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Outbreak of TEM-24-Producing *Enterobacter aerogenes* in an Intensive Care Unit and Dissemination of the Extended-Spectrum β-Lactamase to Other Members of the Family *Enterobacteriaceae*

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We report an outbreak of *Enterobacter aerogenes* in an intensive care unit (ICU) and two medicine departments that produced the extended-spectrum β-lactamase TEM-24, which was difficult to detect by disk agar diffusion. The strains were compared by DNA restriction fragment length polymorphism after pulsed-field gel electrophoresis following cleavage with *Xba*I. This typing method indicated that a single strain, first isolated in the ICU, spread throughout the other medical departments as a result of patient transfer. We also observed the transfer in vivo of the plasmid encoding TEM-24 from the strain of *Enterobacter aerogenes* to different strains of *Escherichia coli* and *Citrobacter freundii* in the ICU. It therefore appears that the epidemic involved results from two events: dissemination of one strain of *Enterobacter aerogenes* and dissemination of the plasmid encoding TEM-24 among various members of the family *Enterobacteriaceae*.

Resistance to broad-spectrum cephalosporins in the *Enterobacter* genus is mostly due to synthesis of an inducible chromosomally encoded cephalosporinase (6, 15, 18). There are many reports of production of extended-spectrum β-lactamases (ESBla) among *Klebsiella pneumoniae* (2, 9, 13, 17, 20) but only a few among *Enterobacter aerogenes* or *Enterobacter cloacae* (7, 8, 19). Detection of ESBla in such strains is more difficult than with other species of *Enterobacteriaceae*, in part because of the production of the cephalosporinase (3). Over a 7-month period, 10 strains of *E. aerogenes* were isolated in three departments: seven in an intensive care unit (ICU), one in a medicine unit (on the same floor as the ICU), and two in a geriatric department (in another town-district). All the strains were resistant to cefotaxime and ceftazidime, and no synergy was observed between the disks of amoxicillin-clavulanic acid and broad-spectrum cephalosporins placed 30 mm apart, as already described (12). During the last 4 months of the outbreak, six *Escherichia coli* strains and one *Citrobacter freundii* strain resistant to ceftazidime and cefotaxime were isolated in the ICU. The aim of this study was to establish by pulsed-field gel electrophoresis (PFGE), as already described for other species (1, 2, 9, 10), if the infections were caused by a single strain of *E. aerogenes*. We also tested the possibility that dissemination of a plasmid could account for resistance to broad-spectrum cephalosporins that appeared in the ICU first among *E. aerogenes* and then in *E. coli* and *C. freundii.*

MATERIALS AND METHODS

Bacterial strains. Ten clinical isolates of ESBla-producing *E. aerogenes* isolated in three departments during 1993 and 1994 were examined: seven originated in the ICU, one originated in a medicine department, and two originated in a geriatric department. Six strains of *E. coli* and a strain of *C. freundii* resistant to broad-spectrum cephalosporins were isolated in the ICU during the last 4 months of the outbreak of *E. aerogenes* (Table 1).

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Nine strains of *E. aerogenes* and seven strains of *E. coli* isolated in other departments of our hospital, as well as *E. aerogenes* reference type strain ATCC 13048, were included for study by PFGE. *E. coli* K-12 C600 resistant to sodium azide was used as a recipient for conjugation experiments with the clinical isolate *E. aerogenes* 3: transconjugants (*E. coli* TR) were selected at a very high frequency on Mueller-Hinton agar containing 256 mg of sodium azide per liter and 8 mg of ceftazidime per liter. The same recipient strain was used for conjugation with *C. freundii. K. pneumoniae* 10031 resistant to rifampin was used for plasmid transfer from *E. coli*, with selection on plates containing 100 mg of rifampin per liter and 2 mg of ceftazidime per liter. *E. coli* K-12 producing the plasmid-mediated β-lactamase TEM-24 (4, 5), kindly provided by D. Sirot, was included in this study for comparison, as well as a strain of *E. coli* containing the TEM-3 ESBla.

Susceptibility testing. Susceptibility tests were performed according to the recommendations of the Société Française de Microbiologie. Disk agar diffusion susceptibility tests were determined on Mueller-Hinton agar. The double-disk synergy test was performed with a 30-mm spacing between the disks as previously described (12) or with disks 20 mm apart. The MICs of β-lactams were determined in Mueller-Hinton broth by a microdilution technique. Inocula of 10^5 to 10^6 CFU/ml were distributed with a replicating device (MIC 2000; Dynatech Laboratories).

Analytical isoelectric focusing. After culture in Trypcase soy broth (bioMérieux) supplemented with yeast extract (5 g/liter) and glucose (10 g/liter), β-lactamases were extracted from bacteria by sonication. Unbroken cells and cell envelopes were removed by centrifugation. Isoelectric focusing was performed on polyacrylamide gels (acrylamide, 7%; bis-acrylamide, 0.2%; Kodak) containing ampholines with a pH range of 3.5 to 10. Migration was carried out at 500 V overnight with a LKB 2117 Multiphor apparatus. The β-lactamase activity was located on the gels by an iodine starch procedure (14). β-Lactamases of known pI (TEM-1, pI 5.4; TEM-2, pI 5.6; TEM-3, pI 6.3; SHV-1, pI 7.7; SHV-4, pI 7.8; TEM-24, pI 6.5) were focused in parallel with the extracts.

Analysis of chromosomal DNA by PFGE. Total DNA was prepared as follows: 0.5 ml of low melting and gelling agarose (Seaplaque FMC, TEBU)—1% wt/vol in TE buffer (10 mM Tris [pH 8], 0.1 mM EDTA)—were mixed with 0.5 ml of bacterial suspension (5×10^9 CFU/ml) harvested on Mueller-Hinton agar and resuspended in TE buffer. The mixture was incubated in 0.5 M EDTA for 1 h and then in 0.5 M EDTA–1% (wt/vol) sodium dodecyl sulfate–1 mg of pronase (Calbiochem) for 48 h at 37°C. Agarose plugs were then washed once for 1 h at 37°C and once for 1 h at room temperature in a solution of phenylmethylsulfonyl fluoride (PMSF; Boehringer)—3.5 mg of PMSF solubilized in 200 μl of isopropanol with 20 ml of TE buffer—and then three times in TE buffer at room temperature. DNA was then digested with 40 U of *Xba*I (Biolabs) according to the manufacturer's instructions. Electrophoresis was performed in a 1% agarose gel (Pharmacia) prepared and run in 0.5× Tris-borate-EDTA buffer on a contour-clamped homogeneous field apparatus (CHEF-DR2; Biorad). The pulse range was 40 to 5 s for 22 h at 180 V for strains of *E. aerogenes* and 50 to 5 s for

TABLE 1. Origin of the members of the *Enterobacteriaceae* producing an extended-spectrum β-lactamase[a]

Patient no.	Strain	Date of isolation (day.mo.yr.)	Ward	Source of isolate	pI of β-lactamases
1	EA	13.09.93	ICU	Blood	6.5, 8.3
2	EA	22.09.93	ICU	Urine	6.5, 8.3
3	EA	11.10.93	ICU	Blood, urine, stool	6.5, 8.3
	EC	01.12.93	ICU	Stool	6.5, 8.3
4	EA	25.10.93	ICU	Sputum	6.5, 8.3
5	EA	16.11.93	MED	Blood	6.5, 8.3
6	EA	13.01.94	GER	Urine	6.5, 8.3
7	EA	14.04.94	ICU	Urine	6.5, 8.3
8	EA	08.12.93	ICU	Stool	6.5, 8.3
9	EA	25.02.94	ICU	Stool	6.5, 8.3
10	EA	04.05.94	GER	Urine	6.5, 8.3
11	EC	10.12.93	ICU	Stool	6.5
12	EC	15.01.94	ICU	Stool	6.5
13	EC	12.03.94	ICU	Urine	6.5
14	EC	22.03.94	ICU	Peritoneal fluid	6.5
15	EC	01.04.94	ICU	Lung	6.5
16	CF	15.04.94	ICU	Urine, stool	6.5, >8.4

[a] Abbreviations: EA, *Enterobacter aerogenes*; EC, *Escherichia coli*; CF, *Citrobacter freundii*; ICU, intensive care unit; MED, medical unit; GER, geriatric department.

20 h at 180 V for strains of *E. coli*. The gel was then stained in ethidium bromide, destained in distilled water, and photographed.

Plasmid DNA preparation and PCR. DNA was extracted from clinical isolates as described previously (16). Plasmid DNA electrophoresis was performed in 0.7% agarose, and the gels were stained with ethidium bromide. PCR was performed with primers (Bioprobe Systems): 5′-d(GGGCAAGAGCAACTCG GT)-3′ and 5′-d(AGACCCACGCTTACCGGT)-3′. The first one, at position 459 according to Sutcliffe numbering (21), is identical to the leading strand of the gene coding for the β-lactamase of the TEM family. The second one, at position 913, identical to the lagging strand, is specific for TEM-24 (4). One microgram of template DNA, prepared as described above, was amplified for 60 rounds as already described (4): denaturation at 94°C for 1 min, annealing at 57°C for 1 min, and polymerization at 72°C for 1 min. Five microliters of PCR products were analyzed by 2% agarose gel electrophoresis.

RESULTS

Susceptibility to β-lactam antibiotics and β-lactamase production. By disk agar diffusion, the strains of *E. aerogenes* appeared more resistant to ceftazidime than to cefotaxime and were also resistant to ticarcillin and piperacillin, a phenotype which does not correspond to overproduction of the chromosomal cephalosporinase. The MICs of β-lactam antibiotics alone or in combination with three β-lactamase inhibitors (clavulanic acid, sulbactam, and tazobactam) were determined for certain strains (Table 2). The most notable synergistic effect was observed with clavulanic acid (2 mg/liter) and ceftazidime on both *E. aerogenes* and *E. coli* TR. By disk diffusion this synergy was detected for *E. aerogenes* only when the amoxicillin-clavulanic acid- and ceftazidime-containing disks were placed 20 mm apart (Fig. 1).

All of the clinical isolates and the transconjugants produced an enzyme with a pI of 6.5 (Table 1).

Plasmid DNA analysis. Agarose gel electrophoresis of plasmidic DNA revealed that the clinical strains and the transconjugants harbored a plasmid with a size of 85 kb, similar to that of the plasmid encoding TEM-24 in *E. coli* (5) (data not shown). In *E. coli* and *K. pneumoniae* transconjugants, β-lactam resistance was cotransferred with resistance to aminoglycosides (amikacin, kanamycin, netilmicin, tobramycin), sulfonamide, trimethoprim, and chloramphenicol. By using TEM-24-specific primers, a DNA fragment of 476 bp was amplified from DNA of all strains producing the enzyme of pI 6.5 (*E. aerogenes* 3, *E. coli* 3, *C. freundii* 16, and *E. coli* containing TEM-24) but was absent from the strain producing TEM-3 (Fig. 2).

TABLE 2. MICs of β-lactam antibiotics alone and in combination with clavulanic acid, sulbactam, or tazobactam for ESBla-producing strains

Antibiotic (plus inhibitor)[a]	MIC (mg/liter) for:			
	E. aerogenes 3	*E. coli*		
		3	TR	TEM-24
Ticarcillin	8,192	4,096	4,096	8,192
+Clav	128	32	32	64
+Sulb	1,024	256	256	1,024
+Tazo	1,024	512	256	512
Piperacillin	128	16	32	64
+Clav	32	2	1	2
+Sulb	32	4	4	4
+Tazo	64	2	4	4
Aztreonam	64	16	16	128
+Clav	32	≤0.125	0.25	0.5
+Sulb	16	0.5	0.5	4
+Tazo	16	1	0.5	4
Cefotaxime	16	1	1	2
+Clav	16	0.06	0.06	0.06
+Sulb	8	0.125	0.125	0.25
+Tazo	8	0.125	0.125	0.25
Ceftazidime	512	128	128	1,024
+Clav	32	0.5	0.5	1
+Sulb	64	4	4	32
+Tazo	32	8	4	16

[a] Clavulanic acid (Clav), sulbactam (Sulb), and tazobactam (Tazo) were used at final concentrations of 2 mg/liter.

On the basis of isoelectrofocusing results, MICs of β-lactams for the strains, susceptibility of the ESBla to β-lactamase inhibitors, and PCR results, it was concluded that the strains of *E. aerogenes*, *E. coli*, and *C. freundii* produced ESBla TEM-24.

Analysis of chromosomal DNA by PFGE. The 10 strains of *E. aerogenes* producing TEM-24 had the same profile, and it was different from those of strain ATCC 13048 and of the non-TEM-24-producing strains (Fig. 3 and 4). The six strains of *E. coli* producing TEM-24 had distinct profiles (Fig. 5).

DISCUSSION

The aim of this study was to determine if the infections observed in the ICU, geriatric department, and medicine de-

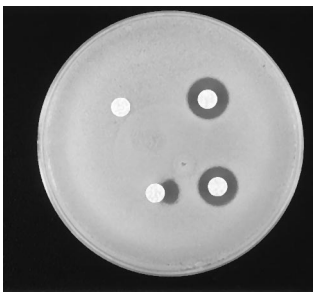

FIG. 1. Detection of ESBla-producing *E. aerogenes* by double-disk agar diffusion. The disks of ceftazidime (left) and amoxicillin-clavulanic acid (right) are separated by 30 mm (top) or 20 mm (bottom).

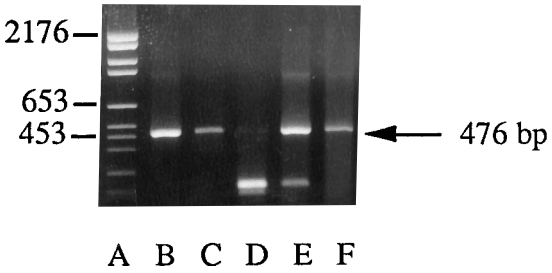

FIG. 2. Agarose gel electrophoresis of PCR products. Lanes: A, size markers (in base pairs); B, *E. aerogenes* 3; C, reference *E. coli* strain producing TEM-24; D, reference *E. coli* strain producing TEM-3; E, *E. coli* 3; F, *C. freundii* 16. The 476-bp DNA fragment is indicated by an arrow on the right.

partment of our hospital caused by TEM-24-producing *E. aerogenes* were due to an epidemic strain and if plasmid dissemination among other members of the *Enterobacteriaceae* also occurred.

Part of the answer was provided by the study of patient transfer between the different departments. The first strains were isolated in the ICU, probably spread between patients by medical procedures. One strain was subsequently isolated in a medicine department, but no patient had been transferred from one unit to the other. Both departments are located on the same floor, the entrances being only a few meters apart and the nurse office being common to the two units. Therefore, proximity can account for dissemination of the strains.

For the two strains from the geriatric department, a relationship was more difficult to establish since this department is located in another area of the city. We discovered only in May 1994 that patient 2 had been transferred to the geriatric department in October 1993 after a stay in the ICU. The two patients (6 and 10) who developed urinary infections caused by a TEM-24-producing *E. aerogenes* strain were in the same bedroom as patient 2.

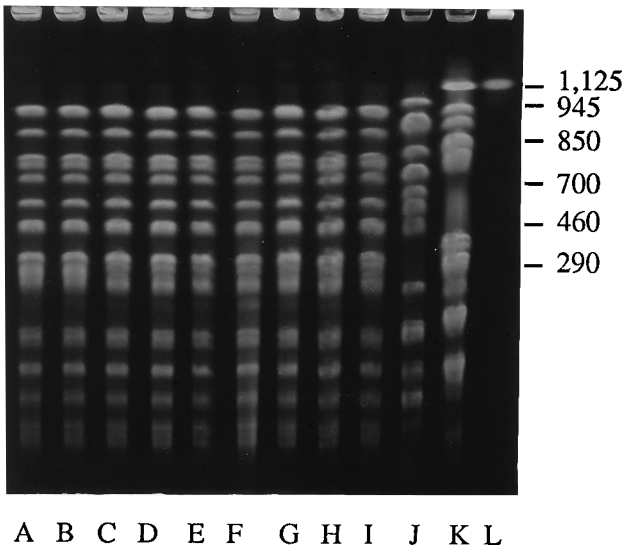

FIG. 3. PFGE of total DNA from *E. aerogenes* cut by *Xba*I. Lanes: A, strain 1 (ICU); B, strain 2 (ICU); C, strain 3 (ICU); D, strain 4 (ICU); E, strain 5 (medical unit); F, strain 6 (geriatric unit); G, strain 7 (ICU); H, strain 8 (ICU); I, strain 10 (geriatric unit); J, unrelated strain of *E. aerogenes*; K, *E. aerogenes* reference strain ATCC 13048; L, *Saccharomyces cerevisiae* chromosomal DNA (sizes are indicated in kilobases).

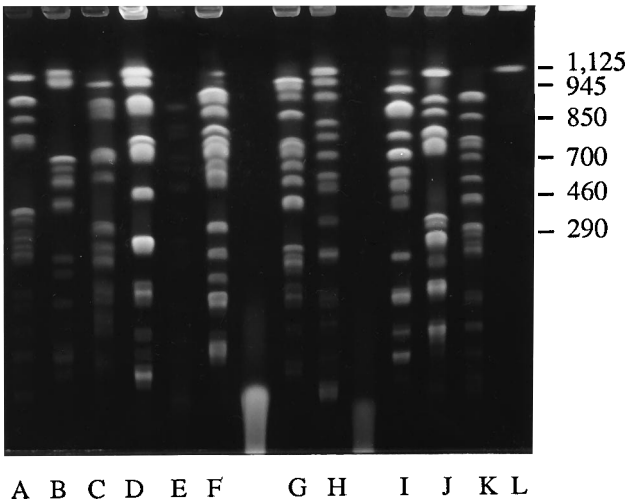

FIG. 4. PFGE of total DNA from *E. aerogenes* cut by *Xba*I. Lanes: A to J, unrelated strains; K, strain 9 (ICU); L, *Saccharomyces cerevisiae* chromosomal DNA (sizes are indicated in kilobases).

According to these findings, spread of a single strain was suggested. This was confirmed by the study of the DNA of the strains by PFGE: the 10 strains had the same profile, whereas a high degree of polymorphism was observed among non-ESBla-producing strains (the reference type strain and unrelated strains). PFGE already proved to be useful for the study of genomic relatedness among non-ESBla-producing *Enterobacter* spp. (10). Our results confirm that it is also a good epidemiological tool for investigation of outbreaks caused by ESBla-producing *E. aerogenes.*

Difficulty in detection of TEM-24 production is probably due to the presence of the chromosomally mediated cephalosporinase (3, 11). The activity of ceftazidime was more efficiently restored by clavulanic acid than the activity of cefo-

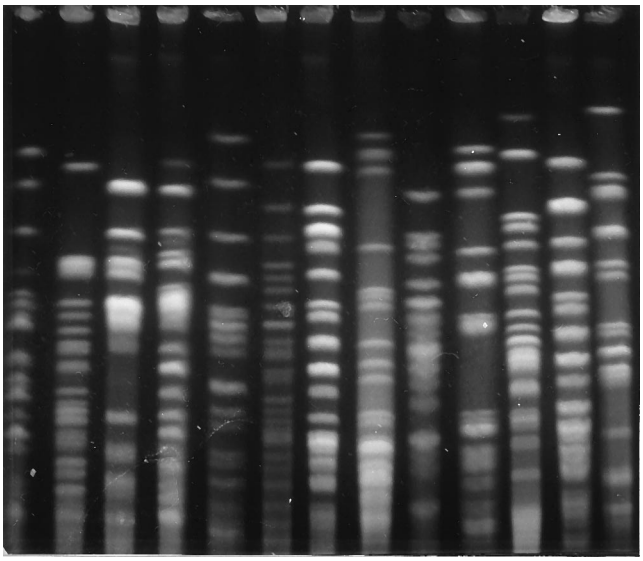

FIG. 5. PFGE of total DNA from *E. coli* cut by *Xba*I. Lanes: A to G, unrelated strains; H, strain 3; I, strain 11; J, strain 12; K, strain 13; L, strain 14; M, strain 15.

taxime or aztreonam, as already described (5). The best detection conditions were with disks of ceftazidime and amoxicillin-clavulanic acid placed 20 mm apart.

This work also stresses the importance of plasmid transfer in vivo. Before the outbreak, we had never isolated extended-spectrum β-lactamase with a pI of 6.5. The first isolates were from *E. aerogenes* (September 1993). Then, over a 3-month period TEM-24 was found only in *E. aerogenes*, and in December 1993 it appeared in *E. coli* and later in *C. freundii* (April 1994). This suggests that the TEM-24-encoding plasmid had been transferred from *E. aerogenes* to other members of the *Enterobacteriaceae*. A single patient, patient 3, was cocolonized in the stool by an *E. aerogenes* and an *E. coli*, both producing TEM-24. Transfer in the digestive tract of the 85-kb plasmid could account for this observation. This notion is supported by the very high frequency of transfer of this plasmid by conjugation under laboratory conditions. The other strains of *E. coli*, as well as the strain of *C. freundii*, were isolated alone, but it is possible that the patients were colonized by *E. aerogenes* or another resistant *E. coli* strain in an anatomical site where no bacteriological investigations were performed. PFGE on those strains of *E. coli* showed that they were genotypically different, excluding the spread of a single strain. Moreover, after the outbreak of *E. aerogenes* ended, TEM-24 was not isolated again in our hospital. This supports the hypothesis of plasmid dissemination from *E. aerogenes* to other members of the *Enterobacteriaceae.*

In conclusion, an outbreak of one strain producing a plasmid-mediated ESBla can be accompanied by an outbreak of this plasmid, and only reliable genotypic typing methods can serve the involved ward and lead to appropriate control measures.

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