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Detection of Extended-Spectrum β-Lactamases in Clinical Isolates of *Klebsiella pneumoniae* and *Escherichia coli*

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Forty clinical isolates of *Escherichia coli* and 141 isolates of *Klebsiella pneumoniae* that either transferred ceftazidime resistance or showed sulbactam enhancement of oxyimino-β-lactam susceptibility were tested by disk diffusion methodology for susceptibility to aztreonam, cefotaxime, ceftazidime, and cefoxitin. With standard 30-μg antibiotic disks, the fraction of these extended-spectrum β-lactamase (ESBL)-producing isolates testing resistant by National Committee for Clinical Laboratory Standards criteria was lowest (24%) with cefotaxime disks. Forty percent of the *E. coli* and 29% of the *K. pneumoniae* isolates appeared susceptible with at least one oxyimino-β-lactam disk. Ceftazidime and aztreonam disks were equivalent in differentiating ESBL production, and both were superior to cefotaxime disks. Over half the *E. coli* and 29% of the *K. pneumoniae* isolates tested cefoxitin resistant. In 30 isolates, cefoxitin resistance was transmissible and due to a plasmid-mediated AmpC-type β-lactamase. With a 5-μg ceftazidime disk, a breakpoint could be chosen with high sensitivity and specificity for ESBL-producing organisms. Present disk diffusion criteria underestimate the prevalence of ESBL-producing strains.

Extended-spectrum β-lactamases (ESBLs) are enzymes that confer resistance to aztreonam, cefotaxime, ceftazidime, and related oxyimino-β-lactams as well as to older penicillins and cephalosporins (4, 5, 14, 16). They arise by mutations in genes for common plasmid-mediated β-lactamases (especially TEM and SHV enzymes) that alter the configuration of the enzyme near its active site to increase the affinity and hydrolytic ability of the β-lactamase for oxyimino compounds while simultaneously weakening the overall enzyme efficiency. Some ESBLs confer high-level resistance to all oxyimino-β-lactams, but for other ESBLs, resistance is only slightly increased or increased selectively for particular β-lactams, which creates a problem for the clinical laboratory, since organisms producing less active ESBLs can fail to reach current National Committee for Clinical Laboratory Standards (NCCLS) breakpoints for resistance yet can cause significant disease (7, 11, 15).

A second type of transmissible resistance to oxyimino-β-lactams arises from plasmid acquisition of a normally chromosomal *ampC* gene. In addition to oxyimino-β-lactam resistance, AmpC β-lactamase also provides resistance to cephamycins such as cefoxitin. *Escherichia coli* and *Klebsiella pneumoniae* isolates with this type of resistance are currently thought to be rare.

To document the detection problem and to devise ways to overcome it, oxyimino-β-lactam-resistant isolates of *K. pneumoniae* and *E. coli* from more than two dozen hospitals in the United States have been characterized for ESBL production, transmissibility of resistance, and susceptibility by Kirby-Bauer disk diffusion methodology to standard 30-μg aztreonam, cefotaxime, and ceftazidime disks. The utility of a 5-μg ceftazidime disk has also been explored.

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MATERIALS AND METHODS

Bacterial isolates. Initially, 169 clinical isolates of *K. pneumoniae* and 111 isolates of *E. coli* were submitted from 29 hospitals in 15 states as resistant to ≥2 μg of aztreonam, ceftazidime, cefotaxime, or ceftriaxone per ml. Sixty-one *E. coli* and 35 *K. pneumoniae* isolates susceptible to oxyimino-β-lactams were obtained from clinical laboratories at the Lahey Hitchcock Clinic and the Bedford Veterans Administration Hospital and retested by disk diffusion to confirm susceptibility. A spontaneous mutant of *E. coli* J53 (F^- *met pro*) (3) resistant to 250 μg of sodium azide per ml (J53 Azi^r) was used as the recipient in resistance transfer experiments.

Susceptibility tests. Disk susceptibility tests were performed and interpreted according to the NCCLS criteria (12), using unsupplemented Mueller-Hinton agar (Difco Laboratories, Detroit, Mich.). Thirty-microgram aztreonam disks were purchased from Difco, and 30-μg disks containing cefotaxime, cefoxitin, or ceftazidime were obtained from Becton Dickinson Microbiology Systems, Cockeysville, Md. Twenty micrograms of sulbactam (Pfizer, Inc. New York, N.Y.) was added to one set of 30-μg oxyimino-β-lactam disks. Five-microgram ceftazidime disks were made by adding ceftazidime (Smith Kline & French Laboratories, Philadelphia, Pa.) to blank disks (Becton Dickinson).

β-Lactamase characterization. The transmissibility of resistance was tested by mating to J53 Azi^r with selection on Trypticase soy agar plates (Becton Dickinson) containing 100 μg of sodium azide (Sigma Chemical Co., St. Louis, Mo.) per ml and 10 μg of ceftazidime per ml or 25 μg of ampicillin (Bristol-Myers Squibb Co., Princeton, N.J.) per ml. β-Lactamase produced by transconjugants or clinical isolates was extracted by lysozyme (Sigma) treatment (1) and subjected to isoelectric focusing on a polyacrylamide gel by a modified version (10) of the method of Matthew et al. (9). Strains carrying plasmids (6) encoding β-lactamases TEM-1 (encoded by R1) (isoelectric point [pI], 5.4), TEM-3 (pCFF04) (pI, 6.3), TEM-4 (pUD16) (pI, 5.9), TEM-26 (pMG225) (pI, 5.6), SHV-2 (pMG229) (pI, 7.6), SHV-3 (pUD18) (pI, 7.0), SHV-4 (pUD21) (pI, 7.75), and SHV-5 (pAFF2) (pI, 8.2) served as the standards.

RESULTS

Study sample. Each submitted isolate was tested for susceptibility by the disk diffusion method with 30-μg disks of aztreonam, cefotaxime, or ceftazidime, with and without 20 μg of sulbactam, and with disks containing 30 μg of cefoxitin or 5 μg of ceftazidime. Sulbactam enhancement of the diameter of the zone of inhibition around any oxyimino-β-lactam disk by at least 5 mm (Fig. 1) was taken as presumptive evidence for the presence of an ESBL on the basis of enhancement observed with strains proven to transfer ceftazidime resistance.

Figure 2 shows the degree of sulbactam enhancement for the 21 *E. coli* and 96 *K. pneumoniae* isolates demonstrated to

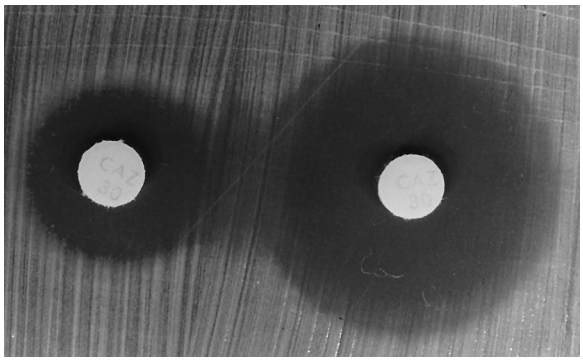

FIG. 1. Response of an ESBL-producing isolate of *K. pneumoniae* with disks containing 30 μg of ceftazidime (left) and 30 μg of ceftazidime plus 20 μg of sulbactam (right).

transfer ceftazidime resistance. All but 17 isolates had a ≥5-mm enhancement of susceptibility in the presence of the β-lactamase inhibitor, while no susceptible control strains showed a ≥5-mm sulbactam effect. With a 5-mm breakpoint for sulbactam enhancement, 39 of the 111 isolates of *E. coli* supplied as resistant and 124 of the 169 submitted isolates of *K. pneumoniae* were designated ESBL producers. Maximal sulbactam enhancement was found with the aztreonam disk in 61 isolates, with the ceftazidime disk in 47 isolates, and with the cefotaxime disk in 29 isolates. For 26 isolates, maximal sulbactam enhancement was the same with aztreonam or ceftazidime.

When the remaining isolates were tested for the ability to transfer ceftazidime resistance by conjugation to *E. coli* J53 Azi^r, 1 additional ESBL-producing *E. coli* and 17 additional ESBL-producing *K. pneumoniae* isolates were detected. The ESBL-producing *E. coli* isolates came from 13 locales, and the ESBL-producing *K. pneumoniae* isolates came from 25 hospitals in the United States. In the study sample, no more than 6 *E. coli* isolates and no more than 16 *K. pneumoniae* isolates came from a single site.

β-Lactamase production by transconjugants was characterized by isoelectric focusing. SHV-type ESBLs, especially SHV-5 and SHV-4, predominated, but SHV-2, SHV-3, and several TEM-type ESBLs were present as well as plasmid-mediated AmpC β-lactamases. Further description of these enzymes will be reported elsewhere.

Susceptibility to 30-μg oxyimino-β-lactam disks. The diameters of zones of inhibition around 30-μg aztreonam, cefotaxime, and ceftazidime disks are shown in Fig. 3 for the 40 isolates of *E. coli* designated ESBL producers and in Fig. 4 for the 141 ESBL-producing isolates of *K. pneumoniae*. The distribution of zone diameters did not differ between strains able or not able to transfer ESBL production. Table 1 shows the distribution of ESBL-positive strains that would be labelled as susceptible, intermediate, or resistant with each type of disk with current NCCLS criteria. It is evident that many ESBL-producing isolates appeared to be susceptible by disk diffusion and that the cefotaxime disk was the least successful in detecting resistance. Overall, 40% (16 of 40) ESBL-producing *E. coli* and 29% (41 of 141) ESBL-producing *K. pneumoniae* isolates tested susceptible with at least one oxyimino-β-lactam disk. Among the 181 ESBL-producing isolates, only one tested susceptible to aztreonam and ceftazidime but resistant to cefotaxime. Aztreonam and ceftazidime disks were equivalent in detecting resistance, but both disks were useful. For example, 5 of 39 ESBL-producing isolates testing ceftazidime susceptible were aztreonam resistant, while 4 of 31 ESBL-producing isolates judged susceptible to aztreonam tested resistant to ceftazidime.

Isolates testing susceptible to oxyimino-β-lactams included isolates producing SHV-2, SHV-3, SHV-4, SHV-5, TEM-12, other TEM-type enzymes, and AmpC-type β-lactamase.

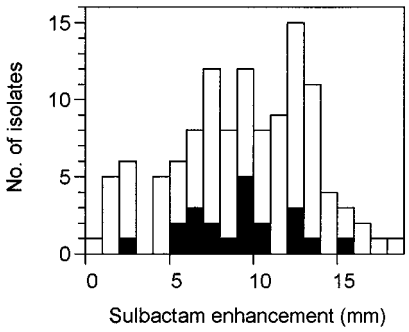

FIG. 2. Maximal sulbactam enhancement with an aztreonam, cefotaxime, or ceftazidime disk for *E. coli* (filled bars) and *K. pneumoniae* (unfilled bars) isolates able to transfer ceftazidime resistance.

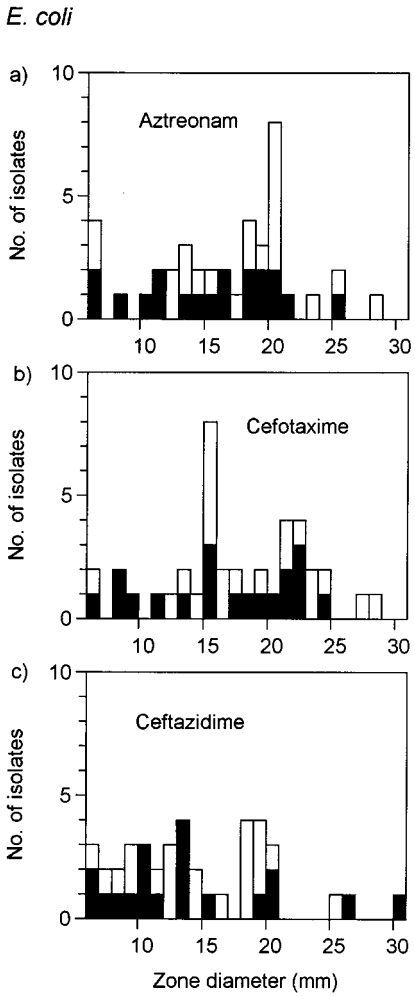

FIG. 3. Distribution of ESBL-producing isolates of *E. coli* with 30-μg antibiotic disks of aztreonam, cefotaxime, or ceftazidime. Filled bars represent isolates unable to transfer ESBL production by conjugation, and unfilled bars denote transfer proficient isolates.

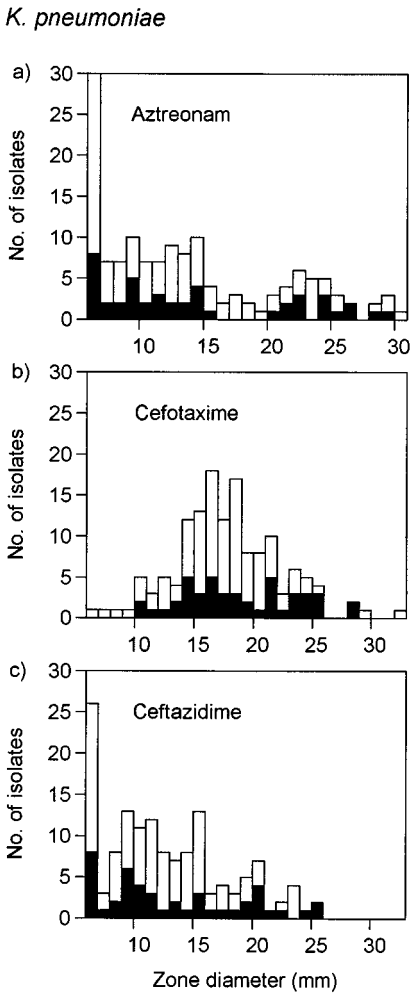

FIG. 4. Distribution of ESBL-producing isolates of *K. pneumoniae* with 30-μg antibiotic disks of aztreonam, cefotaxime, or ceftazidime. Filled bars represent strains unable to transfer ESBL production by conjugation, and unfilled bars denote transfer proficient isolates.

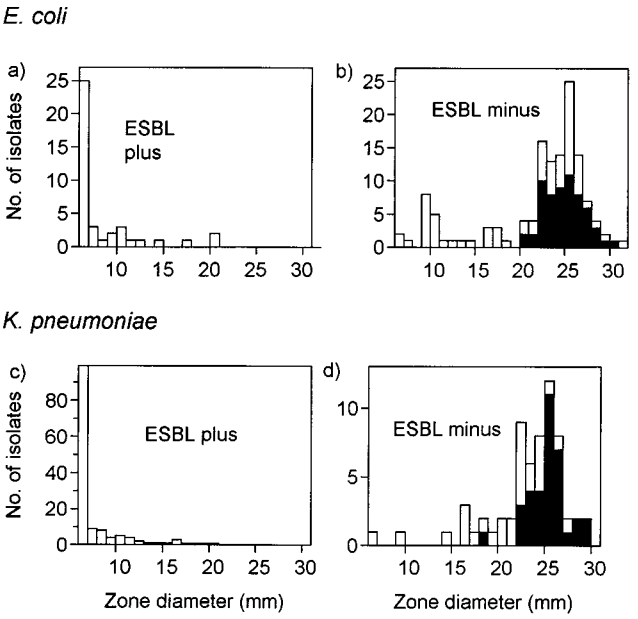

FIG. 5. Inhibition zone diameters using 5-μg ceftazidime disk with isolates of *E. coli* or *K. pneumoniae* producing ESBLs (ESBL plus) or *E. coli* or *K. pneumoniae* not meeting criteria for ESBL production (ESBL minus). Fully susceptible control strains are indicated by filled bars.

Of 71 submitted isolates of *E. coli* that did not meet the criteria for ESBL production, 52 tested susceptible to aztreonam, 61 tested susceptible to cefotaxime, and 67 tested susceptible to ceftazidime. For 28 corresponding isolates of *K. pneumoniae*, 25 were susceptible to aztreonam, 26 were susceptible to cefotaxime, and 27 were susceptible to ceftazidime. Isoelectric focusing indicated that oxyimino-β-lactam-resistant isolates produced large amounts of an AmpC-type enzyme (*E. coli*) or β-lactamases with pIs consistent with those of SHV-2, SHV-3, and SHV-5 (*K. pneumoniae*).

Susceptibility to cefoxitin. Although ESBLs derived from TEM and SHV β-lactamases do not provide resistance to cefoxitin, Table 1 shows that 62 of the 181 ESBL-producing isolates of *K. pneumoniae* and *E. coli* tested resistant to cefoxitin by disk diffusion. Among submitted isolates not shown to produce an ESBL, 10 of 99 isolates were cefoxitin resistant, while 0 of 96 oxyimino-β-lactam-susceptible isolates tested cefoxitin resistant.

Cefoxitin resistance could be transferred from 5 isolates of *E. coli* and from 25 isolates of *K. pneumoniae* originating in nine hospitals. Fourteen of the 17 isolates with ≤5-mm sulbactam enhancement in Fig. 2 were cefoxitin resistant with transmissible AmpC β-lactamase production. The other three isolates produced SHV-type ESBLs. The 16 other isolates with transmissible AmpC-mediated resistance showed sulbactam enhancement of oxyimino-β-lactam susceptibility from 6 to 13 mm.

Susceptibility to 5-μg ceftazidime disks. Figure 5 shows that all isolates of *E. coli* with transmissible oxyimino-β-lactam resistance had inhibition zone diameters of 20 mm or less to a 5-μg ceftazidime disk. Twenty-nine of 71 submitted *E. coli* iso-

TABLE 1. Distribution of ESBL-producing isolates in susceptibility categories by disk diffusion

Species (no. of isolates)	Susceptibility category	No. (%) of ESBL-producing isolates with in each category with 30-μg disks of:			
		Aztreonam	Cefotaxime	Ceftazidime	Cefoxitin
E. coli (40)	Susceptible	4 (10)	6 (15)	14 (35)	18 (45)
	Intermediate	19 (48)	24 (60)	2 (5)	1 (3)
	Resistant	17 (42)	10 (25)	24 (60)	21 (52)
K. pneumoniae (141)	Susceptible	27 (19)	19 (13)	25 (18)	93 (66)
	Intermediate	15 (11)	89 (63)	20 (14)	7 (5)
	Resistant	99 (70)	33 (23)	96 (68)	41 (29)

lates not meeting criteria for ESBL production, but only 2 of 61 susceptible isolates also had inhibition zone diameter of ≤20 mm. Similarly, all isolates of *K. pneumoniae* with transmissible oxyimino-β-lactam resistance had inhibition zone diameters of 20 mm or less to a 5-μg ceftazidime disk, as did 11 of 28 *K. pneumoniae* isolates not demonstrated to produce an ESBL, but only 1 of 35 susceptible isolates. With a breakpoint of 17 mm, 4% of presumptive ESBL isolates would score negative, and there would be no false-positive results among susceptible isolates.

DISCUSSION

Since the test sample came from multiple hospitals and contained organisms producing a variety of enzyme types, it should be representative of the current population of oxyimino-β-lactam-resistant nosocomial *K. pneumoniae* and *E. coli* isolates in the United States. Sulbactam enhancement of oxyimino-β-lactam susceptibility was used to screen for ESBL production. On the basis of the responses of isolates shown to transfer resistance to ceftazidime, isolates with at least a 5-mm sulbactam enhancement of the inhibition zone diameter around a conventional aztreonam, cefotaxime, or ceftazidime disk were designated presumptive ESBL producers. This criterion, however, is neither wholly specific nor completely sensitive for ESBL production. Three isolates with transmissible SHV-type ESBLs gave a negative response, and 16 of 30 isolates with transmissible AmpC-type β-lactamase had a positive sulbactam enhancement test. Consequently, lack of sulbactam enhancement cannot be relied on to differentiate *K. pneumoniae* and *E. coli* isolates producing AmpC-type enzymes from those producing TEM- or SHV-type ESBLs. Sixty percent of isolates with a positive sulbactam enhancement test could transfer resistance to ceftazidime. In the remainder, the responsible β-lactamase gene may be carried on the chromosome or on a transfer-deficient plasmid. The same location may be responsible for the few isolates testing intermediate or resistant with 30-μg aztreonam, cefotaxime, or (least often) ceftazidime disks that failed to demonstrate either transmissibility or sulbactam enhancement. Isoelectric focusing indicated that they produced β-lactamases consistent with SHV-type ESBLs or, for *E. coli*, large amounts of AmpC-type β-lactamase.

The problem in detecting ESBL-producing organisms by disk diffusion with conventional 30-μg disks is that depending on the test antibiotic, as few as 23% isolates (*K. pneumoniae* with cefotaxime) meet current NCCLS criteria for resistance while as many as 35% isolates (*E. coli* with ceftazidime) appear susceptible (Table 1). False-susceptible results were found with all ESBL types (SHV, TEM, and AmpC) in the sample. Several reasons for this are known. TEM-type ESBLs with single amino acid substitutions (such as TEM-7 or TEM-12) have only low-level oxyimino-β-lactam activity (4). Other ESBLs have greater relative oxyimino-β-lactam activity but pay a price for their expanded spectrum in lower intrinsic enzyme efficiency (2). A strong β-lactamase gene promoter can compensate for lower specific activity but may be lacking in less-resistant clinical isolates. Such strains can still, however, cause disease, and one factor in their success is the enhancement of resistance that occurs with increased inoculum (4).

An unanticipated finding was the high frequency of cefoxitin resistance in the study sample. In 32 of 62 isolates, cefoxitin resistance could not be transferred. Seven such *E. coli* isolates were shown to produce large amounts of AmpC β-lactamase by isoelectric focusing, presumably from alterations in the control of the *ampC* chromosomal gene (13, 17). *K. pneumoniae* strains lack this gene and may instead have altered expression of the porin channel through which cefoxitin penetrates the outer cell membrane. Such porin loss can also augment the level of resistance due to ESBL production and limit the access of sulbactam or other β-lactamase inhibitors to the enzyme (8).

One way to increase the sensitivity of ESBL detection is with a 5-μg ceftazidime disk. All designated ESBL-producing *E. coli* and *K. pneumoniae* isolates had inhibition zone diameters of 20 mm or less with this disk (Fig. 5). Ninety-six percent would be detected with an inhibition zone diameter of 17 mm or less, while all susceptible control isolates had zone diameters of 18 mm or more. Disks containing cefpodoxime also appear promising for ESBL detection (18).

Further studies are needed to establish the optimal technique for detecting ESBL production. Meanwhile, it should be recognized that present disk diffusion criteria underestimate the prevalence of these strains and that cefotaxime disks are inferior in sensitivity to aztreonam or ceftazidime disks for their detection.

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