Survey of Plasmid-Associated Genetic Markers in *Enterobacteriaceae* with Reduced Susceptibilities to Cephalosporins

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Clinical isolates of *Enterobacteriaceae* with reduced susceptibilities to cephalosporins were collected from 1993 to 2000. The organisms were screened for the extended-spectrum β -lactamase (ESBL) phenotype, and plasmid extracts were screened for genetic markers by hybridization. A *bla*_{TEM} probe was derived from pUC19; other probes were derived from pACM1, the plasmid responsible for the first known appearance of an ESBL in our institution. These probes included *bla*_{SHV}, *int*, *aac(3)-Ia*, *dfrA1*, IS6100, *tetA*, IncM markers, and Anon 13, a marker for the *Klebsiella pneumoniae* chromosomal sequences that flank *bla*_{SHV-5}. There were 42 hybridization patterns among 237 isolates. Patterns designated pACM1-like occurred in 44% of the isolates (eight species) and were always associated with the clavulanic acid (CA)-susceptible ESBL phenotype. The TEM marker was not predictive of the ESBL phenotype. Mapping indicated the presence of an SHV marker and up to 7.5 kb of its flanking chromosomal sequences in three non-IncM plasmids obtained in transformation experiments. We theorize that this DNA segment spread to other plasmids from pACM1-like sources. CA insensitivity became more frequent with time and was usually associated with either the TEM marker or the absence of both *bla* markers. One plasmid-encoded enzyme with characteristics of an AmpC β -lactamase was observed in a transformant lacking both TEM and SHV markers. Although SHV type ESBLs were a continuing source of reduced susceptibility to cephalosporins in our institution, organisms with different resistance mechanisms were added to the hospital microflora in later years. These changes might be related, in part, to ESBL control strategies implemented in 1995.

Members of the family *Enterobacteriaceae* can acquire resistance to the expanded-spectrum cephalosporin β -lactams by a number of mechanisms, and among the more important are the plasmid-encoded extended-spectrum β -lactamases (ESBLs) and AmpC β -lactamases. These resistance factors have compromised antimicrobial therapy in medical institutions worldwide (1, 10). ESBLs became a major source of cephalosporin resistance following the introduction and widespread use of oxyimino-cephalosporins (e.g., ceftazidime) in the 1980s (1). Originally, the ESBL designation referred to mutants of the broad-spectrum TEM and SHV β -lactamases, but new enzyme families (e.g., OXA and CTX-M) have been added to the list (1). ESBLs are generally not active against cephamycins (cefoxitin and cefotetan) and are susceptible to the β -lactamase inhibitors clavulanic acid, sulbactam, and tazobactam (1). By the end of the 1980s, a second wave of transmissible cephalosporin resistance became apparent. New plasmid-encoded cephalosporinases active against cephamycins and less susceptible to inhibitors were derived from chromosomally located AmpC genes (10). The proliferation of plasmidic AmpCs followed that of ESBLs, in part because cephamycins and β -lactam-inhibitor combinations (e.g., piperacillin-tazobactam) were used to counter the threat of ESBL-mediated resistance (10).

In order to guide appropriate antimicrobial therapy, it is important to recognize and distinguish ESBLs and plasmidic AmpC β -lactamases in the clinical laboratory. Since the dis-

covery of ESBLs, traditional susceptibility tests have been adapted to identify the typical oxyimino-cephalosporin-resistant and inhibitor-susceptible phenotype. The value of these tests is compromised by the fact that ESBLs express different affinities for any single cephalosporin chosen for screening, by the presence of multiple β -lactamases in one host, and by other host factors, including the presence of inducible chromosomal class C β -lactamases (1). The test currently recommended by the National Committee for Clinical Laboratory Standards (NCCLS) is approved only for *Klebsiella* spp. and *Escherichia coli*, organisms that lack chromosomal *ampC* and the ability to induce *ampC*, respectively. AmpC production does not always result in predictable phenotypes (10), and there are no simple tests for detecting plasmidic AmpCs on a routine basis.

Our hospital first detected reduced susceptibility to ceftazidime and other oxyimino-cephalosporins in a strain of *Klebsiella oxytoca* that caused an outbreak in a neonatal intensive care unit in 1993. The phenotype was mediated by the SHV-5 ESBL encoded by the conjugative plasmid pACM1 (17). Although the neonatal intensive care unit outbreak was controlled, resistance or reduced susceptibilities to oxyimino-cephalosporins have been observed in isolates of *Enterobacteriaceae* from adult patient care units since that time. For the purpose of guiding antimicrobial therapy, these isolates have been flagged as ESBL producers, with a statement indicating possible resistance to penicillins and cephalosporins. The present study was undertaken to reevaluate the ESBL status of these isolates, to examine plasmid genotypes associated with ESBL and other resistance phenotypes, and to determine if any changing trends in the genotypes and/or phenotypes are associated with clinical interventions designed to curb ESBL resistance.

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FIG. 1. (A) *EcoRI* map of pACM1. *EcoRI* sites are represented by black lines perpendicular to the circle. Relevant genes (arrows) and probe fragments (gray blocks) are shown; smaller probes and genes appear as gray lines. The fragment represented by the white block has not been cloned but is known to contain at least one *EcoRI* site. *rep* indicates the location of the IncM replicon. (B) Enlarged view of the indicated segment of pACM1. The *ClaI* site indicated in bold is overlapped by a *dam* methylase site. Segments below the map indicate the minimum region of homology between pACM1 and the *K. pneumoniae* chromosome (I), pACM120 (II), pACM150 (III), and pACM170 (IV). (C) *EcoRI* gel fingerprint for pACM1. Fragment sizes in kilobases are shown on the right. Corresponding fragments in panels A and C are identified by letters.

A means of surveying the genetic markers of resistance plasmids from large numbers of clinical isolates was developed, with pACM1 as the prototype for comparison. Most of pACM1 has been cloned, and significant features (Fig. 1) have been identified (11–14). This IncM plasmid encodes the SHV-5 ESBL on a 7.9-kb segment of DNA derived from the chromosome of *Klebsiella pneumoniae* (15). DNA probes representing this chromosomal segment (Fig. 1B, fragment I), resistance genes, mobile elements, and IncM markers were created from fragments of pACM1 and the cloning vector pUC19. The dot blot format was used to maximize the number of isolate-probe combinations that could be tested at one time.

MATERIALS AND METHODS

Patients. The study population included adult patients admitted to a tertiary care academic medical center with approximately 600 beds. The study specimens were collected from 1993 through 2000.

Collection criteria for bacterial isolates. Routine clinical isolates of *Enterobacteriaceae* identified as probable ESBL producers based on standard disk diffusion assays (8) were collected and frozen at -70°C . Isolates with reduced susceptibilities to cephalosporins were considered ESBL producers if the inhibition zone diameter for cephalexins (cefotaxime and cefotetan) was greater than that for the broad-spectrum cephalosporins (ceftazidime and ceftriaxone), and increased zones of inhibition were observed when β -lactamase inhibitors were added to β -lactam antimicrobials (ticarcillin with clavulanate or piperacillin with tazobactam.) Only those isolates retaining the ability to grow on agar containing ceftazidime (4 $\mu\text{g}/\text{ml}$) after thawing were included in the study.

Reevaluation of ESBL status. We updated the ESBL status of flagged isolates by the method recommended by NCCLS document M100-S12 (9), dated January 2002. The test compares the zones of inhibition around standard ceftazidime and cefotaxime disks to those around ceftazidime and cefotaxime disks with 10 μg of

clavulanic acid added. For research purposes, this test was applied to all study isolates regardless of species.

Dot blot hybridization. Testing of batches of plasmid DNAs (5 to 18 per batch) was conducted occasionally during the survey period and completed in 2001. Plasmids were extracted from clinical isolates by alkaline lysis (6). The dot blot procedure, including the labeling of probes (Fig. 1; Table 1) and hybridization, was done according to the Genius System user's guide for filter hybridization, version 2.0 (Boehringer Mannheim, Indianapolis, Ind.) with the reagents of the DIG DNA labeling and detection kit (Roche). Various combinations of the IncM marker probes (Anons 4 to 9 and *priL*), most commonly Anon 5, Anon 9, and *priL* or Anon 5 and *priL*, were used on different batches of DNAs.

pACM1 and pUC19 each served as the positive control for their own probe(s) and as the negative control for the probe(s) from the other source. Reactions of clinical isolates were visually scored as positive or negative based on control reactions and internal consistency; i.e., if one isolate had strong reactions to some of the probes but weak reactions to others, the weak reactions were scored as negative. If the isolate had weak reactions to some probes and no strong reactions, the weak reactions were scored as positive. This kept the scoring conservative and eliminated false positives due to the presence of incorrect but related genes, partially deleted target sequences, and sequences (e.g., the SHV-1 gene in *K. pneumoniae*) in residual chromosomal DNA, without prejudicing very low copy number plasmids.

Hybridization conditions of 50% formamide and 55°C were originally designed to prevent the cross-reaction, observed at 42°C , of the SHV probe with a membrane-bound TEM target (17). (Although the SHV probe cross-reacted with a TEM target at 42°C , the TEM probe did not cross-react with an SHV target at the same temperature [unpublished observation].) Although the *bla* probes should distinguish one gene family from another, they are not intended to detect the small number of nucleotide substitutions that distinguish members of the same family. Other probes that did not bind well to positive controls at 55°C were used at empirically determined lower temperatures (Table 1).

Molecular analysis of resistance plasmids. Hybridization results prompted further investigation of a sample of isolates. Resistance plasmids were separated from other cellular plasmids by transformation of *E. coli* DH5 α , with either

TABLE 1. Gene probes from pACM1 (unless otherwise noted)

Name	Fragment and/or description (reference)	T ^a (°C)
SHV	<i>bla</i> _{SHV-5} (14)	55
TEM	<i>bla</i> _{TEM-1} , 684-bp <i>SspI</i> - <i>BglI</i> fragment of pUC19	42
IS6100	Insertion sequence (14)	55
<i>int</i>	<i>intI1</i> , type I integrase gene (11)	55
<i>tet</i>	<i>tetA</i> from Tn1721 (14)	42
Anon 4	Putative IncM marker (14)	42
Anon 5	Putative IncM marker (14)	55
Anon 7	Probe overlapping IncM <i>rep</i> region (14, 15)	55
Anon 9	Putative IncM marker (14)	55
<i>priL</i>	IncM primase gene, formerly Anon 10 (13, 14)	55
Anon 13	<i>K. pneumoniae</i> chromosomal sequence near <i>bla</i> _{SHV-5} ; includes <i>fucA</i> and other sequence (15)	55
<i>aac(3)-Ia</i>	403-bp <i>AspI</i> - <i>EcoRV</i> fragment, gentamicin resistance gene cassette (11)	55
<i>dfiA1</i>	490-bp <i>HpaI</i> fragment, trimethoprim resistance gene cassette (11)	55

^a T, hybridization temperature.

ceftazidime (4 µg/ml) or ampicillin (50 µg/ml) for selection. Plasmids were extracted from transformants with reagents in the QIAprep Spin miniprep kit (Qiagen, Valencia, Calif.), but isopropanol precipitation of plasmid DNA (16) was substituted for the spin column procedure. Plasmids were compared by restriction digestion, Southern transfer, and hybridization. β-Lactamases from donors and transformants were detected with nitrocefin on isoelectric focusing gels; isoelectric points (pIs) were estimated relative to those of control β-lactamases, including SHV-5, TEM-3 (2), and TEM-5 (2). These techniques have been described previously (16, 17). MICs of ceftazidime were determined with Etest strips as per the manufacturer's instructions (AB Biodisk North America, Inc.). Conjugation experiments were performed with DH5α transformants as donors and DH5α(pACYC184) (3) as the recipient as previously described (14). Transconjugants were selected on plates containing chloramphenicol (20 µg/ml) and ceftazidime (4 µg/ml) or tetracycline (15 µg/ml) and ampicillin (50 µg/ml).

RESULTS

Two hundred thirty-seven isolates from 208 patients were included in the survey. The majority (131) were *K. pneumoniae*; other species included *K. oxytoca* (30), *E. coli* (27), *Enterobacter cloacae* (27), *Citrobacter freundii* (8), *Serratia marcescens* (7), *Morganella morganii* (2), *Enterobacter agglomerans* (2), *Enterobacter aerogenes* (1), *Enterobacter* sp. (1), and *Klebsiella rhinoscleromatis* (1). Duplicates (subsequent isolates of the same species and hybridization pattern from the same patient in the same calendar year) were not included. All isolates met the NCCLS initial screening criteria for ESBLs, having zones of inhibition of ≤22 mm for ceftazidime and ≤27 mm for cefotaxime. Phenotypes were designated as ESBL or non-ESBL based on the ESBL confirmatory test. Forty isolates (17%) were assigned the latter phenotype, including 17 *K. pneumoniae*, 12 *E. coli*, 5 *Enterobacter cloacae*, 2 *C. freundii*, and 2 *M. morganii* isolates and 1 *K. oxytoca* and 1 *Enterobacter aerogenes* isolate.

It was noted that for any given isolate, the IncM markers (Anons 4 to 9 and *priL*) were either all positive or all negative, so the data for these probes were combined into one category (IncM). Forty-two different hybridization patterns were observed (Table 2). Patterns that were positive for SHV, *int*, IS6100, *tet*, and the IncM probes at minimum were defined as pACM1-like patterns and were designated by a Y prefix. Isolates with Y patterns were considered to be potential carriers of plasmids similar to pACM1. These accounted for 105 (44%) of the isolates tested, including eight species from five genera. One hundred thirty-two isolates produced N patterns lacking one or more of the required pACM1 gene markers. The most

common single hybridization patterns were N29 and Y6, the pattern exhibited by the pACM1 control, with 42 and 39 isolates, respectively. *int* was the most frequent marker, occurring in 89% of the isolates tested. IS6100 and SHV each occurred in 78% of the isolates. The 42 patterns were placed in hybridization pattern groups I to VI based on their β-lactamase markers and their classification as pACM1-like or non-pACM1-like (Table 2).

The yearly incidence of pattern groups for various species is shown in Fig. 2. The data for early (January to June) and late (July to December) 1995 were separated to assess the effects of a multidisciplinary program, instituted in June, designed to reduce the incidence of antimicrobial resistance. Interventions included the final phase of an active infection control program and a change in the hospital formulary that encouraged the use of piperacillin-tazobactam as an alternative to expanded-spectrum cephalosporins (M. H. Miller et al., unpublished data). The following trends were observed. The frequency of *Klebsiella* isolates with the ESBL phenotype decreased over the 8-year period, particularly after June 1995. In contrast, *Enterobacter cloacae* isolates with the ESBL phenotype became more frequent. *E. coli* also became more common, but isolates with the non-ESBL phenotype outnumbered those with the ESBL phenotype in the last 3 years. Approximately one-third of *K. pneumoniae* isolates from late 1995 to 2000 had the non-ESBL phenotype. With one exception (a *C. freundii* isolate from early 1995), all non-ESBL phenotypes appeared after June 1995.

In 1993 all isolates had pACM1-like hybridization patterns (groups V and VI). Thereafter, *K. pneumoniae* isolates were associated with a variety of pattern groups, while *K. oxytoca* isolates continued to be associated almost exclusively with pACM1-like patterns. The incidence of pACM1-like hybridization patterns among all isolates decreased to lows of 10 and 22% in 1999 and 2000, respectively. All pACM1-like patterns were associated with the ESBL phenotype. Ninety-two percent of all isolates with the ESBL phenotype belonged to one of the SHV-positive hybridization groups (III through VI); 90% of isolates with the non-ESBL phenotype belonged to hybridization groups I and II, which lack SHV.

There was a significant spike in the number of *K. pneumoniae* isolates in 1994 and early 1995 that can be attributed to isolates belonging to hybridization group IV. All of the group IV isolates in 1994 and 14 of 15 in 1995 had the N29 pattern.

TABLE 2. Hybridization patterns

Group ^a	Pattern ^b	Hybridization probe ^c									No. of isolates
		SHV	TEM	IS <i>6100</i>	<i>int1</i>	<i>tetA</i>	IncM	Anon 13	<i>aac(3)-Ia</i>	<i>dfpA1</i>	
I	N1										10
	N2				•						4
	N3			•							1
	N4			•		•					1
	N5			•	•						16
	N6			•	•		•				1
	N7			•	•	•	•		•	•	1
II	N8		•								4
	N9		•			•					1
	N10		•		•						3
	N11		•		•	•					5
	N12		•		•	•				•	1
	N13		•	•			•				3
III	N14	•									1
	N15	•						•			2
	N16	•					•				1
	N17	•			•			•			8
	N18	•			•			•		•	1
	N19	•			•		•	•		•	1
	N20	•		•		•	•	•			1
	N21	•		•	•						1
	N22	•		•	•				•		1
	N23	•		•	•			•			6
	N24	•		•	•			•	•	•	4
IV	N25	•	•					•			1
	N26	•	•				•	•			1
	N27	•	•		•			•			7
	N28	•	•		•			•		•	1
	N29	•	•	•	•			•			42
	N30	•	•	•	•			•	•	•	1
	N31	•	•	•	•		•				1
V	Y1	•		•	•	•	•			•	4
	Y2	•		•	•	•	•		•		21
	Y3	•		•	•	•	•		•	•	4
	Y4	•		•	•	•	•	•		•	1
	Y5	•		•	•	•	•	•	•		23
	Y6	•		•	•	•	•	•	•	•	39
VI	Y7	•	•	•	•	•	•		•		5
	Y8	•	•	•	•	•	•		•	•	2
	Y9	•	•	•	•	•	•	•		•	1
	Y10	•	•	•	•	•	•	•	•		3
	Y11	•	•	•	•	•	•	•	•	•	2
Total no. of isolates		186	84	185	210	115	115	145	106	63	
% of isolates		78	35	78	89	49	49	61	45	27	

^a Group based on *bla* markers and similarity to pACM1.^b N and Y indicate non-pACM1-like and pACM1-like patterns, respectively.^c Positive and negative are indicated by dots and blanks, respectively.

The frequency of this species-pattern association dropped sharply in the postintervention time period (Fig. 2). A significant clonal outbreak of *K. pneumoniae* was identified by pulsed-field gel electrophoresis of total DNA from a sample (66 isolates from 65 patients) of *K. pneumoniae* from 1994 to 1996 (Miller et al., unpublished data). About half of the isolates were indistinguishable and were designated clone A. The

N29 pattern was associated with 27 of 30 clone A isolates and with a single isolate >90% similar to clone A. The N23 pattern, similar to N29 but lacking TEM, was associated with two clone A isolates. (One clone A isolate was not included in the present study because it failed to grow on ceftazidime agar after thawing.) Among the 35 (mostly unique) isolates not related to clone A, there were 24 (69%) with pACM1-like

FIG. 2. The distribution of hybridization pattern groups and phenotypes (ESBL or non-ESBL) over time for each species. Species represented by fewer than 10 isolates were included in the category "other species" (other spp.). Data for 1995 were divided into E95 (January to June) and L95 (July to December) periods to illustrate the effects of anti-ESBL interventions begun in June. Hybridization (Hyb.) groups were as follows: (I) not pACM1-like, negative for both TEM and SHV; (II) not pACM1-like, TEM positive, and SHV negative; (III) not pACM1-like, TEM negative, and SHV positive; (IV) not pACM1-like, positive for both TEM and SHV; (V) pACM1-like, SHV-positive; and (VI) pACM1-like, positive for both TEM and SHV.

hybridization patterns, 1 with the N29 pattern, and 10 with other non-pACM1-like patterns.

We were particularly interested in the coincidence of the SHV and Anon 13 markers, both from the same region of the *K. pneumoniae* chromosome, and the IncM markers, repre-

senting a particular family of plasmids. The relationships among these markers changed with time (Fig. 3). In 1993, the SHV gene was associated with IncM markers in all isolates, although Anon 13 was absent in 38% of them. In following years, SHV began to appear without IncM markers but almost

FIG. 3. Coincidence of IncM and Anon 13 markers in SHV-positive isolates. For each year the percentage of SHV-positive isolates that were also positive for IncM, Anon 13, or both markers is indicated. All other markers were ignored.

always with Anon 13. During the entire study period, only three SHV-positive isolates lacking both Anon 13 and IncM markers were observed. Anon 13 was never present in any isolate without SHV. Thus, the linkage between SHV and Anon 13 persisted outside the IncM plasmid family.

Transformation experiments. Transformation experiments confirmed that Y hybridization patterns predicted the presence of plasmids structurally similar to pACM1. Donors included *K. pneumoniae* (4), *K. oxytoca* (1), *E. coli* (1), *Enterobacter cloacae* (1), *S. marcescens* (1), and *C. freundii* (1). Hybridization patterns Y1, Y5, Y6, and Y7 were transferred without loss of markers to DH5 α recipients. All plasmids produced *EcoRI* fingerprints similar to that of pACM1 (Fig. 1C), and all transformants produced a β -lactamase that comigrated with the SHV-5 control at pI 8.2. Additional β -lactamases that focused at pIs 5.4 and 6.9 were encoded on pACM130, a Y7 plasmid from *C. freundii*. Southern blotting and fragment cloning indicated that these β -lactamases were encoded on an 8-kb insert in the F fragment (Fig. 1A and C).

Another goal of transformation was to characterize the plasmids and plasmid-encoded β -lactamases of a sample of isolates with non-pACM1-like hybridization patterns. Plasmids obtained from four ESBL-producing *K. pneumoniae* isolates were compared to pACM1 (Table 3). The donors' ESBL phenotype and hybridization patterns were transferred intact to recipients. IncM-positive pACM140 resembled a pACM1-like plasmid with major deletions to the left of SHV (though its ability to conjugate was not impaired). The other three, all SHV positive but IncM negative, resembled neither pACM1 nor one another. The local regions flanking the SHV gene in each

plasmid were compared to the *bla*_{SHV-5} region of pACM1 (Fig. 1B). Southern blots of digested plasmids were hybridized with SHV or Anon 13 to identify homologous fragments of equal sizes. The regions of homology in the non-IncM plasmids (Table 3; Fig. 1B) lie within the sequence that is derived from the *K. pneumoniae* chromosome in pACM1. All four plasmids encoded an enzyme that focused at pI 8.2. pACM150 and pACM120 were also TEM positive and encoded an enzyme, probably TEM-1, that focused at pI 5.4. No ESBL phenotypes not associated with SHV genes were successfully transferred; transformants that acquired only a probable TEM-1 were not reported.

pACM150 was derived from a clone A *K. pneumoniae* isolate collected in 1994. Although conjugative, it produced 300 times fewer transconjugants than pACM1 under experimental conditions (Table 3). This might explain why the N29 pattern was associated almost exclusively with clone A. pACM120 was derived from a non-clone A donor collected in 1996. Although it had the same markers, it appeared to be structurally distinct from pACM150.

One transformation was successful in transferring a clavulanic acid-insensitive cephalosporinase to DH5 α . pACM160 was isolated from a clinical *E. coli* donor with the N9 pattern. pACM160 retained only the *tet* marker from the donor but encoded a non-SHV, non-TEM β -lactamase of pI 9.1. In addition to a reduced susceptibility to ceftazidime, this enzyme conferred on the host cefoxitin resistance and a reduced susceptibility to cefotetan (data not shown). We therefore hypothesize that the plasmid-encoded enzyme is an AmpC.

DISCUSSION

Application of recent NCCLS standards for the ESBL phenotype confirmed the ESBL producer status for most of the isolates collected by using less stringent criteria established in 1993. Most organisms with the ESBL phenotype had plasmid-borne SHV genes, indicating that SHV variants are the major ESBLs in our institution. The ESBL phenotype was historically associated with the SHV-5 β -lactamase in our hospital. Although sampling was limited, enzymes of pI 8.2 were found in all SHV-positive experimental transformants. While there are at least four other SHV enzymes with the same pI (<http://www.lahey.org/studies/webt.html>), most are considered derivatives of SHV-5. We therefore conclude that SHV-5 or deriv-

TABLE 3. Characteristics of pACM1 and plasmids from ESBL-producing *K. pneumoniae* donors with non-pACM1-like hybridization patterns^a

Plasmid	Hybridization pattern	Size (kb)	Restriction fragment fingerprint ^b	Homology to pACM1 near SHV gene (kb) ^c	β -Lactamase pI ^d	MIC (μ g/ml) of CAZ ^e	Transconjugants per donor ^f
pACM1	Y6	88	A-1	=	8.2	16	4.1×10^{-4}
pACM140	N16	55	A-2	55	8.2	16	7.4×10^{-4}
pACM170	N22	35	B	2.5	8.2	32	None
pACM150 ^g	N29	160	C	5.8	5.4, 8.2	6	1.3×10^{-6}
pACM120	N29	70	D	7.5	5.4, 8.2	12	None

^a After transformation of *E. coli* DH5 α .

^b *EcoRI* and additional enzymes used.

^c =, not applicable.

^d Approximate pI.

^e CAZ, ceftazidime. MIC for the DH5 α host is 0.125 μ g/ml.

^f From *E. coli* DH5 α donor to DH5 α (pACYC184) recipient.

^g From clone A outbreak strain.

atives thereof were the most frequent ESBL in our hospital during the study.

As time passed, however, there was a shift toward non-ESBL phenotypes in species without inducible chromosomal AmpCs, as well as an increase in species, especially *Enterobacter cloacae*, that have the chromosomal gene. This suggests that resistance mechanisms other than ESBL production were becoming more relevant. These mechanisms might include the production of plasmidic AmpCs or overproduction of the chromosomal enzyme. Transformation experiments revealed at least one probable plasmid-mediated AmpC cephalosporinase among the clinical isolates. With one exception, the appearance of clavulanate-insensitive organisms followed the establishment of ESBL control strategies in 1995 and might be related, in part, to increased use of β -lactam-inhibitor combinations. Other demographic or epidemiological factors that might have influenced the shift were not investigated for this study.

In our study, the TEM marker was not significantly associated with the ESBL phenotype. We suspect that most of the TEM genes in our survey are of the broad-spectrum TEM-1 variety, based on our molecular analyses and the observation that TEM-1 is the most commonly encountered β -lactamase in gram-negative organisms (1). Jacoby observed that ESBL-producing isolates often produce TEM-1 as well and demonstrated that adding *bla*_{TEM-1} plasmids to ESBL-producing strains rendered the strains less susceptible to β -lactam- β -lactamase inhibitor combinations (5). In theory, the combination of an ESBL with an AmpC β -lactamase could provide a similar advantage. Many *Enterobacter cloacae* isolates in the survey had SHV, TEM, or both markers and expressed the ESBL inhibitor-susceptible phenotype in vitro; the effect of inhibitors might differ in vivo.

Nearly half of the 42 hybridization patterns were observed only once, and many species-hybridization pattern associations were observed only once in any year. This indicates that many of the organisms and plasmids were transient in the hospital microflora. In a 12-year survey involving PCR detection of ESBLs, Coque et al. (4) reached a similar conclusion. The 1994 to 1995 outbreak of *K. pneumoniae* was the exception, and the June 1995 interventions were apparently effective in curtailing the spread of the pattern N29 clone A strain.

The SHV and Anon 13 markers appear to be part of an element that has been mobilized from the *K. pneumoniae* chromosome and disseminated into various plasmid families. The temporal shift in marker associations suggests that, locally, the SHV-Anon 13 element disseminated from IncM plasmids to plasmids of other incompatibility groups. Alternately, non-IncM plasmids carrying the same element might have entered the local gut flora independently. Additional studies are needed to determine whether the SHV-5-Anon 13 segment of pACM1 has the features of a transposon, which can mobilize itself.

IS26 has been associated with *bla*_{SHV-5} in pSEM (18) and *bla*_{SHV-2a} in the chromosome of *Pseudomonas aeruginosa* RP-1 (7). A study of sequences flanking other plasmid-borne SHV genes would be of value. Unfortunately, one of the disadvantages of direct sequencing of PCR-amplified *bla* genes is that no information about flanking sequences is obtained. Some local restriction mapping with gene probes would be helpful.

The dot blot method was of great value in making a broad survey of genetic markers in a large collection of isolates, predicting plasmids structurally related to pACM1, and selecting interesting plasmids for additional molecular analysis. The discriminatory power of dot blots could be greatly enhanced by the addition of probes for other resistance genes and plasmid incompatibility groups.

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