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TEM Derivative-Producing *Enterobacter aerogenes* Strains: Dissemination of a Prevalent Clone

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TEM-24 (CAZ-6) extended-spectrum β-lactamase (ESBL) was detected in 1988 in Clermont-Ferrand, France, in *Klebsiella pneumoniae* (bla_{TEM-24}) and *Enterobacter aerogenes* ($bla_{TEM-24b}$), and since 1994, a TEM-24-producing *E. aerogenes* clonal strain has been observed elsewhere in the country. To determine if the spread of this clonal strain was restricted to TEM-24-producing *E. aerogenes* strains, 84 *E. aerogenes* strains (non-TEM/SHV-producing strains, TEM-1- or -2-producing strains, and different ESBL-producing strains), isolated from 1988 to 1999 in Clermont-Ferrand ($n = 59$) and in 11 other French hospitals in 1998 ($n = 25$), were studied. A clonal strain was found for TEM-24- but also for TEM-3- and TEM-1- or 2-producing isolates. This study shows that there is a clonal strain dependent on acquisition of the TEM-type enzyme (TEM-24 and other TEM types).

In 1988, TEM-24 (CAZ-6) extended-spectrum β-lactamase (ESBL) encoded by the bla_{TEM-24} gene was reported in *Klebsiella pneumoniae* in Clermont-Ferrand, France (5). The enzyme spread quickly in *Enterobacter aerogenes* (7) but was encoded by the $bla_{TEM-24b}$ gene. Since 1994, there have been several reports of a TEM-24-producing *E. aerogenes* clone in France (4, 9, 11, 14, 15) and in Belgium (8).

The aim of this study was to determine if the dissemination of the clone dated from 1988 and whether the other TEM derivates were produced by the same *E. aerogenes* clone.

Eighty-four clinical nonrepetitive isolates of *E. aerogenes* were randomly selected among samples taken from January 1988 to December 1999 at the teaching hospital of Clermont-Ferrand ($n = 59$) and at 11 other French hospitals ($n = 25$) in 1998. These strains, identified by the rapid Id 32E System (bioMérieux, La Balme les Grottes, France) according to the manufacturer's instructions, were as follows (Table 1): 60 ESBL-producing strains (22 for TEM-3, 1 for TEM-8, 33 for TEM-24, and 4 for SHV-4) identified by isoelectric focusing and direct sequencing of PCR products, 12 TEM-1- or -2-producing strains (11 for TEM-1 and 1 for TEM-2), and a control group with 12 non-TEM/SHV-producing strains (intrinsic resistance to β-lactams amoxicillin, amoxicillin-clavulanate, cephalothin, and cefoxitin) identified by resistance phenotype and isoelectric focusing. Of the 60 ESBL-producing strains, 35 strains were from Clermont-Ferrand (up to two isolates selected a year and by ESBL type) and 25 strains were from 11 other French hospitals (up to two isolates selected by the center and by ESBL type). For TEM-1- or -2-producing strains and non-TEM/SHV-producing strains, one isolate a year was selected from Clermont-Ferrand.

The 84 strains were typed by ribotyping and enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR). With both methods, bacterial DNA was prepared by growing *E. aerogenes* overnight in Mueller-Hinton broth at 37°C. Cells were obtained by centrifugation at 4,000 × *g* for 5 min at 4°C. The pellet was resuspended in 300 μl of a solution containing Tris-HCl, pH 7.5, sucrose, lysozyme, and 120 μl of 0.5 M EDTA, pH 8.0. The suspension was mixed and allowed to stand on ice for 10 min. The cells were lysed by adding 1.4 ml of 6 M guanidium chloride followed by 100 μl of 7.5 M ammonium acetate and by incubation at room temperature with shaking for 1 h. Contaminating proteins were digested with 15 μl of proteinase K (10 mg/ml) and 200 μl of 10% (wt/vol) Sarkosyl for 60 min at 60°C. High-molecular-weight DNA was precipitated with 100% ethanol and recovered by centrifugation. The DNA was resuspended in a solution containing 6 mM Tris-HCl, pH 7.5, 6 mM NaCl, and 0.1 mM EDTA.

For ribotyping, we used the procedure of Bingen et al. (1) for restriction fragment length polymorphisms of ribosomal DNA region analysis with restriction endonuclease *Eco*RI. Sixteen profiles (A to P) were obtained from the 84 *E. aerogenes* isolates (data not shown), of which the most frequent was

TABLE 1. Distribution of β-lactamases produced by 84 *E. aerogenes* strains selected from Clermont-Ferrand hospital and 11 other centers

β-Lactamase[a] or characteristic	No. of strains from:		Total no.
	Clermont-Ferrand (1988–1999)	Other centers[b] (1998)	
TEM-24*	14	19	33
TEM-8*	1		1
TEM-3*	18	4	22
SHV-4*	2	2	4
TEM-1	11		11
TEM-2	1		1
Non-TEM/SHV	12		12
Total	59	25	84

[a] Asterisks indicate extended-spectrum β-lactamases.
[b] Aix-en-Provence, Grenoble, Lille, Lyon, Montpellier, Mulhouse, Nice, Perpignan, Paris, Toulouse, Valenciennes.

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TABLE 2. Distribution of the main profile obtained by ribotyping and ERIC-PCR according to different β-lactamases produced

Enzyme (*n*)[a]	Profile A[b] [no. (%)]	Profile 1[c] [no. (%)]
TEM-24* (33)	33 (100)	33 (100)
TEM-3* (22)	16 (73)	17 (77)
TEM-8* (1)	1	
SHV-4* (4)		1
TEM-1 or 2 (12)	11 (92)	12 (100)
Non-TEM/SHV (12)	1	4
Total (84)	62 (74)	67 (80)

[a] Asterisks indicate extended-spectrum β-lactamases. *n*, no. of strains.
[b] Ribotyping.
[c] ERIC-PCR.

profile A, found in all hospitals (Table 2). It was observed in 62 isolates (74%). All 33 *E. aerogenes* TEM-24-producing strains (100%), 16 of 22 TEM-3-producing strains (73%), the one strain producing TEM-8, and 11 of 12 TEM-1- or TEM-2-producing strains (92%) had this profile, but only one non-TEM/SHV-producing strain did.

For ERIC-PCR, each 50-μl reaction mixture contained 50 pmol of each primer (Eurogentec): ERIC1R (5′-ATGTAAG CTCCTGGGGATTCA-3′) and ERIC2(5′-AAGTAAGTGA CTGGGGTGAGCG-3′), 500 ng of DNA, 0.2 mM of each of 4 dNTPs, 1.5 U of *Taq* DNA polymerase (Appligene) in a buffer with 10% dimethyl sulfoxide. PCR amplifications were performed in an automated thermal cycler (Perkin-Elmer/Cetus DNA Thermal Cycler) with initial denaturation (95°C, 7 min) followed by 30 cycles of denaturation (90°C, 30 s), annealing (52°C, 1 min), and extension (65°C, 8 min) with a single final extension (65°C, 16 min). Twenty-five microliters of each PCR was then electrophoresed directly on a 1% agarose gel containing 0.5 × Tris borate-EDTA and 0.5 μg of ethidium bromide/ml. Ten profiles (1 to 10) were obtained from the 84 *E. aerogenes* isolates, of which the most frequent was profile 1, found in all hospitals (Table 2). It was observed in 67 isolates (80%). All 33 *E. aerogenes* TEM-24-producing strains (100%), 17 of 22 TEM-3-producing strains (77%), 1 of the 4 SHV-4-producing strains, and all TEM-1- or TEM-2-producing strains (100%) had this profile, but only 4 non-TEM/SHV-producing strains did.

The results obtained with the two techniques were concordant, and by combining both methods (ribotyping and ERIC-PCR) we obtained 18 profiles, of which the most frequent was profile A/1 (100% TEM-24, 73% TEM-3, 92% TEM-1 or -2, and one non-TEM/SHV-producing strain). The 12 strains without acquired β-lactamases were distributed in 12 different types. This variety of types and the low frequency of type A/1 among non-TEM/SHV-producing strains of *E. aerogenes* were confirmed by studies in progress (data not shown).

Sixty four of 84 strains were studied for plasmid DNA analysis: the 12 TEM-1 or -2 strains, 12 non-TEM/SHV-producing strains, and 40 of 60 ESBL-producing strains were selected by ESBL type, year, and center (Table 3). The size of the plasmids was estimated after plasmid DNA extraction by the method of Kado and Liu (10) and electrophoretic migrations in a 1% agarose gel in comparison with standard plasmids (Rsa, 39 kb; TP114, 61.5 kb; CF204, 85 kb; CF604, 180 kb). Labeling of TEM probe was performed by random priming with the 2,4-dinitrophenyl-DNA-labeling kit purchased from Appligene Oncor (Illkirch, France). Hybridization with TEM probe and revelation were performed as previously described (3) according to the manufacturer's recommendations on DNA extracts denatured and immobilized on Nytran filters.

TABLE 3. Plasmid content of 64 selected *E. aerogenes* strains

Enzyme (*n*)[a]	Year(s)	Profile R/E[b]	Presence of plasmid and hybridization[c]		No. of isolates by year
			180 kb	85 kb	
TEM-24 (24)	88/94	A/1	+	⊕	2/1
	91	A/1	−	⊕	1
	89/90/91	A/1	⊕	+	1/1/1
	89/90/92/94/96/98[e]/99	A/1	⊕	−	1/1/1/1/2/10/1
TEM-3 (12)	88/89/96/99	A/1	−	⊕	1/1/1/1
	90/94/98	A/1	+	⊕	1/1/1
	91/98	A/1-E/2	⊕	+	1/2
	92/97	A/1	⊕	−	1/1
TEM-8 (1)	91	A/4	+	⊕	1
SHV-4 (3)	93/94/98	B/1-E/4-F/2	⊕	−	1/1/1
TEM-1/2 (12)	93	B/1	⊕	−	1
	88–92/94–99	A/1	⊕	−	1/1
Non-TEM/SHV (12)	90	A/1	+	−	1
	88/89/91–99	11 different profiles[d]	−	−	1/1/1

[a] *n*, no. of strains.
[b] R/E, Ribotyping/ERIC-PCR.
[c] Plasmid presence (+) and positive hybridization with TEM probe (⊕).
[d] B/8-G/1-H/7-I/5-J/1-K/2-L/9-M/1-N/7-O/3-P/3.
[e] 1998: the year of the multicentric study (strains producing TEM-24 isolated from 10 of 12 different centers).

TABLE 4. Associated resistance markers in tranconjugants of 24 selected *E. aerogenes* strains

Enzyme (*n*)[a]	Yr	Center[b]	Profile (R/E[c])	Presence of plasmid of size (kb)		Presence of associated resistance markers[e]				
				180	85	AAC6′-I type[d]	Tc	Cm	Su	Tp
TEM-24 (15)	1988	CF	A/1		+	+	+	−	+	−
	1991	CF	A/1		+	+	+	−	+	+
	1992	CF	A/1	+		+	−	+	+	−
	1994	CF	A/1	+		+	−	−	+	+
	1994	CF	A/1	+		+	+	−	+	−
	1998	LI	A/1	+		+	−	+	+	+
		MO	A/1	+		+	−	+	+	+
		PA	A/1	+		+	−	+	+	+
		TO	A/1	+		+	−	+	+	+
		NI	A/1	+		+	−	+	+	+
TEM-24 (15)	1998	LY	A/1	+		−	−	−	+	+
		AP	A/1	+		+	−	+	+	+
		PE	A/1	+		+	−	+	+	+
		MU	A/1	+		+	−	+	+	+
		VA	A/1	+		+	−	+	+	+
TEM-3 (4)	1988	CF	A/1		+	+	+	−	+	−
	1990	CF	A/1		+	+	+	−	+	−
	1994	CF	A/1		+	+	+	−	+	−
	1998	LI	C/2	+		+	−	+	+	+
TEM-1 or -2 (4)	1988[f]	CF	A/1	+		−	−	+	+	+
	1989	CF	A/1	+		−	−	+	+	+
	1993	CF	B/1	+		−	−	+	+	+
	1999	CF	A/1	+		−	−	+	+	+
Non-TEM/SHV (1)	1990	CF	A/1	+		−	−	+	+	+

[a] *n*, no. of strains.
[b] CF, Clermont-Ferrand; LI, Lille; MO, Montpellier; PA, Paris; TO, Toulouse; NI, Nice; LY, Lyon; AP, Aix-en-Provence; PE, Perpignan; MU, Mulhouse; VA, Valenciennes.
[c] R/E, Ribotyping/ERIC-PCR.
[d] AAC6′-I type: resistance phenotype T^R G^S N^R A^R.
[e] Cm, chloramphenicol; Su, sulphonamides; Tc, tetracycline; Tp, trimethoprim.
[f] TEM-2-producing strain.

All of the *E. aerogenes* strains producing ESBL harbored one or two large plasmids (180 and/or 85 kb). These plasmids were found in only one non-TEM/SHV-producing strain, which had the profile A/1 (180 kb). Hybridization with the TEM probe showed that the *bla* genes were always on large plasmids: 85 kb (11 strains) or 180 kb (38 strains). The TEM-24-encoding gene was originally located in 1988 on an 85-kb plasmid. Since then it has always been observed on a 180-kb plasmid, except for one strain in 1991. The TEM-3-encoding gene was located alternately on 85- or 180-kb plasmids. TEM-1- or -2-encoding genes were always located on 180-kb plasmids.

For 24 of 64 strains selected according to plasmid content, ESBL type, year, and center, a transfer experiment by conjugation was performed for 40 min at 37°C as previously described (16) with *Escherichia coli* K12 C600 resistant to rifampin used as the recipient strain. The 180-kb plasmid (n = 19) encoding TEM-24 (13 of 15 strains), TEM-3 (1 of 4), TEM-1 (3 of 3), or TEM-2 (1 of 1) and present in one non-TEM/SHV-producing strain had the following associated resistance markers (Table 4): sulfonamides (19 of 19), trimethoprim (17 of 19), and chloramphenicol (16 of 19). The 85-kb plasmid (n = 5) encoding TEM-24 or TEM-3 always had sulfonamides and tetracycline resistance as associated markers (5 of 5 strains). The AAC6′-I type was associated with ESBL in all but one strain (LY-98).

For digestion of plasmids and hybridization with the TEM probe, 10 strains were selected according to plasmid size, ESBL type, and cotransferred resistances. The β-lactamase-encoding plasmids were extracted by the alkaline lysis method (2) and digested with *Eco*RI and *Hin*dIII restriction endonucleases according to the recommendations of the manufacturer (Boehringer Mannheim, Meylan, France). The five profiles obtained with 180-kb plasmids encoding TEM-1, TEM-2, TEM-3, and TEM-24 and that of the non-TEM/SHV-producing strain were similar. The three profiles obtained with the 85-kb plasmid encoding TEM-3 or TEM-24 were the same. Whatever the size of the plasmid, the TEM probe hybridized on the same large fragment (>10 kb) (data not shown).

By a combination of both typing methods, we showed the existence of an epidemic clone (A/1) not only for TEM-24-producing *E. aerogenes* isolates but also for the other TEM-type enzyme-producing *E. aerogenes* strains. That suggests a possible "in-clone" evolution of β-lactamase in this species. TEM-24 seemed to be less frequently encountered in other *Enterobacteriaceae* than in *E. aerogenes*, as reported in a 1998 survey (6), probably in correlation with the epidemic spread of

E. aerogenes. However, dissemination of TEM-24 by in vivo transfer of encoding plasmid among different species of *Enterobacteriaceae*, including the *E. aerogenes* clonal strain, had been reported in France previously (12, 13, 14, 15). All strains belonging to type A/1 harbored one or two plasmids. These large plasmids (180 or 85 kb) with multiple resistance markers conferred to this host (clone A/1) resistance under selective antibiotic pressure. This selective factor could be responsible for the exceptional adaptation of the *E. aerogenes* clone A/1 to the hospital environment and for its dissemination via patient transfer from one hospital to another. When there is transfer in vivo, plasmids encoding β-lactamases transfer from different species of *Enterobacteriaceae* into the different clones of *E. aerogenes*, among which only clone A/1 is able to maintain the plasmid. To test this hypothesis, experiments are currently being performed in our laboratory to transfer these large plasmids into the different clones of. *E. aerogenes.*

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