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High-Level Resistance to Cefotaxime and Ceftazidime in *Klebsiella pneumoniae* Isolates from Cleveland, Ohio

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Two isolates of *Klebsiella pneumoniae* possessing both TEM-1 and SHV-2 β-lactamases were isolated from patients at the Cleveland Clinic in 1988. The β-lactamases were discriminated and identified by using substrate hydrolysis data and an isoelectric focusing procedure in which the gel was overlaid with β-lactamase inhibitors.

Klebsiella pneumoniae produces a chromosomally mediated β-lactamase, usually at a low level. Depending on the level of expression, this enzyme tends to confer resistance to penicillins but not to cephalosporins. Production of the plasmid-mediated β-lactamases TEM-1 and SHV-1 usually confers resistance to penicillins and older cephalosporins and sometimes to cefamandole and cefoperazone. Strains producing these enzymes usually remain susceptible to cefotaxime, ceftazidime, ceftriaxone, and aztreonam. However, since 1983 strains of *K. pneumoniae* have been reported to be resistant or to have reduced susceptibility to these four agents (14). Such strains produce derivatives of TEM and SHV β-lactamases. To date, the occurrence of strains producing these extended-spectrum β-lactamases has been predominantly in countries outside the United States.

Since 1988, there have been seven reports of extended-spectrum β-lactamases being detected in the United States (6, 8, 9, 13, 15, 16, 18). These β-lactamases were produced by members of the family *Enterobacteriaceae*, predominantly *K. pneumoniae*, isolated from patients in the northeastern quadrant of the United States. At least eight enzyme types, based on isoelectric points, have been reported, but only three, TEM-10 (14), TEM-12 (18), and MIR-1 (13), have been described in detail. Only one report describes an enzyme with features consistent with SHV-2 (9). We report two strains of *K. pneumoniae*, isolated from patients at the Cleveland Clinic in 1988, that produce both TEM-1 and SHV-2.

The two strains of *K. pneumoniae*, JW1 and JW2, were isolated in June and August 1988 from patients at the Cleveland Clinic, Cleveland, Ohio. They were isolated from urine and from a miscellaneous specimen (i.e., not blood, urine, or respiratory secretions). Antibiotic susceptibility was determined by standard disk diffusion (10) and agar dilution (11) procedures. For the latter, an inoculum of 10^4 CFU per spot was used. β-Lactamases were initially investigated by using the double-disk-potentiation test of Jarlier et al. (7) and then by spectrophotometric assay using 50 and 100 μM antibiotic solutions and crude β-lactamase preparations derived from sonicated bacterial cultures (12). β-Lactamases were also characterized on isoelectric focusing gels by simultaneously determining their pIs and substrate (1) or inhibitor (17) profiles. Plasmid DNA was prepared by the alkaline extraction method of Birnboim and Doly (2). Transformation procedures were performed by both the one-step transformation method (3) and the Hanahan method (5), attempting to select transformants on Luria-Bertani agar (Difco) containing 25 μg of ampicillin per ml. Mating experiments were performed on 0.45-μm filter disks for 2 to 18 h at 37°C with either *Escherichia coli* W3110 (Nal^r *lac*) or *E. coli* C600N (Nal^r *lac*) as recipient. Attempts to mobilize plasmid DNA were carried out in triparental matings using the in *trans* conjugative properties of pRK2013 in *E. coli* AC80 (*thr leu met hsdR hsdM*) (4). For selection of β-lactamase-producing transconjugants, nalidixic acid (14 μg/ml) was incorporated into solid media in combination with one or more of the following: ampicillin (40 or 100 μg/ml), ceftazidime (2 μg/ml), gentamicin (8 μg/ml), and tetracycline (20 μg/ml).

The two isolates appeared to be identical in comparisons of their biochemical reactions, antibiograms, β-lactamases (Table 1; see Fig. 2), and plasmid profiles (see below). This suggested that they were the same strain. They were multiply antibiotic resistant and among the β-lactams were susceptible to imipenem, cefoxitin, and cefoperazone-sulbactam (2:1) and showed diminished susceptibility to cefotaxime and ceftazidime (MIC = 16 μg/ml) and aztreonam (MIC = 8 μg/ml). Both isolates were susceptible to ciprofloxacin and amikacin but were resistant to gentamicin, kanamycin, ampicillin, ticarcillin, piperacillin, amoxicillin-clavulanate, ticarcillin-clavulanate, cefamandole, cefoperazone, tetracycline, sulfamethoxazole-trimethoprim, and chloramphenicol.

The positioning of an amoxicillin-clavulanate disk near disks containing cefotaxime, ceftazidime, and cefoxitin potentiated the activities of cefotaxime and (only marginally) ceftazidime but not cefoxitin when the agents were tested against lawn cultures of JW1 and JW2. For these organisms, the disk locations recommended by Jarlier et al. (7), i.e., 30 mm apart, did not detect the potentiation. It was necessary to place the disks closer together, with the optimal distances from the amoxicillin-clavulanate disk being 17 mm for cefotaxime and 19 mm for ceftazidime (Fig. 1). Hydrolysis assays showed that cefotaxime was hydrolyzed more rapidly than ceftazidime and aztreonam by the crude enzyme preparations (Table 1). This type of substrate profile was consistent with reports of TEM-3, TEM-4, CAZ-2, and SHV extended-spectrum β-lactamases (14).

Isoelectric focusing showed that both isolates produced

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TABLE 1. Substrate hydrolysis of β-lactamase preparations

Enzyme prepn	Hydrolysis (nmol of substrate hydrolyzed/min/mg of protein)			
	Nitrocefin (100 μM)	Cefotaxime (100 μM)	Ceftazidime (50 μM)	Aztreonam (100 μM)
JW1	287	10	0.17	—[a]
JW2	278	16	0.13	—
TEM-1	685	—	—	—
SHV-1	747	—	—	—
SHV-2	108	10	0.13	—

[a] —, rate of hydrolysis < 0.1.

two β-lactamases with pI values of 5.4 and 7.6 (Fig. 2), values consistent with TEM-1 (pI 5.4) and SHV-1 (pI 7.6) or SHV-2 (pI 7.6). OXA-5 (pI 7.62) from *E. coli* J53(pSC86) was distinctly different from the pI 7.6 enzyme in that it did not align with it on the gel (data not shown). It was therefore necessary to further investigate the pI 7.6 enzyme by adopting inhibitor (17) and substrate-based (1) techniques for the characterization of β-lactamases after isoelectric focusing on polyacrylamide gels. In the inhibitor-based procedure, we used well-characterized TEM-1 [from *E. coli* RTEM(R6K)], SHV-1 [from *E. coli* J53(R1010)], and SHV-2 (from *Klebsiella ozaenae* 2180) β-lactamase preparations as controls and simultaneously examined the susceptibility to inhibition of the β-lactamases of JW1 and JW2 when they were overlaid by solutions of potassium clavulanate (1,000 μM), cloxacillin (1,000 μM), cephalothin (10,000 and 20,000 μM), cefotaxime (10,000 and 20,000 μM), ceftazidime (10,000 and 20,000 μM), and aztreonam (10,000 μM). The TEM-1 control and the pI 5.4 enzyme of the JW isolates behaved identically in these tests (Fig. 2). Both the pI 7.6 enzyme of the JW isolates and the SHV-2 control were substantially inhibited by aztreonam in comparison to the SHV-1 control (Fig. 2). The other inhibitors failed to discriminate between SHV-1 and SHV-2.

The substrate-based technique (1) comprised overlaying a focused isoelectric focusing gel with Mueller-Hinton agar containing cefotaxime (1 μg/ml) followed by incubation at 35°C for 2 h, after which the plate was inoculated with a lawn culture of *E. coli* ATCC 25922 (cefotaxime MIC, 0.06 to 0.25 μg/ml) and incubated overnight at 35°C. Growth occurred above the SHV-2 and pI 7.6 bands of the JW strains but not above the pI 5.4, TEM-1, or SHV-1 bands. This procedure

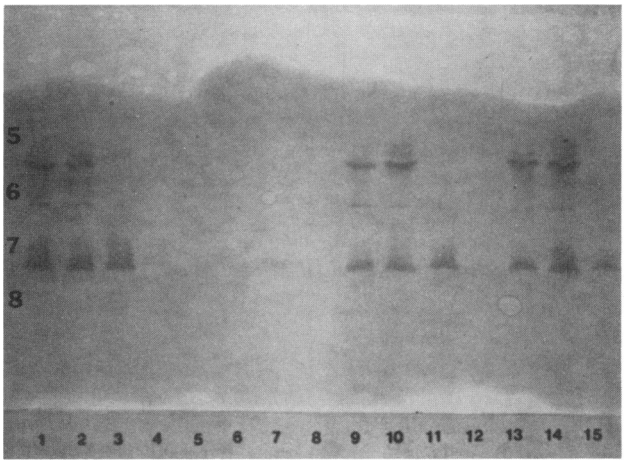

FIG. 2. Isoelectric focusing of β-lactamases using nitrocefin agar for detection and inhibitor-based detection system. Lanes 1 to 3 were untreated before being overlaid with nitrocefin agar. Lanes 5 to 7 were treated with 1,000 μM potassium clavulanate, lanes 9 to 11 were treated with 1,000 μM cloxacillin, and lanes 13 to 15 were treated with 10,000 μM aztreonam before being overlaid with nitrocefin agar. The enzymes in each lane were as follows: JW1 (pI 5.4, 7.6) in lanes 1, 5, 9, and 13; TEM-1 (pI 5.4) and SHV-1 (pI 7.6) in lanes 2, 6, 10, and 14; and SHV-2 (pI 7.6) in lanes 3, 7, 11, and 15. Lanes 4, 8, and 12 are blank. Numbers at left indicate pH. Apparent bands at pH 6.3 represent the location of sample application. Identification of the SHV-2-like nature of the pI 7.6 enzyme of JW1 was achieved by comparing this enzyme in lane 1 (no inhibitor) and in lane 13 (aztreonam applied as inhibitor). The partial inhibition of this enzyme by aztreonam in lane 13 is indicated by the fainter appearance of the pI 7.6 band and by the distinctive disappearance of the band's tail. This effect is identical to the partial inhibition by aztreonam of SHV-2 (compare bands and tail in lanes 3 and 15) and in direct contrast to the absence of inhibition by aztreonam of either band or tail of SHV-1 (compare lanes 2 and 14).

confirmed that it was the pI 7.6 enzyme and not the pI 5.4 enzyme of the JW strains that hydrolyzed cefotaxime and that this enzyme resembled SHV-2 but not SHV-1.

Plasmid profiles showed both JW1 and JW2 to contain four plasmids of approximate sizes 2.7, 3.4, 6.4, and 9.3 kb. No large conjugative plasmids were detected. Repeated at-

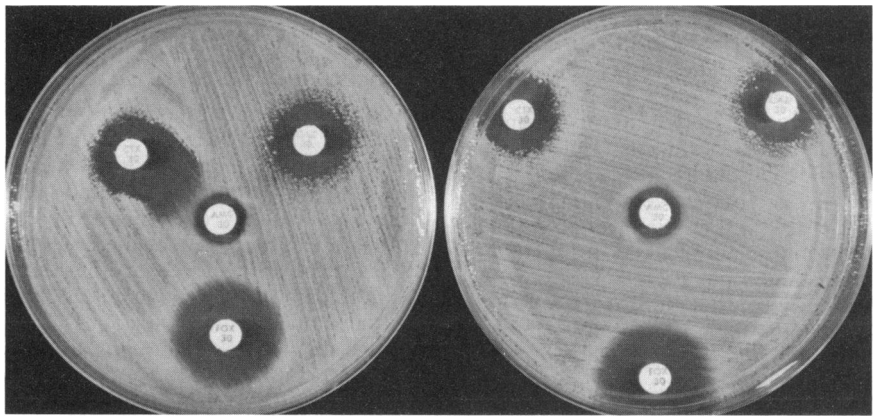

FIG. 1. Double-disk-potentiation test showing 30-mm disk spacing recommended by Jarlier et al. on the right (7) and a closer, more optimal disk arrangement on the left, indicating potentiation of cefotaxime activity. The three antibiotic disks strategically placed around a disk of amoxicillin-clavulanate (AMC 30) are cefotaxime (CTX 30) (left), ceftazidime (CAZ 30) (right), and cefoxitin (FOX 30) (bottom).

tempts to transfer the β-lactamase genes of JW1 and JW2 into *E. coli* by both conjugation and transformation of plasmid DNA were unsuccessful. The difficulties experienced in transferring the genes for these β-lactamases into *E. coli* raise the possibility that they may be chromosomally located.

Thus, on the basis of the rate of cefotaxime hydrolysis, isoelectric points, and discriminatory inhibition of SHV-2 by aztreonam, we conclude that strains JW1 and JW2 produce both TEM-1 and SHV-2 β-lactamases. In this example, the inhibitor and substrate studies on isoelectric focusing gels proved to be crucial to the identification of the β-lactamases of bacteria producing more than one enzyme.

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