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Molecular Epidemiology of *Enterobacteriaceae* Isolates Producing Extended-Spectrum β-Lactamases in a French Hospital

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In 2002, 80 isolates of *Enterobacteriaceae* producing extended-spectrum β-lactamases (ESBLs) were collected from infected patients in our hospital. *Enterobacter aerogenes* was the most common bacterium isolated from all specimens (36.5%). The ESBLs were predominantly (90%) TEM derivatives (TEM-24, TEM-3). Pulsed-field gel electrophoresis highlighted that *E. aerogenes*, *Klebsiella pneumoniae*, and *Citrobacter koseri* had a clonal propagation.

Over the last 20 years, there has been an increased resistance to β-lactams because of the secretion of extended-spectrum β-lactamases (ESBLs) mediated by plasmids. This type of resistance is now observed in all species of *Enterobacteriaceae* and is currently disseminated throughout the world (22, 29, 35). From 1991 to 1993, we described the first ESBL-producing *Enterobacteriaceae* strains isolated in our hospital, a 1,588-bed university hospital in southern France.

To evaluate the epidemiological evolution of *Enterobacteriaceae* producing ESBL in our hospital from 1993 onward, a prospective study was conducted from April 2002 to March 2003 (20). We screened 3,063 nonrepetitive clinical isolates of *Enterobacteriaceae* recovered consecutively from infection sites of hospital patients. Antibiotic susceptibility testing was performed on Muller-Hinton agar with antibiotic disks from Pasteur Diagnostics (Marne-la-Coquette, France), placed at defined points, with the Vitek 2 GNS-F7 card (bioMérieux, Marcy-l'Etoile, France). ESBL production was tested with the double-disk synergy test (31). Strains were studied whenever the synergy test was positive. Duplicates isolated from the same patient were excluded. Isolates from superficial wounds, those from stool, ear, nose, and throat specimens, and those not involved in infections as defined by the Centers for Disease Control and Prevention criteria were excluded (17).

The β-lactamases were characterized by isoelectric focusing, performed with polyacrylamide gels as previously described. Standard enzymes (including TEM-1, TEM-3, TEM-24, SHV-5, and CTX-M-1) were used as pI markers (6). The ESBL that was neither a TEM nor an SHV derivative was identified by direct sequencing of the PCR product obtained with specific primers CTX-MF (5′-GCGATGTGCAGCACCAGTAA-3′) and CTX-MR (5′-GGTTGAGGCTGGGTGAAGTA-3′), which were previously described (19). DNA sequencing of both strands of the PCR products was performed with an ABI 1377 automated sequencer with the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit with AmpliTaq DNA polymerase FS (Perkin-Elmer/Applied Biosystems, Foster City, Calif.) at C. Chanal's laboratory.

The clonality of the strains was examined by pulsed-field gel electrophoresis (PFGE) with a CHEF DRII system (Bio-Rad SA, Ivry-sur-Seine, France) as previously described (20). The *Enterobacter aerogenes*, *Escherichia coli*, *Klebsiella pneumoniae*, and *Citrobacter koseri* chromosomal DNAs were digested overnight with the restriction enzyme XbaI (Promega, Madison, Wis.), whereas the *Proteus mirabilis* and *Providencia stuartii* DNAs were digested with SmaI (Promega). Electrophoresis was performed at 6 V/cm for 30 h; the pulse time ranged from 40 to 5 s for *E. aerogenes*, *K. pneumoniae*, *C. koseri*, and *E. coli* strains and from 25 to 5 s for *P. mirabilis* and *P. stuartii*. Because a single base mutation in the chromosomal DNA of an isolate is sufficient to introduce differences in three fragments of its restriction pattern, isolates with restriction patterns showing the same differences in one to three fragments were considered to belong to the same genotype (32). The PFGE patterns were analyzed with the GelCompar computer software for Windows, version 3.5 (Applied Maths, Kortrijk, Belgium), and compared by the algorithmic clustering method known as the unweighted pair group method using arithmetic averages with the Dice coefficient of similarity. Isolates were considered to be within a cluster if the coefficient of similarity was >80%.

Out of the 3,063 *Enterobacteriaceae* strains isolated, 80 produced an ESBL, i.e., 2.62%, in accordance with other French publications (1, 2, 11, 15), and corresponded to: *E. aerogenes* (n = 29 [36.3%]), *K. pneumoniae* (n = 15 [18.8%]), *E. coli* (n = 13 [16.2%]), *C. koseri* (n = 12 [15%]), *P. mirabilis* (n = 6 [7.5%]), *P. stuartii* (n = 4 [5%]), and *K. oxytoca* (n = 1 [1.2%]). No epidemic was reported during the surveillance period. The prevalence of the ESBL production in the various species was 20.34% (12 of 59) for *C. koseri*, 17.9% (29 of 162) for *E. aerogenes*, 8.24% (15 of 182) for *K. pneumoniae*, 7.55% (4 of 53) for *P. stuartii*, 2.33% (6 of 258) for *P. mirabilis*, 1.22% (1 of 82) for *K. oxytoca*, and 0.71% (13 of 1,827) for *E. coli*. These results were close to those found in other French hospitals, except for *E. aerogenes* (17.9% in our hospital compared to

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TABLE 1. Characteristics of ESBL-producing *Enterobacteriaceae* strains isolated in a French university hospital in 2002

Species (total no. of isolates)	Total no. (%) of ESBL-producing isolates	PFGE profile[a]	Specimen(s)[b]	Unit or ward (no.)	β-Lactamase content[c]	Antibiotype[d]
E. aerogenes (162)	29 (36.3)	EAI_A (9)	Pus (5), urine (3), respiratory tract (1)	Medicine (4), ICU (2), surgery (2), geriatric	TEM-24, AmpC	KTNt(A)-NAL, OFX, NOR, CIP, PEF-SXT TET CHL (6)
						KTGNt(A)-NAL, OFX, NOR, CIP, PEF-SXT TET CHL (3)
		EAI_B (6)	Pus (3), urine (3)	Medicine (5), geriatric	TEM-24, AmpC	KTNt(A)-NAL, OFX, NOR, CIP, PEF-SXT TET CHL (5)
						KTGNt(A)-NAL, OFX, NOR, CIP, PEF-SXT TET CHL (1)
		EAI_C (4)	Urine (2), cutaneous (1), respiratory tract (1)	ICU (4)	TEM-24, AmpC	KTNt(A)-NAL, OFX, NOR, CIP, PEF-SXT TET CHL (1)
		EAI_E (4)	Urine (3), pus (1)	Medicine, surgery, geriatric, recovery	TEM-24, AmpC	KTNt(A)-NAL, OFX, NOR, CIP, PEF-SXT TET CHL
		EAI_G (3)	Urine (2), pus (1)	Medicine, geriatric, recovery	TEM-24, AmpC	KTNt(A)-NAL, OFX, NOR, CIP, PEF-SXT TET CHL
		EAI_F	Pus	Surgery	TEM-24, AmpC	KTNt(A)-NAL, OFX, NOR, CIP, PEF-SXT TET CHL
		EAI_D	Urine	Recovery	TEM-24, AmpC	K-NAL, OFX, NOR, CIP, PEF-TET CHL
		EAI_H	Urine	Geriatric	TEM-24, AmpC	KTNt(A)-NAL, OFX, NOR, CIP, PEF-SXT TET CHL
K. pneumoniae (182)	15 (18.8)	KPI	Urine	Recovery	TEM-24, TEM-1	KTNt(A)-NAL-SXT TET CHL
		KPII	Urine	Medicine	TEM-3	KTNt(A)-NAL-SXT TET CHL
		$KPIII_A$ (8)	Urine (5), cutaneous (1), pus (1), respiratory tract (1)	ICU (4), geriatric (2), surgery, recovery	TEM-3, TEM-1, SHV-1	KTGNt(A)-NAL, OFX, NOR, CIP, PEF-SXT TET CHL
		$KPIII_C$ (2)	Pus, respiratory tract	Surgery, medicine	TEM-3, TEM-1	KTGNt(A)-NAL, OFX, NOR, CIP, PEF-SXT TET CHL
		$KPIII_B$	Urine	Medicine	TEM-3	KTGNt(A)-NAL, OFX, NOR, CIP, PEF-SXT TET CHL
		$KPIII_D$	Urine	Medicine	TEM-3, TEM-1, SHV-1	KTGNt(A)-NAL, OFX, NOR, CIP, PEF-SXT TET CHL
		$KPIII_E$	Urine	Recovery	TEM-3, TEM-24	KTNt(A)-NAL, OFX, NOR, CIP, PEF-SXT TET CHL
E. coli (1,827)	13 (16.2)	ECI_A (2)	Pus, urine	Medicine, geriatric	TEM-24, TEM-1	KTNt(A)-NAL, OFX, NOR, PEF-SXT TET CHL
		$ECII_A$ (2)	Pus, urine	Medicine, surgery	CTX-M-3, TEM-1	KTGNt(A)-NAL, OFX, NOR, CIP, PEF-SXT TET CHL
		EC1	Respiratory tract	Medicine	TEM-24	KTNt(A)-NAL-SXT TET CHL
		EC2	Urine	Medicine	CTX-M-15, TEM-1/OXA-1	KTGNt(A)-NAL, OFX, NOR, CIP, PEF-SXT TET CHL
		EC3	Urine	Medicine	CTX-M-15, OXA-1	KTGNt(A)-NAL, OFX, NOR, PEF-SXT TET CHL
		EC4	Urine	Medicine	CTX-M-15, OXA-1	KTGNt(A)-NAL, OFX, NOR, PEF-SXT TET CHL
		EC5	Pus	Surgery	CTX-M-14, TEM-1	K(A)-NAL-SXT TET CHL
		EC6	Cutaneous	ICU	CTX-M-14, TEM-1	K(A)-NAL-SXT TET CHL
		EC7	Venereal	ICU	CTX-M-15, TEM-1/OXA-1	KTGNt(A)-NAL, OFX, NOR, PEF-TET CHL
		EC8	Urine	Geriatric	TEM-24	KTNt(A)-NAL, OFX, NOR, CIP, PEF-SXT TET CHL
		EC9	Urine	Surgery	TEM-3	KTNt(A)-NAL-TET CHL
C. koseri (59)	12 (15)	CKI_A (4)	Urine	Medicine (2), geriatric, ICU	TEM-24	KTNt(A)-NAL, OFX, NOR, CIP, PEF-SXT TET CHL
		CKI_D (4)	Urine	Geriatric (2), medicine (2)	TEM-3	KTNt(A)-NAL, OFX, NOR, CIP, PEF-SXT TET CHL
		CKI_E (2)	Urine	Recovery, ICU	TEM-3	K(A)-NAL, OFX, NOR, CIP, PEF-SXT TET CHL
		CKI_B (1)	Urine	Surgery	TEM-3	KTNt(A)-NAL, OFX, NOR, CIP, PEF-SXT TET CHL
		CKI_C (1)	Pus	Medicine	TEM-3	K(A)-NAL, OFX, NOR, PEF-SXT TET CHL
P. mirabilis (258)	6 (7.5)	PM1	Urine	Medicine	TEM-3	K(A)-NAL, OFX, NOR, CIP, PEF-SXT TET CHL
		PM2	Urine	Medicine	TEM-3, TEM-1	K(A)-NAL, OFX, NOR, CIP, PEF-SXT TET CHL
		PM3	Urine	ICU	TEM-3	K(A)-NAL, OFX, NOR, CIP, PEF-SXT TET CHL
		PM4	Urine	Geriatric	TEM-3	KTGNt(A)-NAL, OFX, NOR, CIP, PEF-SXT TET CHL
		PM5	Cutaneous	Geriatric	TEM-3	KTNt(A)-NAL, OFX, NOR, CIP, PEF-SXT TET CHL
		PM6	Cutaneous	Geriatric	TEM-3	KTNt(A)-NAL, OFX, NOR, CIP, PEF-SXT TET CHL
P. stuartii (53)	4 (5)	PS1	Urine	Geriatric	TEM-24	KTGNt(A)-NAL, OFX, NOR, CIP, PEF-SXT TET CHL
		PS2	Pus	Recovery	TEM-24	KTGNt(A)-NAL, OFX, NOR, PEF-SXT TET CHL
		PS3	Urine	ICU	TEM-24	KTGNt(A)-NAL, OFX, NOR, CIP, PEF-SXT TET CHL
		PS4	Urine	Recovery	TEM-24	KTGNt(A)-NAL, OFX, NOR, CIP, PEF-TET CHL
K. oxytoca (82)	1 (1.2)	KO1	Urine	Medicine	TEM-3	KTNt(A)-NAL-SXT TET CHL

[a] PFGE profiles were generated after restriction digestion of chromosomal DNA with the restriction enzyme XbaI.
[b] The number of isolates is given in parentheses if more than one isolate was recovered.
[c] AmpC and SHV-1, species-specific cephalosporinase and SHV-1 like chromosomal penicillinase, respectively.
[d] Resistance to different antimicrobial agents. Abbreviations: T, tobramycin; A, amikacin; G, gentamicin; K, kanamycin; Nt, netilmicin; SXT, cotrimoxazole; NAL, nalidixic acid; OFX, ofloxacin; NOR, norfloxacin; CIP, ciprofloxacin; IPM, imipenem; TET, tetracycline; CHL, chloramphenicol. Parentheses indicate a low level of resistance.

high levels ranging from 31.9 to 53.5% in other hospitals) (5, 11, 25). The majority of strains were isolated from urinary specimens (n = 51 [63.8%]) (Table 1). Out of the 80 ESBL-producing strains isolated, 11.25% were found in the recovery unit, 20% were found in the intensive care unit (ICU), 20% were found in the geriatric unit, and 36.25% were found in the medicine unit (Table 1). However, among the *Enterobacteriaceae* strains isolated in each unit, the proportion of ESBL-producing strains was 7.3% (9 of 123) in the geriatric unit and 6.75% (16 of 237) in the recovery unit. This rate was only 1.8% in the medicine unit (29 of 1,596). Indeed, 32 (41.6%) out of 77 patients had stayed in an ICU in the 6 months prior to isolation of the ESBL-producing bacteria. The propagation of the ESBL-producing strain could be correlated to time spent in an ICU, as already described by others (12, 36). Contrary to previous studies, ESBL strains were not detected in pediatric patients (21, 28). *E. aerogenes* was the predominant bacterium, and this has been the trend in France since 1993, while ESBL-producing *K. pneumoniae* isolates are decreasing (3, 5, 20). This phenomenon has also been observed in other countries such as the United States and Spain, although not in Italy (10, 29, 30). Furthermore, we isolated few strains of *K. pneumoniae* in geriatric wards (13.3%, 2 out of 15), where the first ESBL-producing *Enterobacteriaceae* strains were described in our hospital (20).

The following five different ESBLs were characterized: TEM-24 (n = 38 [47.5%]), TEM-3 (n = 34 [42.5%]), CTX-M-15 (n = 4 [5%]), and CTX-M-3 and CTX-M-14 (n = 2 [2.5%] each) (Table 1). *E. aerogenes* and *P. stuartii* secreted exclusively TEM-24, and *P. mirabilis*, *C. koseri*, and *K. oxytoca*, secreted exclusively TEM-3. *K. pneumoniae* mainly produced TEM-3 (n = 14, [93.3%]). Lastly, *E. coli* produced the greatest range of ESBLs, especially the CTX-M type. Since 1988, members of the family *Enterobacteriaceae* producing TEM-24, particularly *E. aerogenes*, have spread massively throughout several European countries such as France, Belgium, Italy, and Spain (7, 9, 13, 14, 16, 18, 23, 25, 27). TEM-24 has been found in other strains that produced ESBL (*E. coli* and *P. stuartii*) and confirmed plasmidic diffusion of this β-lactamase, without providing evidence of epidemic outbreaks. However, the production of TEM-24 in *K. pneumoniae* only concerned 13.3% of the *K. pneumoniae* isolates producing ESBLs, while in a neighboring geographic region these bacteria remained at epidemic proportions (18). In France, TEM-3 is secreted largely by *Klebsiella* spp., *P. mirabilis*, and *C. koseri*, while in other countries, other ESBLs are in the majority (4, 8, 11, 24, 26, 30, 34). ESBLs in the CTX-M group (CTX-M-3, CTX-M-14, and CTX-M-15) were only observed in *E. coli* strains. The majority of these enzymes have been found in South America, Australia, Japan, South Africa, Israel, and Eastern Europe, while a recent study confirmed their absence in the United States (11, 26, 33). It could therefore be concluded that these enzymes are responsible for an increased role in resistance mechanisms especially for *E. coli*.

In our study, PFGE analysis showed that *E. aerogenes*, *K. pneumoniae*, and *C. koseri* had a clonal propagation. All of the results are summarized in Table 1. Eight clusters, each containing isolates with coefficients of similarity of more than 80%, were identified among *E. aerogenes* isolates. An example of patterns obtained with XbaI are shown in Fig. 1. Four clusters were identified among *K. pneumoniae* isolates. However, a high level of genetic heterogeneity was found in two isolates. Thus, five clusters were identified among *C. koseri* strains.

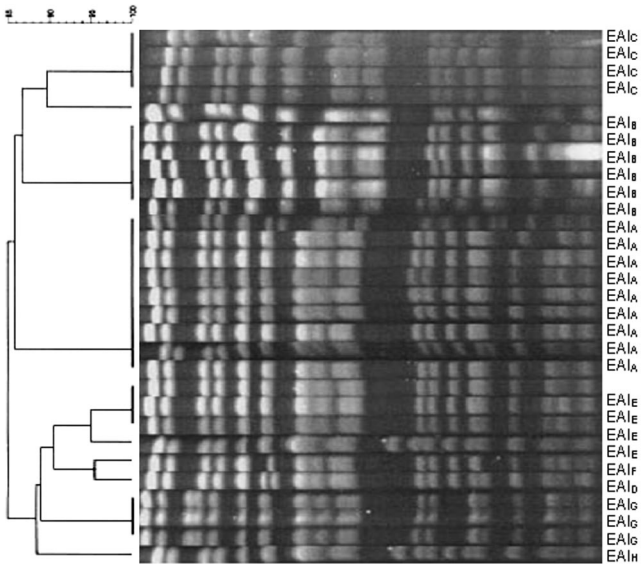

FIG. 1. Dendrogram and PFGE of XbaI-digested genomic DNAs from ESBL-producing *E. aerogenes* from our university hospital. Strains were clustered by the unweighted pair group method using arithmetic averages (UPGMA). The scale indicates the percentage of genetic similarity. Max. tol., maximum tolerance in percentage of the curve to match bands; Min. surf., minimum surface area of a band.

Ten years after our principal study, six new varieties of *Enterobacteriaceae* were identified as producing ESBLs. We noted a complex evolution: the persistence of TEM-3 as the major ESBL secreted by *K. pneumoniae*, dissemination of clonal strains of *E. aerogenes* producing TEM-24, diffusion of these resistance mechanisms to other microorganisms such as *E. coli* and *P. stuartii*, isolation of *E. coli* producing CTX-M, and dissemination of ESBL-producing strains throughout the hospital. This type of propagation in the hospital environment is rapid and alarming, despite the introduction of procedures aimed at limiting patient-to-patient diffusion of multiresistant bacteria, as well as a concerted policy regarding the use of extended-spectrum β-lactams (1, 5, 11). These surveillance measures, combined with effective screening, should assist in the fight against the worrying propagation of these microorganisms.

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