≤8.0	31
TEM-3	8.0	16.0 (I)	>32.0 (I)	≤8.0	14 (I)
TEM-7	0.12	0.125	≤4.0	≤8.0	31
TEM-12	0.06	0.047	8.0	≤8.0	32
TEM-26	0.25	0.5	≤4.0	≤8.0	29
SHV-2	8.0	8.0	32.0 (I)	≤8.0	18 (I)
SHV-4	0.5	0.5	≤4.0	≤8.0	28
MIR-1	0.5	2.0	≤4.0	≤8.0	25
Cefuroxime					
R[−]	8.0	4.0	4.0	≤4.0	23
TEM-3	128.0 (R)	256.0 (R)	>16.0 (R)	≥32.0 (R)	6 (R)
TEM-7	8.0	3.0	4.0	≤4.0	24
TEM-12	8.0	4.0	≤2.0	≤4.0	24
TEM-26	16.0 (I)	8.0	8.0	≤4.0	22

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TABLE 1—*Continued*

β-Lactam and β-lactamase	MIC (μg/ml)				Disk diffusion zone diam (mm)
	Agar dilution	E test strip	MicroScan microdilution	Vitek automated	
SHV-2	32.0 (R)	24.0	>16.0 (R)	10.0 (I)	13 (R)
SHV-4	8.0	6.0	4.0	8.0	23
MIR-1	64.0 (R)	32.0 (R)	16.0 (I)	12.0 (I)	10 (R)
Cephalothin					
R^-	8.0	8.0	8.0	5.0	20
TEM-3	≥256.0 (R)	≥256.0 (R)	>64.0 (R)	≥32.0 (R)	6 (R)
TEM-7	16.0 (I)	24.0 (I)	32.0 (R)	7.0	17 (I)
TEM-12	8.0	6.0	16.0 (I)	7.0	20
TEM-26	16.0 (I)	16.0 (I)	32.0 (R)	7.0	18
SHV-2	≥256.0 (R)	≥256.0 (R)	>64.0 (R)	≥32.0 (R)	6 (R)
SHV-4	128.0 (R)	96.0 (R)	64.0 (R)	≥32.0 (R)	12 (R)
MIR-1	≥256.0 (R)	≥256.0 (R)	>64.0 (R)	≥32.0 (R)	6 (R)

[a] R, resistant, I, intermediate.

confers resistance to cefotetan and cefoxitin as well as to the oxyimino compounds.

Susceptibility tests with *E. coli*. Table 1 shows the results of susceptibility tests on the *E. coli* derivatives that make extended-spectrum β-lactamases. By agar dilution, R^+ derivatives (except that which makes the TEM-12 enzyme) had decreased susceptibilities to aztreonam, cefotaxime, and ceftriaxone, but only with ceftazidime, cefuroxime, and cephalothin did some of the strains reach the breakpoint for resistance. The strain making MIR-1 had diminished susceptibility to cefotetan, but only with cefoxitin was the criterion for resistance met. The E test result was equal to or within plus or minus one doubling dilution of the agar dilution result in 86% of the tests. Like the agar dilution test result, the disk diffusion zone diameters for β-lactamase-producing strains were generally smaller than that for the R^- parent, although with TEM-7 and TEM-12 the diminution was evident only with ceftazidime. However, only with TEM-3, TEM-26, and SHV-2 was the interpretive standard for intermediate susceptibility or resistance met with at least two oxyimino-β-lactams. The 18-h MicroScan microdilution test detected resistance to an oxyimino drug in 10 of 28 tests, whereas the 4-h Vitek automated procedure did so in only 3 of 28 tests, all with ceftazidime. Of the oxyimino-β-lactams tested, ceftazidime was the most successful in detecting resistance, but resistance caused by SHV-2, SHV-4, or MIR-1 would have been missed if reliance were placed only on this drug. None of the test-drug combinations detected resistance caused by TEM-12 β-lactamase.

Susceptibility tests with *K. pneumoniae*. Results with *K. pneumoniae* (Table 2) were quite similar to those with *E. coli*. By agar dilution, oxyimino-β-lactam MICs were higher for derivatives producing an extended-spectrum β-lactamase, but the resistance breakpoint was reached for some enzymes only with ceftazidime, cefuroxime, and cephalothin and, for the strain producing MIR-1, with cefoxitin. Again, there was good agreement between MICs estimated by the E test or by agar dilution. A few more of the disk diffusion zone diameters were out of the susceptible range for the *K. pneumoniae* derivatives than for the *E. coli* derivatives, but no β-lactam disk detected resistance caused by TEM-7 or TEM-12. MicroScan microdilution produced an intermediate or resistant reading to an oxyimino agent in 11 of 28 tests, while 6 of 28 tests were similarly positive by the Vitek technique. Ceftazidime was again the most effective test oxyimino compound in detecting resistance. Resistance produced by SHV-4 was more easily detected in *K. pneumoniae* than in *E. coli*, while the opposite was true for TEM-3.

DISCUSSION

The mutations that broaden the specificities of extended-spectrum β-lactamases also tend to lower their efficiencies as enzymes and often result in enhanced but low-level resistance to oxyimino-β-lactams (11, 12). By agar dilution, the *E. coli* or *K. pneumoniae* strains that produced the seven enzymes tested in the present study would be considered susceptible to cefotaxime, ceftriaxone, and, with the exception of SHV-4 in *K. pneumoniae*, aztreonam. Agar dilution testing with ceftazidime detected resistance caused by TEM-3, TEM-26, and SHV-4, but strains that made TEM-7, TEM-12, SHV-2, and MIR-1 still appeared to be susceptible to ceftazidime. Disk diffusion zone diameters tended to be smaller with strains that produced an extended-spectrum β-lactamase but reached the limit for susceptibility in only 7 of 28 tests in *E. coli* and 11 of 28 tests in *K. pneumoniae*. A similar detection frequency was obtained by the conventional 18-h MicroScan microdilution procedure, while the rapid 4-h Vitek automated susceptibility method did well only with ceftazidime.

It is reasonable to wonder whether such a low level of in vitro resistance is clinically important. A number of patients infected with strains that make extended-spectrum β-lactamases have failed to respond to treatment with oxyimino-β-lactams (1, 9, 15, 21, 26, 27, 29). This failure may relate to the dramatic rise in MIC that occurs as the inoculum is increased (3, 7, 24). In animal models of infection, treatment with cefotaxime, ceftazidime, or ceftriaxone has been unsuccessful, despite antibiotic levels in serum in excess of the MIC determined at the conventional inoculum of 10^5 CFU/ml (3, 7, 24). Hence, the clinical laboratory should use the most reliable procedures available to detect the presence of extended-spectrum enzymes.

Disk diffusion, microdilution, or rapid automated testing may uncover the stronger extended-spectrum β-lactamases. Since the β-lactamase genes are often found on plasmids encoding resistance to aminoglycosides, sulfonamide, tetracycline, and other antibiotics, the finding of unusual resistance to these agents in *E. coli* or *K. pneumoniae* isolates should alert the laboratory to the need for further studies. Such rules can be incorporated into an automated expert system (10). Extended-spectrum enzymes in the TEM or SHV families become even

TABLE 2. Detection of extended-spectrum β-lactamases in *K. pneumoniae*[a]

β-Lactam and β-lactamase	MIC (μg/ml)				Disk diffusion zone diam (mm)
	Agar dilution	E test strip	MicroScan microdilution	Vitek automated	
Aztreonam					
R⁻	0.03	0.16	≤8.0	≤8.0	33
TEM-3	1.0	2.0	≤8.0	≤8.0	26
TEM-7	0.12	0.38	≤8.0	≤8.0	32
TEM-12	0.06	0.125	≤8.0	≤8.0	33
TEM-26	2.0	2.0	16.0 (I)	12.0 (I)	24
SHV-2	1.0	1.0	≤8.0	≤8.0	30
SHV-4	16.0 (I)	32.0 (R)	>16.0 (R)	≥32.0 (R)	12 (R)
MIR-1	0.5	0.5	≤8.0	≤8.0	27
Cefotaxime					
R⁻	0.03	0.032	≤4.0	≤4.0	30
TEM-3	4.0	8.0	≥32.0 (R)	≤4.0	19 (I)
TEM-7	0.12	0.19	≤4.0	≤4.0	30
TEM-12	0.06	0.064	≤4.0	≤4.0	31
TEM-26	0.25	0.38	≤4.0	≤4.0	29
SHV-2	4.0	12.0 (I)	8.0	≤4.0	18 (I)
SHV-4	2.0	8.0	32.0 (I)	≤4.0	18 (I)
MIR-1	1.0	8.0	≤4.0	≤4.0	22 (I)
Cefotetan					
R⁻	0.06		≤4.0	≤16.0	29
TEM-3	0.125		≤4.0	≤16.0	29
TEM-7	0.125		≤4.0	≤16.0	31
TEM-12	0.06		≤4.0	≤16.0	32
TEM-26	0.06		≤4.0	≤16.0	31
SHV-2	0.125		≤4.0	≤16.0	32
SHV-4	0.125		≤4.0	≤16.0	30
MIR-1	2.0		≤4.0	≤16.0	20
Cefoxitin					
R⁻	4.0	2.0	4.0		
TEM-3	2.0	2.0	≤2.0		
TEM-7	2.0	3.0	≤2.0		
TEM-12	2.0	2.0	≤2.0		
TEM-26	2.0	2.0	≤2.0		
SHV-2	4.0	2.0	≤2.0		
SHV-4	4.0	2.0	≤2.0		
MIR-1	32.0 (R)	48.0 (R)	>16.0 (R)		
Ceftazidime					
R⁻	0.12	0.25	≤2.0	≤8.0	29
TEM-3	4.0	8.0	16.0 (I)	14.0 (I)	20
TEM-7	8.0	16.0 (I)	16.0 (I)	14.0 (I)	20
TEM-12	2.0	3.0	4.0	≤8.0	23
TEM-26	32.0 (R)	64.0 (R)	>16.0 (R)	≥32.0 (R)	15 (I)
SHV-2	4.0	8.0	4.0	≤8.0	24
SHV-4	16.0 (I)	96.0 (R)	≥16.0 (R)	≥32.0 (R)	14 (R)
MIR-1	2.0	1.5	≤2.0	≤8.0	24
Ceftriaxone					
R⁻	0.03	0.064	≤4.0	≤8.0	29
TEM-3	4.0	12.0 (I)	32.0 (I)	≤8.0	19 (I)
TEM-7	0.12	0.25	≤4.0	≤8.0	28
TEM-12	0.06	0.125	≤4.0	≤8.0	29
TEM-26	0.5	1.0	≤4.0	≤8.0	16 (I)
SHV-2	8.0	≥32.0 (I)	16.0 (I)	≤8.0	18 (I)
SHV-4	4.0	≥32.0 (I)	32.0 (I)	≤8.0	16 (I)
MIR-1	1.0	3.0	≤4.0	≤8.0	21
Cefuroxime					
R⁻	4.0	2.0	≤2.0	≤4.0	24
TEM-3	32.0 (R)	32.0 (R)	≥16.0 (R)	5.0	16 (I)
TEM-7	4.0	3.0	≤2.0	≤4.0	24
TEM-12	4.0	3.0	≤2.0	≤4.0	24
TEM-26	4.0	3.0	≤2.0	≤4.0	25

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TABLE 2—*Continued*

β-Lactam and β-lactamase	MIC (μg/ml)				Disk diffusion zone diam (mm)
	Agar dilution	E test strip	MicroScan microdilution	Vitek automated	
SHV-2	32.0 (R)	24.0 (I)	>16.0 (R)	≤8.0	15 (I)
SHV-4	16.0 (I)	32.0 (R)	>16.0 (R)	6.0	14 (R)
MIR-1	64.0 (R)	48.0 (R)	>16.0 (R)	≤4.0	6 (R)
Cephalothin					
R⁻	4.0	3.0	≤4.0	≤2.0	24
TEM-3	128.0 (R)	192.0 (R)	>64.0 (R)	≥32.0 (R)	8 (R)
TEM-7	16.0 (I)	24.0 (I)	16.0 (I)	16.0 (I)	19
TEM-12	8.0	8.0	32.0 (R)	14.0 (I)	20
TEM-26	8.0	8.0	≤4.0	3.0	21
SHV-2	≥256.0 (R)	≥256.0 (R)	>64.0 (R)	≥32.0 (R)	6 (R)
SHV-4	256.0 (R)	≥256.0 (R)	>64.0 (R)	≥32.0 (R)	6 (R)
MIR-1	≥256.0 (R)	≥256.0 (R)	>64.0 (R)	≥32.0 (R)	6 (R)

[a] I, intermediate, R, resistant.

more susceptible than the parental types to inhibition by agents such as clavulanate or sulbactam, so that a double-disk diffusion test with an aztreonam or ceftazidime disk placed near one containing the inhibitor provides one technique for confirming the presence of these enzymes in the clinical laboratory (1, 14). A recently described three-dimensional test is reported to be even more sensitive (28). Cefotaxime and ceftazidime with and without clavulanate or sulbactam have been incorporated into experimental panels for the Vitek (8) and ATB CMI (bioMérieux, France) (25) systems, with greatly improved levels of detection of TEM- and SHV-type extended-spectrum β-lactamases. The false-positive susceptibility rate could also be reduced by lowering the breakpoints used to define resistance to oxyimino-β-lactams. A substantial number of clinical isolates would need to be evaluated with the new breakpoints and tested for the presence of an extended-spectrum β-lactamase to document the sensitivities and specificities of any new standards.

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