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Epidemiology of Conjugative Plasmid-Mediated AmpC β-Lactamases in the United States

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A sample of 752 resistant *Klebsiella pneumoniae*, *Klebsiella oxytoca*, and *Escherichia coli* strains from 70 sites in 25 U.S. states and the District of Columbia was examined for transmissibility of resistance to ceftazidime and the nature of the plasmid-mediated β-lactamase involved. Fifty-nine percent of the *K. pneumoniae*, 24% of the *K. oxytoca*, and 44% of the *E. coli* isolates transferred resistance to ceftazidime. Plasmids encoding AmpC-type β-lactamase were found in 8.5% of the *K. pneumoniae* samples, 6.9% of the *K. oxytoca* samples, and 4% of the *E. coli* samples, at 20 of the 70 sites and in 10 of the 25 states. ACT-1 β-lactamase was found at eight sites, four of which were near New York City, where the ACT-1 enzyme was first discovered; ACT-1 β-lactamase was also found in Massachusetts, Pennsylvania, and Virginia. FOX-5 β-lactamase was also found at eight sites, mainly in southeastern states but also in New York. Two *E. coli* strains produced CMY-2, and one *K. pneumoniae* strain produced DHA-1 β-lactamase. Pulsed-field gel electrophoresis and plasmid analysis suggested that AmpC-mediated resistance spread both by strain and plasmid dissemination. All AmpC β-lactamase-containing isolates were resistant to cefoxitin, but so were 11% of strains containing transmissible SHV- and TEM-type extended-spectrum β-lactamases. A β-lactamase inhibitor test was helpful in distinguishing the two types of resistance but was not definitive since 24% of clinical isolates producing AmpC β-lactamase had a positive response to clavulanic acid. Coexistence of AmpC and extended-spectrum β-lactamases was the main reason for these discrepancies. Plasmid-mediated AmpC-type enzymes are thus responsible for an appreciable fraction of resistance in clinical isolates of *Klebsiella* spp. and *E. coli*, are disseminated around the United States, and are not so easily distinguished from other enzymes that mediate resistance to oxyimino-β-lactams.

Plasmid-mediated AmpC-type enzymes are less common than are extended-spectrum β-lactamases (ESBLs) as a mechanism for resistance to ceftazidime and other oxyimino-β-lactams in *Klebsiella pneumoniae* and *Escherichia coli*; such enzymes are important to recognize since they provide an even broader spectrum of resistance (15, 19). ESBLs are predominantly derived from members of the TEM-, SHV-, and OXA-β-lactamase families by one or more amino acid substitutions that facilitate the accommodation between the enzyme's active site and oxyimino-β-lactam substrates (3). AmpC β-lactamase genes are native to the chromosome of many gram-negative bacilli but are missing from *Klebsiella* spp. and are poorly expressed in *E. coli*. Acquisition of AmpC-type genes by plasmids in *K. pneumoniae* and *E. coli* has been known since 1989, but only recently has their occurrence in multihospital samples from the United States been published (5, 15). The aims of this study were to establish the frequency of this resistance mechanism in isolates of *K. pneumoniae*, *Klebsiella oxytoca*, and *E. coli* from various sites in the United States, to identify the particular AmpC enzyme types involved, to gain insight into how the involved plasmids spread, and to explore criteria for distinguishing AmpC-type from ESBL-determined resistance.

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MATERIALS AND METHODS

Strains. Clinical isolates of *K. pneumoniae*, *K. oxytoca*, and *E. coli* came from several sources. Each came from a unique patient. A total of 340 isolates from 34 sites were provided as part of a study of potential ESBL-producing strains though the auspices of Merck & Co. Health Science Associates. The criteria for strain inclusion were a MIC of ≥2 μg/ml of ceftazidime, ceftriaxone, cefotaxime, aztreonam, or ceftizoxime. A total of 229 isolates from 12 sites were part of a study whose goal was to validate NCCLS criteria for ESBL detection (17). A total of 127 isolates suspected of ESBL production from 22 sites were provided by James W. Snyder, Louisville, Ky. An additional 56 resistant strains came from various U.S. sources. Most of the strains were collected in 1994 and 1995, with collection dates ranging from 1992 to 2000. *E. coli* DH5α pCLL3414 encoding cloned ACT-1 β-lactamase was provided by Patricia Bradford (4).

Susceptibility tests. Disk susceptibility tests were performed and interpreted according to NCCLS criteria (16) by using Mueller-Hinton agar (Becton Dickinson and Co., Sparks, Md.) and antibiotic disks from the same source. Ten micrograms of clavulanic acid (GlaxoSmithKline, Research Triangle Park, N.C.) was added to 30 μg of cefotaxime or ceftazidime disks to test β-lactamase inhibition. A ≥5-mm increase in zone diameter in comparison to a disk without inhibitor was interpreted as a positive test result (16). Resistance to mercuric chloride was evaluated using disks containing 10 μg of $HgCl_2$.

β-Lactamase characterization. Transmissibility of resistance was tested by mating clinical isolates to *E. coli* J53 Azi^r (8) with selection on trypticase soy agar plates (Becton Dickinson and Co.) containing 200 μg of sodium azide (Sigma, St. Louis, Mo.) per ml and 10 μg of ceftazidime (Eli Lilly & Co., Indianapolis, Ind.) per ml. Transconjugants were shown to contain only a single plasmid by using the plasmid isolation procedure of Takahashi and Nagano (22). β-Lactamase was extracted with lysozyme (Sigma) treatment (1), concentrated with a Micron YM-10 device (Millipore, Bedford, Mass.), and subjected to isoelectric focusing on a polyacrylamide gel either by a modified version (14) of the method of Matthew et al. (13) or by using the PhastSystem (Amersham Biosciences, Pis-

TABLE 1. Primers used in this study for PCR amplification and sequencing

β-Lactamase gene	Sequence (5′–3′)[a]	Location of 5′ end relative to *bla*	GenBank accession no.
ACT-1	CCTTGAACTGCTATTACGGA	26 bp upstream	U58495
	CGCCACCCGGCAATGTTTAC	19 bp downstream	U58495
FOX-5	ATGCCAATTTCATTCACCAC	32 bp upstream	X77455
	ATKTGGAMGCCTTGAACTCG	25 bp downstream	X77455
CMY-2	CGGACACCTTTTTGCTTTTAATTAC	37 bp upstream	X91840
	GAAAGAAAGGAGGCCCAATA	39 bp downstream	X91840
DHA-1	GTCCGGTGAATCTGACGATA	57 bp upstream	AJ237702
	TTAAATTACGGCCCCGGCGT	43 bp downstream	AJ237702

[a] K represents G or T, and M stands for A or C.

cataway, N.J.) as described by Huovinen (6) with enzyme localization with nitrocephin. Strains producing β-lactamases with isoelectric points (pIs) of 5.4 (TEM-1), 5.9 (TEM-4), 6.3 (TEM-3), 7.0 (SHV-3), 7.6 (SHV-1), and 8.2 (SHV-12) were used as pI standards.

***bla* sequencing.** Early in the course of the study, *bla* genes were identified by cloning and sequencing. Plasmid DNA was extracted (2), cut with *Eco*RI restriction endonuclease, ligated into chloramphenicol-resistant vector pBC SK (Stratagene, La Jolla, Calif.), and transformed into *E. coli* XL1-Blue MRF′ Kan (Stratagene), selecting on plates containing 30 μg of cefoxitin per ml and 30 μg of chloramphenicol per ml. Candidate clones were characterized by susceptibility testing, β-lactamase isoelectric focusing, plasmid extraction, and restriction analysis. Recombinant plasmids with inserts of minimal size were sequenced, starting from the vector ends and proceeding along both DNA strands by primer walking using cycle sequencing (Perkin-Elmer Cetus, Norwalk, Conn.). Later in the course of the study, genes were identified after amplification with the PCR primers shown in Table 1 and sequenced using the amplifying primers as well as additional primers designed from the known *bla* gene sequence.

Pulsed-field gel electrophoresis. Pulsed-field gel electrophoresis of DNA from clinical isolates was performed after cleavage with restriction endonuclease *Xba*I (12) using the CHEF-DR II System (Bio-Rad, Hercules, Calif.).

RESULTS

Characteristics of the sample. Clinical isolates of *K. pneumoniae*, *K. oxytoca*, and *E. coli*, all with increased resistance to oxyimino-β-lactams, were obtained at 70 sites from 25 states and the District of Columbia. Resistance to cephamycin was not a prerequisite for selection. A total of 517 *K. pneumoniae*, 58 *K. oxytoca*, and 177 *E. coli* samples collected between 1992 and 2000 were confirmed to be oxyimino-β-lactam resistant and were tested for the ability to transfer ceftazidime resistance to *E. coli* J53 Azi^r. Transconjugants producing AmpC-type β-lactamases were identified by resistance to cefoxitin, isoelectric focusing, cloning or PCR amplification, and ultimately sequencing. The remaining transconjugants were readily inhibited by clavulanic acid and produced enzymes with pIs consistent with membership in the SHV or TEM ESBL families, a finding that was confirmed for selected strains by sequencing.

As shown in Table 2, 303 (59%) of the *K. pneumoniae*, 16 (28%) of the *K. oxytoca*, and 75 (42%) of the *E. coli* isolates transferred resistance to ceftazidime (R^+). Plasmids encoding AmpC-type β-lactamases (R^+AmpC^+) were found in 44 (8.5%) of the *K. pneumoniae* isolates, 4 (6.9%) of the *K. oxytoca* isolates, and 7 (4%) of the *E. coli* isolates. The frequency of plasmid-mediated AmpC-type resistance was 7.3% in the sample of 752 isolates and 14% among the R^+ strains. R^+AmpC^+ strains were detected at 20 (29%) of 70 sites and in 10 of 25 states. The other R^+ strains produced a variety of SHV- and TEM-type ESBLs (R^+ESBL^+).

Phenotypic tests were helpful, but not definitive, for distinguishing R^+AmpC^+ from R^+ESBL^+ strains. All of the R^+AmpC^+ clinical isolates tested resistant to cefoxitin by the disk method, but so did 11% of R^+ESBL^+ strains, including 30 strains of *K. pneumoniae* and 9 strains of *E. coli*. Augmentation of the zone diameter by at least 5 mm in the presence of clavulanic acid was observed in 10 of 54 R^+AmpC^+ strains with a ceftazidime disk and in 3 additional strains with a cefotaxime disk, thus yielding an overall positive result for this ESBL confirmatory test of 24% (16). A positive inhibitor test result, however, persisted in only four transconjugants from these 13 strains, and each of these transconjugants produced an ESBL enzyme in addition to an AmpC enzyme. Hence, the misleading response of the clinical isolates to clavulanic acid was due to coexistence of multiple enzymes and not to an intrinsic property of the AmpC β-lactamases themselves.

AmpC identification. Initially, β-lactamase genes were identified by cloning and sequencing. Plasmid DNA from transconjugants was digested with *Eco*RI and cloned into the *Eco*RI site of vector pBC SK carrying chloramphenicol resistance. The inserts, ranging in size from 4.5 to more than 10 kb, were sequenced by primer walking along both nucleotide strands. Strains producing ACT-1, CMY-2, and DHA-1 were identified in this way, as was the first strain producing FOX-5. Subsequent FOX-5 strains were presumptively identified by a characteristic pattern of multiple bands on isoelectric focusing and confirmed by PCR amplification and sequencing. The sequence found for ACT-1 differed by six nucleotides from that originally published (4), but the same changes were found in the ACT-1 gene from the original outbreak; therefore, GenBank entry U58495 has been updated. Table 3 shows the properties of the R^+AmpC^+ strains so identified.

TABLE 2. Frequency of ESBL-based resistance transmissible by conjugation (R^+ESBL^+), transmissible AmpC-type β-lactamase (R^+AmpC^+), and nontransmissible resistance (R^-) in 752 oxyimino-β-lactam-resistant clinical isolates

Clinical isolate	No. of isolates transmitting resistance		
	R^+ESBL^+	R^+AmpC^+	R^-
K. pneumoniae	259	44	214
K. oxytoca	12	4	42
E. coli	68	7	102
Total	339	55	358

TABLE 3. Plasmid-mediated AmpC-type β-lactamases

β-Lactamase	Sampling location	Strain	Isolation date (mo/yr)	Source	Associated resistances[c]	β-Lactamase bands on IEF[d]	Plasmid designation
ACT-1	Medical College of Virginia, Richmond	*K. pneumoniae*	12/93	Urine	CmKmSmSuTmHg	5.4, 8.5+	pMG278
ACT-1	Cabrini Medical Center, New York, N.Y.	*K. pneumoniae*	3/94	Throat	CmKmSmSuHg	5.4, 8.5+	pMG249
ACT-1	Boston University Medical Center, Boston, Mass.	*E. coli*	5/94	Sputum	CmKmSmSuHg	5.4, 8.5+	pMG245
ACT-1	Westchester County Medical Center, Valhalla, N.Y.	*K. pneumoniae*	8/94	Blood	CmKmSmSuHg	5.4, 8.5+	pMG246
ACT-1	Bronx-Lebanon Hospital, New York, N.Y.	*K. pneumoniae*	8/94	Sputum	CmKmSmSuHg	5.4, 8.5+	pMG251
ACT-1	St. Elizabeth's Medical Center, Brighton, Mass.	*K. pneumoniae*	4/95	Blood	CmGmKmSmSuTmHg	8.5+	pMG287
ACT-1	VA Medical Center, New York, N.Y.	*K. pneumoniae*	1995	NA[a]	CmKmSmSuHg	5.4, 8.5+	pMG260
ACT-1	University of Pennsylvania, Philadelphia	*K. pneumoniae*	Bef[b] 4/00	NA	CmKmSmSuTmHg	5.4, 8.5+	pMG279
FOX-5	University of Alabama, Birmingham	*K. pneumoniae*	9/94	Urine	CmKmQnSmSuTpHg	5.7, 6.0–7.6	pMG252
FOX-5	Coral Gables, Fla.	*E. coli*	Bef 1/97	Urine	CmKmSmSuTpHg	5.7, 6.0–7.6	pMG280
FOX-5	North Florida, Fla.	*E. coli*	Bef 1/97	Urine	SuHg	6.0–7.6	pMG281
FOX-5	Dallas, Tex.	*K. pneumoniae*	Bef 1/97	Urine	SuHg	6.0–7.6	pMG282
FOX-5	San Antonio, Tex.	*K. pneumoniae*	Bef 1/97	Urine	CmKmSmSuTpHg	5.7, 6.0–7.6	pMG283
FOX-5	Fort Worth, Tex.	*K. pneumoniae*	Bef 1/97	Urine	CmKmSmSuTpHg	5.7, 6.0–7.6	pMG284
FOX-5	Greensboro, N.C.	*K. pneumoniae*	Bef 1/97	Urine	CmKmSmSuTpHg	5.7, 6.0–7.6	pMG285
FOX-5	Strong Memorial Hospital, Rochester, N.Y.	*K. pneumoniae*	3/99	Blood	CmKmSmSuTpHg	5.4, 5.7, 6.0–7.6	pMG286
CMY-2	University of Michigan, Ann Arbor	*E. coli*	8/94	Wound	CmKmSmSuTc	5.4, 8.5+	pMG250
CMY-2	Duke University Medical Center, Durham, N.C.	*E. coli*	Bef 7/96	NA	CmSmSuTcTpHg	8.5+	pMG248
DHA-1	University of California Medical Center, Los Angeles	*K. pneumoniae*	9/94	Urine	CmKmSmSuTmTpHg	6.3, 7.8	pMG247
Unknown	University of Alabama, Birmingham	*E. coli*	6/94	Urine	GmKmSmSuTcTmTpHg	8.5+	pMG288

[a] NA, not available.
[b] Bef, before.
[c] Resistance abbreviations: Cm, chloramphenicol; Gm, gentamicin; Hg, mercuric chloride; Km, kanamycin; Qn, quinolone; Sm, streptomycin; Su, sulfonamide; Tc, tetracycline; Tm, tobramycin; Tp, trimethoprim.
[d] IEF, isoelectric focusing.

ACT-1 β-lactamase. With the exception of one isolate from Richmond, Va., all the strains producing ACT-1 came from hospitals in the northeastern United States, especially around New York City but also Boston and Philadelphia. The ACT-1 β-lactamase-encoding plasmids at New York sites carried identical resistance markers, but plasmids from Virginia, Brighton, Mass., and Philadelphia carried additional resistances. All were about 200 kb in size. By pulsed-field gel electrophoresis, the ACT-1$^+$ *K. pneumoniae* strains from Bronx-Lebanon Hospital, Cabrini Medical Center, and the VA Medical Center in New York City were indistinguishable, while the ACT-1$^+$ *K. pneumoniae* isolate from Westchester County Medical Center differed by more than 10 bands.

FOX-5 β-lactamase. Strains producing FOX-5 β-lactamase came from states in the Southeast, with the exception of an isolate from Rochester, N.Y. At two of the eight sites, plasmids lacked resistance to four markers carried by other FOX-5 plasmids and also failed to express a pI-5.7 β-lactamase identified as PSE-1 by sequencing DNA from plasmid pMG252 (data not shown). By pulsed-field gel electrophoresis, the FOX-5$^+$ *K. pneumoniae* strains from San Antonio, Dallas, and Fort Worth, Tex., were indistinguishable but differed from the FOX-5$^+$ strain from Greensboro, N.C., in at least 10 fragments. The FOX-5$^+$ plasmid from Birmingham, Ala., was unique in carrying the *qnr* gene for quinolone resistance (7, 11). The FOX-5 plasmids were also about 200 kb in size.

Other AmpC-type β-lactamases. *E. coli* strains from Ann Arbor, Mich., and Durham, N.C., were found to carry CMY-2 β-lactamase, while a *K. pneumoniae* isolate from Los Angeles, Calif., produced DHA-1. The AmpC-type enzyme in one strain has not yet been identified. With this strain, a PCR product was not produced with any of the primer pairs shown in Table 1.

DISCUSSION

The first R$^+$AmpC$^+$ strains detected in the United States came from Providence, R.I., in 1988 and 1989 and produced MIR-1 β-lactamase, an enzyme that has not subsequently been found elsewhere (18).

The next enzyme, ACT-1 β-lactamase, was reported in isolates at the New York Hospital Medical Center in Queens in March 1994 (4). As indicated in Table 3, ACT-1$^+$ strains were already then present at other sites around New York City and as far away as Virginia; they have subsequently been found in Boston and Philadelphia. ACT-1 has also been reported at two New York City hospitals by Moland et al. (15) and was found previously to be inducible (21). Pulsed-field gel electrophoresis studies suggest that a single ACT-1$^+$ strain of *K. pneumoniae* has been primarily responsible for the spread of this enzyme around New York City.

FOX-5 β-lactamase was reported in 2001 in a *K. pneumoniae* isolate from New York City (20) but was already widely distributed around the United States. For this study, FOX-5-producing strains were found beginning in July 1994 from isolates collected in Alabama, Florida, North Carolina, and Texas, and also at Rochester, N.Y. Moland et al. detected additional FOX-5-producing strains in Kentucky, Maryland, and Washington (15). Pulsed-field gel electrophoresis and plasmid studies indicate that, as with ACT-1, spread can occur by FOX-5$^+$ strain dissemination (as occurred at three sites in Texas) as well as by plasmid dissemination to different strains; the studies also indicate that plasmids encoding FOX-5 are not homogeneous in resistance markers.

Other AmpC types were less common. CMY-2 was found in *E. coli* isolates from Michigan and North Carolina and also by Moland et al. in *K. pneumoniae* isolates from Oregon (15). DHA-1 was detected in a *K. pneumoniae* isolate from California in this study and in another *K. pneumoniae* strain from Florida by Moland et al. (15).

In the course of this study, a plasmid-mediated AmpC enzyme was found in 8.5% of ceftazidime-resistant U.S. *K. pneumoniae* isolates compared to a frequency of 11% found by Moland et al. with somewhat different selection criteria (15). The R$^+$AmpC$^+$ isolates were all cefoxitin resistant, but so were 11% of strains with transmissible ESBLs, presumably because these strains had additional mutations affecting porin channels for antibiotic uptake (9) or because they concomitantly expressed an AmpC β-lactamase. Response to the β-lactamase inhibitor clavulanic acid was somewhat helpful in differentiating R$^+$AmpC$^+$ from R$^+$ESBL$^+$ strains, but 23% of R$^+$AmpC$^+$ clinical isolates gave a positive reaction. Some plasmid-mediated AmpC enzymes were inhibited by clavulanic acid, although less so than was true for ESBLs (19), but the most likely explanation for the response to clavulanic acid is the production of both an ESBL and an AmpC enzyme in these clinical isolates. Detecting R$^+$AmpC$^+$ isolates may be clinically important not only because of their broader cephalosporin resistance but also because carbapenem resistance can arise in such strains by further mutations, resulting in reduced porin expression (4, 10).

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