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Coexistence of SHV-4- and TEM-24-Producing *Enterobacter aerogenes* Strains before a Large Outbreak of TEM-24-Producing Strains in a French Hospital

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In 1996, a monitoring program was initiated at the teaching hospital of Amiens, France, and carried out for 3 years. All extended-spectrum β-lactamase (ESBL)-producing *Enterobacter aerogenes* isolates recovered from clinical specimens were collected for investigation of their epidemiological relatedness by pulsed-field gel electrophoresis and enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) and determination of the type of ESBL harbored by isoelectric focusing and DNA sequencing. Molecular typing revealed the endemic coexistence, during the first 2 years, of two clones expressing, respectively, SHV-4 and TEM-24 ESBLs, while an outbreak of the TEM-24-producing strain raged in the hospital during the third year, causing the infection or colonization of 165 patients. Furthermore, this strain was identified as the prevalent clone responsible for outbreaks in many French hospitals since 1996. This study shows that TEM-24-producing *E. aerogenes* is an epidemic clone that is well established in the hospital's ecology and able to spread throughout wards. The management of the outbreak at the teaching hospital of Amiens, which included the reinforcement of infection control measures, failed to obtain complete eradication of the clone, which has become an endemic pathogen.

Enterobacter aerogenes is an opportunistic pathogen. It has been associated with significant nosocomial infections, including urinary tract infections, especially in catheterized patients, respiratory tract infections, and bacteremia, particularly in elderly or debilitated patients. This species is naturally resistant to aminopenicillins and older cephalosporins due to a chromosomal cephalosporinase but remains susceptible to oxyimino cephalosporins. However, overproduction of the AmpC β-lactamase (5) or plasmid-mediated extended-spectrum β-lactamases (ESBLs) can confer resistance to extended-spectrum cephalosporins (35).

Outbreaks of multiresistant *E. aerogenes* infections have emerged during the past decade in many countries. They were investigated by using molecular typing methods such as pulsed-field gel electrophoresis (PFGE) (2, 15, 24, 32), random amplified polymorphic DNA analysis (4, 8, 12, 13, 23), enterobacterial repetitive intergenic consensus sequence PCR (ERIC-PCR) (8, 13, 15, 21), and ribotyping (4, 21, 23). In some studies, the β-lactam resistance was characterized, giving ESBL identification (4, 8, 11, 14, 32, 35). The outbreaks occurred in the United States (21, 31, 35), Belgium (15, 24), and Austria (2). In France, ESBL-producing *E. aerogenes* (ESBL-EA) has become a threat since an epidemic clone producing TEM-24 ESBL has spread to nearly all French teaching hospitals, including the hospital of Amiens (8). This prevalent clone was highly resistant to all antibiotics except gentamicin, isepamicin, imipenem, and the latest cephalosporins, such as cefepime and cefpirome. Furthermore, the emergence in France of strains resistant to all β-lactams after the use of imipenem led to a therapeutic dilemma, as no antibiotic alternatives were available (7, 8).

The emergence of ESBL-EA in the hospital of Amiens, France, was detected in 1995. The increasing number of isolates found during the following months caused us to survey the situation. Our monitoring program was initiated in October 1996 and carried out for 3 years. All strains of ESBL-EA isolated from clinical specimens were collected for determination of their epidemiological relatedness by by two molecular typing methods, ERIC-PCR and PFGE analysis. In addition, the ESBLs were characterized by the determination of their isoelectric points and by determination of the nucleotide sequences of the genes that encode them.

MATERIALS AND METHODS

Hospital presentation. The university-affiliated hospital center of Amiens, France, is a 1,750-bed teaching hospital with mainly medical (695 beds) and surgical (450 beds) care units and several intensive care units (ICU; 80 beds). The hospital is divided into two main geographical sites (a north site and a south site). The distance between these sites is about 3.5 miles.

Data collection. The surveillance program was initiated in October 1996 after the detection and isolation of several ESBL-EA iolates in the hospital and carried on until August 1999. We included in the analysis all clinical samples from any body site that was positive for an ESBL-EA isolate. The genus and species were determined biochemically with the API 20E (bioMérieux, Marcy l'étoile, France). On the basis of an agar disk diffusion assay (1), the strains were found to be resistant to expanded-spectrum cephalosporins, and ESBL production was detected by the double-disk synergy test (25). Duplicates isolated from the same patient were excluded. We calculated the incidence rate of hospital-acquired ESBL-EA infection or colonization as the number of newly infected or colonized patients per 1,000 patient days (PD).

Six strains of TEM-24-producing *E. aerogenes* belonging to the prevalent clone described by Bosi et al. (8) were kindly provided by C. Bollet (Marseille, France) to be included in PFGE and ERIC-PCR studies.

Escherichia coli XL-1 blue (Stratagene, St-Quentin-en-Yvelines, France) was used as the host for plasmid transfer experiments.

PFGE analysis. PFGE was performed with all of the ESBL-EA strains isolated during the outbreak period. Macrorestriction analysis of chromosomal DNA was

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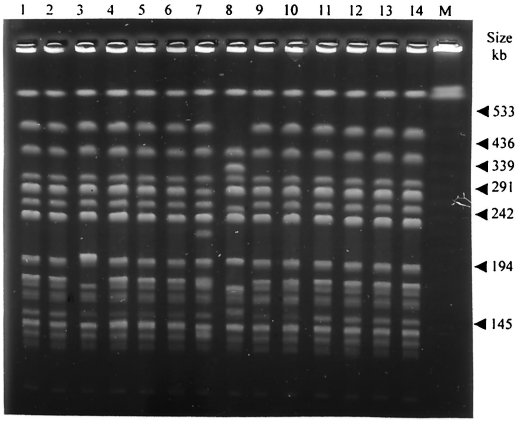

FIG. 1. Representative PFGE fingerprints obtained after digestion with *Xba*I of 14 clinical isolates of *E. aerogenes* belonging to the minor clone (lanes 1 to 14). Lane M contains molecular size markers (lambda ladder).

done with PFGE by published procedures with *Xba*I (New England Biolabs, Boston, Mass.) (32). Restriction fragments of DNA were separated by PFGE with a GenPath apparatus (Bio-Rad S.A., Ivry-sur-Seine, France). Electrophoresis was performed at 6 V/cm and 14°C. The run time was 19.7 h, with pulse times ranging from 5 to 25 s. A lambda ladder (Bio-Rad) was used for molecular size markers. The gels were stained with ethidium bromide and photographed.

ERIC-PCR analysis. After an overnight culture at 37°C on blood sheep agar medium, the total cellular DNA of one colony was extracted by the Chelex technique (16) and the DNA concentration was determined by UV spectrophotometry. Random amplified polymorphic DNA analysis with primer ERIC-2 was performed as previously described (13). Amplified products were monitored in 1.5% agarose gels in Tris-acetate-EDTA buffer, stained with ethidium bromide, and photographed on a UV light transilluminator. Strains were considered to be different if their profiles differed by two or more bands according to previous studies (39, 44).

Plasmid DNA purification and transformation experiment. Plasmid DNA was purified from bacterial cells by the alkaline lysis method (6) with the QIAGEN Plasmid Midi Kit (Qiagen, Courtaboeuf, France). Transformation experiments were performed as described by Sambrook et al. (40). Transformants were selected on Mueller-Hinton agar plates containing amoxicillin (50 μg/ml). This antibiotic was obtained from Sigma (Sigma-Aldrich, St-Quentin-Fallavier, France).

PCR detection of the bla_{TEM} and bla_{SHV} genes. Plasmid DNAs extracted from transformant cells were used as templates in specific PCRs for the detection of the bla_{TEM} and bla_{SHV} genes. Primers A and B (10) were used for amplification of the bla_{TEM} gene; primers 1 and 3 were used for amplification of the gene coding for the SHV β-lactamase (37).

A Perkin-Elmer 9600 apparatus was used, and the reactions were run under the following conditions: 30 cycles of 1 min at 95°C, 1 min at 42°C, and 1 min at 72°C and, finally, 3 min at 72°C for the bla_{TEM} amplification and 5 min at 95°C, followed by 30 cycles of 1 min at 95°C, 1 min at 55°C, and 1 min at 72°C and, finally, 3 min at 72°C for the bla_{SHV} amplification. The resulting PCR products were run in 1.5% agarose gels.

The PCR product was sequenced by automated fluorescent sequencing by the dye terminator method (Perkin-Elmer, Courtaboeuf, France) with oligonucleotides A, B, C, and D (10) for the bla_{TEM} gene or with oligonucleotides 1, 3, 8, and 13 for the bla_{SHV} gene (37).

β-Lactamase study. Following Trypticase soy broth culture (bioMérieux), β-lactamases were extracted from bacteria by sonication. Unbroken cells and cell envelopes were removed by centrifugation. Detection of β-lactamases and determination of pIs by analytical isoelectric focusing in polyacrylamide gels (pH range, 3.5 to 9.5) were performed as reported elsewhere (29), and the β-lactamase activity was localized by the use of an iodine starch method in agar gel (27). β-Lactamases whose pIs are known (TEM-1, pI 5.4; TEM-2, pI 5.6; TEM-3, pI 6.3; TEM-24, pI 6.5) were focused in parallel with the extracts.

RESULTS

Strain collection. During the study period, a total of 743 strains of *E. aerogenes* were isolated (7 to 50 strains per month). Two hundred thirty-seven clinical isolates of ESBL-EA (0 to 33 per month), each one from a different patient, were detected and collected. Among the *E. aerogenes* isolates, the percentage of ESBL-EA strains varied from 0 (November 1996 and January 1997) to 66 (January 1999). These strains were recovered from 103 urine cultures, 45 stool cultures, 18 surgical wounds, 15 tracheal aspirations, 14 sputum samples, 8 central venous catheters, 8 bronchial aspirations, 8 skin swabs, 7 fluid collection cultures, 6 blood cultures, 2 vaginal swabs, 1 bronchoalveolar fluid sample, 1 nasal swab, and 1 peritoneal exudate sample. All strains were fully susceptible to imipenem on the basis of the agar disk diffusion method.

PFGE pattern and ERIC-PCR analysis. A major PFGE pattern was found in 209 isolates (0 to 33 per month). Although slight differences in the restriction patterns of some of them were found, they were considered subtypes of the epidemic clone (42). A minor PFGE pattern was also found in the analysis of 28 ESBL-EA isolates (0 to 3 per month). It differs from that of the major clone by more than seven bands. Fourteen strains belonging to the minor clone and 14 strains belonging to the major clone were selected to represent the PFGE patterns shown, respectively, in Fig. 1 and 2A. ERIC-PCR was applied to all of the ESBL-EA isolates. The results obtained with this technique were concordant with those of the PFGE analysis. Only two clones were identified among all of the ESBL-EA isolates. Representative ERIC-PCR profiles of the minor and major clones are shown, respectively, in Fig. 3 and 4A.

Identification of ESBLs. The plasmid contents of EAA56 and EAA89, which belong, respectively, to the major and the minor clones isolated in Amiens, were used for the transformation experiment. The transformant *E. coli* XLA56 expressed a single β-lactamase with an estimated pI of 6.5, in agreement with the pI of TEM-24. The PCR product of the bla_{TEM} gene was detected. Nucleotide sequence analysis showed that it differed from the TEM-2 sequence by four substitutions leading to the amino acid replacements Glu→Lys-104, Arg→Ser-164, Ala→Thr-237, and Glu→Lys-239 (positions are numbered in

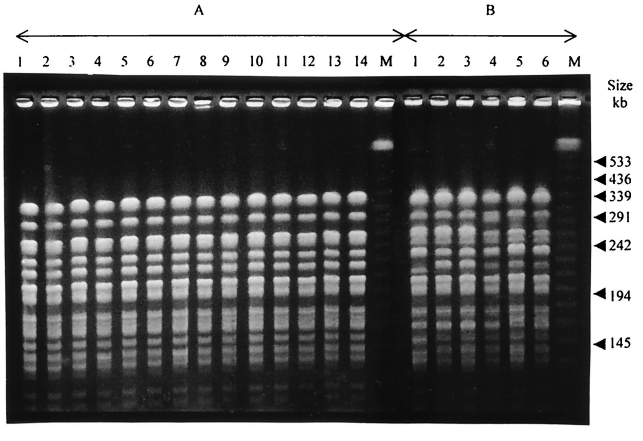

FIG. 2. Representative PFGE fingerprints obtained after digestion with *Xba*I of 14 clinical isolates of *E. aerogenes* belonging to the major clone (A) and six strains of *E. aerogenes* belonging to the clone prevalent in France (B). Lanes M contain molecular size markers (lambda ladder).

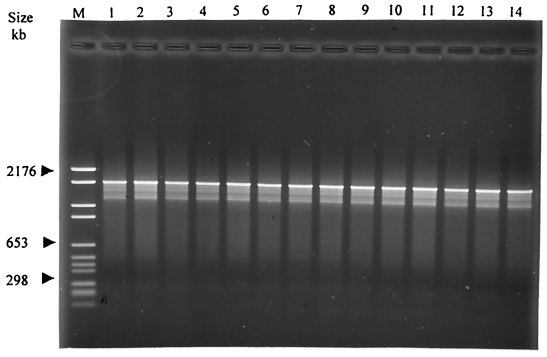

FIG. 3. Representative ERIC-PCR patterns of 14 clinical isolates of *E. aerogenes* belonging to the minor clone (lanes 1 to 14). Lane M contains molecular size markers (marker VI).

accordance with the system of Ambler et al. [3]) and by one silent mutation at position 925 (A→G) (according to Sutcliffe's numbering system [41]). These substitutions are identical to those previously described for TEM-24, except for the cytidine at position 682, which is identical to the $bla_{\text{TEM-2}}$ gene, instead of the silent mutation (C→T) as described previously (10).

The transformant *E. coli* XLA89 expressed a single β-lactamase with a pI of 7.9. The PCR product of the bla_{SHV} gene was detected. Nucleotide sequence analysis showed that it differed from the SHV-1 sequence by three substitutions leading to the amino acid replacements Arg→Leu-205, Gly→Ser-238, and Glu→Lys-240 and by two silent mutations at positions 722 (T→C) and 796 (C→G) (positions are numbered in accordance with the coding sequence of SHV-1 [30]). These mutations are identical to those previously described for SHV-4 (22, 26), except for the silent mutations.

Evolution of ESBL-EA incidence and geographical clusters. During the study period, 6,922 to 9,330 admittances per month were observed and represented 39,234 to 47,485 days of hospitalization per month. The incidence rates of the minor and major clones are presented in Table 1. The ESBL-EA incidence remained constant until August 1998 (Fig. 5). From September 1998 to January 1999, the incidence increased dramatically. This increase was exclusively due to the major clone, whereas the minor clone disappeared. The incidence reached its highest level in January 1999 (0.72/1,000 PD). From February to May 1999, the incidence decreased to the rate which had been observed in May 1998 (0.16/1,000 PD) and then increased again during the summer months.

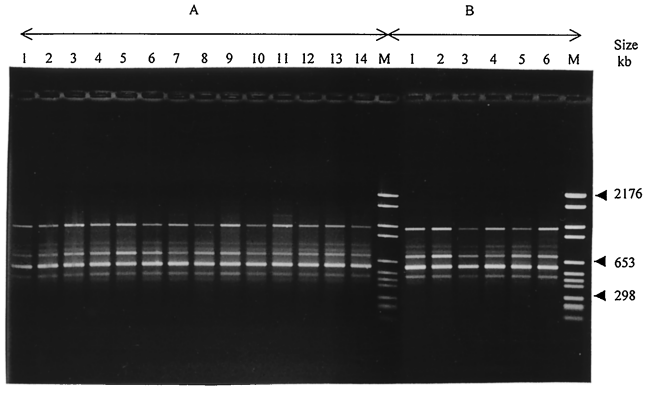

FIG. 4. Representative ERIC-PCR patterns of 14 clinical isolates of *E. aerogenes* belonging to the major clone (A) and six strains of *E. aerogenes* belonging to the clone prevalent in France (B). Lanes M contain molecular size markers (marker VI).

TABLE 1. Numbers of isolates per month and incidence rates of TEM-24- and SHV-4-producing *E. aerogenes* strains over the course of the study period

Mo	No. of *E. aerogenes* isolates			Incidence/1,000 PD	
	Total non-ESBL and ESBL producers	TEM-24 producers	SHV-4 producers	TEM-24	SHV-4
10-96	13	2	3	0.044	0.066
11-96	12	0	0	0	0
12-96	16	1	1	0.023	0.023
01-97	7	0	0	0	0
02-97	20	1	2	0.024	0.047
03-97	18	1	1	0.022	0.022
04-97	19	0	1	0	0.023
05-97	9	1	0	0.023	0
06-97	9	2	1	0.045	0.022
07-97	15	1	2	0.024	0.047
08-97	17	1	0	0.025	0
09-97	15	3	1	0.071	0.024
10-97	14	3	2	0.066	0.044
11-97	21	3	0	0.068	0
12-97	22	2	1	0.046	0.023
01-98	22	2	2	0.045	0.045
02-98	15	1	1	0.024	0.024
03-98	22	2	2	0.043	0.043
04-98	17	4	1	0.089	0.022
05-98	21	6	1	0.133	0.023
06-98	26	4	0	0.090	0
07-98	16	2	1	0.047	0.023
08-98	15	1	1	0.025	0.025
09-98	26	13	0	0.306	0
10-98	16	8	0	0.175	0
11-98	29	16	0	0.361	0
12-98	35	15	0	0.356	0
01-99	50	33	0	0.724	0
02-99	41	18	0	0.420	0
03-99	24	17	0	0.358	0
04-99	26	5	1	0.113	0.023
05-99	25	7	0	0.158	0
06-99	33	12	0	0.272	0
07-99	23	13	2	0.316	0.049
08-99	34	10	0	0.250	0

During all the entire study period, SHV-4-producing strains were recovered individually in different wards with no geographical connection. The geographical distribution of the major clone is described in Fig. 6. From October 1996 to April 1998, there was no evidence of geographical clustering. From May 1998 to August 1998, several strains of the TEM-24-producing clone were recovered in an ICU and in a geriatric ward. Since September 1998 and through the outbreak of the TEM-24-producing clone, geographical clustering between the north site (19 strains) and the south site (53 strains) was evident. Indeed, during this 5-month period, the incidence was significantly higher at the south site (0.78/1,000 versus 0.28/1,000 PD; $P < 0.0001$). Moreover, most of these strains were isolated in two geriatric wards (22 strains; incidence, 5.6/1,000 PD) and one medical ICU (15 strains; incidence, 16.1/1,000 PD).

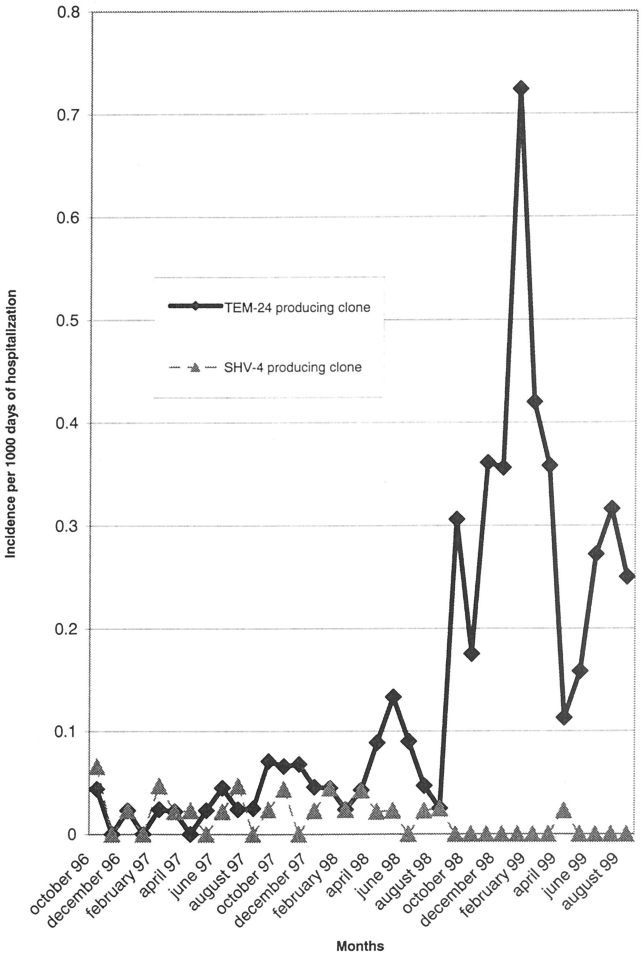

FIG. 5. Monthly evolution of the incidence of ESBL-EA producing strains isolated during the study period per 1,000 PD.

Genotypic comparison of the major epidemic clone from Amiens hospital and the clone prevalent in France. The PFGE and ERIC-PCR patterns of the six *E. aerogenes* strains provided by C. Bollet are presented in Fig. 2B and 4B, respectively. The TEM-24-producing *E. aerogenes* strains isolated at the Amiens hospital had PFGE and ERIC-PCR profiles identical to those of the clone prevalent in France that was previously described by Bosi et al. in 1999 (8).

DISCUSSION

The epidemiological situation concerning ESBL-producing enterobacteria is very dynamic and constitutes a growing worldwide problem (9, 22). The first nosocomial outbreaks caused by ESBL-producing strains occurred in 1985 in France (26, 34). *Klebsiella pneumoniae* was the ESBL-producing enterobacterium most frequently isolated from clinical specimens, but *E. aerogenes* has recently emerged as an important hospital opportunist.

The first ESBL-EA strains were isolated and characterized in 1988 at the teaching hospital of Clermont-Ferrand, France (14). It was found that the ESBL harbored by these strains was a TEM-24 enzyme. Since that time, several outbreaks have been reported. The overview of epidemiological studies suggests two opposite situations in the world: the epidemic situation that occurred in the United States (21, 31, 35) and Belgium (15, 24), characterized by sporadic outbreaks without any linkage and the French situation, characterized by the clonal dissemination of an ESBL-EA strain in nearly all of the hospitals in the country (8). A chronological review of the investigations conducted in France will give better insight into the purpose of this study. At the St-Marguerite hospital in Marseille, France, Davin-Regli et al. (13) conducted a 1-year prospective epidemiological study in 1994 and found a prevalent clone producing an ESBL among 185 clinical isolates. In 1996, Arpin et al. (4) reported an outbreak at the Pellegrin hospital in Bordeaux, France, caused by several clones of ceftazidime-resistant *E. aerogenes* producing a TEM-type or an SHV-type ESBL. In 1996, Neuwirth et al. (32) reported the characterization of 10 clinical isolates of *E. aerogenes* with the same PFGE pattern, collected during 1993 and 1994 at the Bocage hospital in Dijon, France, and producing a TEM-24 ESBL. All of these reports induced Bosi et al. to establish the prevalence of the TEM-24-producing clone in France (8); a representative selection of *E. aerogenes* isolates sent from 23 French hospital laboratories was analyzed. The prevalent *E. aerogenes* clone was isolated in all but two hospitals, confirming the hypothesis that this strain, bearing the large conjugative plasmid with ESBL and aminoglycoside resistance genes, had been transferred from one hospital to the others. The long-term clonal dissemination of TEM-24 ESBL in French hospitals was recently confirmed (19).

In our study, we used two molecular typing methods, PFGE and ERIC-PCR, already successfully used in previous studies (2, 8, 13, 15, 21, 24), to analyze all of the ESBL-EA strains isolated from clinical specimens at the Amiens teaching hospital during a 3-year study. The results provided by the two techniques were concordant for all of the strains, which indicates that ERIC-PCR is not only an easy and rapid method with which to test *E. aerogenes* strains but also a reproducible and discriminatory method.

From October 1996 to August 1998, two clones, producing SHV-4 and TEM-24 ESBL, respectively, were isolated with a low and constant rate of incidence. However, during the last year of the study, from September 1998 to August 1999, the incidence of the TEM-24 clone increased dramatically with the concomitant disappearance of the SHV-4 clone. The study revealed two successive periods: the endemic presence of two clones during the first 2 years and the outbreak of the TEM-24-producing clone during the last year.

This study demonstrates that the strain can be maintained over prolonged periods of time in the hospital environment and can cause clonal outbreaks which lead to the disappearance of other ESBL-EA clones. Moreover, a molecular epidemiological relationship was found between strains isolated in Amiens and strains isolated in other regions of France. These results suggest the clonal spread of the clone prevalent in France, described by Bosi et al. in 1999 (8), within our hospital. Unfortunately, it is impossible to determine when this strain appeared in the hospital because the monitoring of ESBLs started only with the collection of these isolates.

The TEM-24-producing *E. aerogenes* strain had probably been maintained in the environment, causing infections in pre-

Sites	Wards	10/96	11/96	12/96	01/97	02/97	03/97	04/97	05/97	06/97	07/97	08/97	09/97	10/97	11/97	12/97	01/98	02/98	03/98
	M1																		
	M2																		1
North	M3								1					1					
	S1									1									
Site	S2																		
	S3						1												
	S4											1					1	1	
	ICU1													1	1		1		
	M4	1	1											1					
	M5												2						1
South	M6																		
	M7																		
Site	M8																		
	S5					1	1				1				1				
	Ger										1					1	1		
	ICU2															1			

Sites	Wards	04/98	05/98	06/98	07/98	08/98	09/98	10/98	11/98	12/98	01/99	02/99	03/99	04/99	05/99	06/99	07/99
	M1						1										
	M2									1						1	
North	M3	1					3		1	1	1						1
	S1								3	1	1	2					3
Site	S2																2
	S3							1		2						1	
	S4											1			1		
	ICU1						1		1		1			1	1		1
	M4						1		1			2	3	1		1	
	M5									1							
South	M6									1							
	M7						1	1									
Site	M8	1	4						1	2	2	1				1	1
	S5						1		3	1			2	1	1		1
	Ger	1	1	4	2	1	4	4	4	3	7	5	10	3	5	3	2
	ICU2		1						2	4	9	3	3		1	3	

M : medical wards S : surgical wards ICU : Intensive care units Ger : Geriatric wards

FIG. 6. Geographical distribution of the TEM-24-producing clone in the hospital wards during the study period.

disposed patients. The evolution of the incidence and the geographical clustering of TEM-24-producing *E. aerogenes* isolates reveals the appearance of few strains in geriatric wards prior to the outbreak. Geriatric wards, where critically ill patients with low levels of resistance to exogenous colonization are cared for, are thought to be a reservoir for epidemic multidrug-resistant enterobacteria. Indeed, the investigation of Wiener et al. demonstrated the high prevalence of ESBL-producing enterobacteria in nursing homes (43). Patients admitted to geriatric wards require frequent care, which involves numerous interactions with staff members. The investigation of Denman et al. conducted in long-term care facilities in Maryland, where most of the patients were elderly, revealed breaches of handwashing and glove use protocols potentially resulting in microbial transmission (17). This important prevalence of fecal microorganism carriage among elderly patients might be responsible for outbreaks within geriatric wards, as described by Jalaluddin et al. (24) and Rice et al. (38). The spread of epidemic strains from nursing homes to other units, especially surgical units and ICUs, can be suspected. ICUs, where patients have predisposing factors such as foreign devices, compromised immunity, and broad-spectrum antibiotic treatment, are considered to serve as breeding grounds for epidemic multidrug-resistant bacteria leading to outbreaks (2, 4, 12, 13, 15, 21, 23).

The predominance of TEM-24-producing *E. aerogenes* among all of the ESBL-EA isolates found is probably due to virulence determinants such as antibiotic resistance or surface factors involved in epithelial cell surface adherence. A 150-kb plasmid, extracted from *K. pneumoniae*, and encoding an SHV-4 ESBL, has been found to produce a surface protein which facilitates adhesion to intestinal cells (18), but the prevalent TEM-24 clone has not been shown to harbor such an adhesive factor. Moreover, there is no antibiotic resistance difference between the two epidemic ESBL-EA clones isolated at the Amiens hospital, unless the prevalent clone possesses a chromosomally encoded derepressed cephalosporinase (8), unlike the SHV-4 clone.

Since *K. pneumoniae* was the first ESBL-producing enterobacterium identified; many epidemiological studies were dedicated to producing outbreaks caused by ESBL-*K. pneumoniae*. Complete eradication of the smallest outbreaks was achieved, but management of large nosocomial outbreaks, by reinforcement of hygiene measures or restricted use of oxyimino β-lactams, failed to eliminate the epidemic clone (33). In our hospital, we observed the same situation regarding TEM-24-producing *E. aerogenes*. In February 1999, 6 months after the beginning of the outbreak, a program intended to control the diffusion of multiresistant bacteria was implemented. It was based on the barrier precautions defined by the Centers for Disease Control and Prevention (20), particularly hand disinfection (with antiseptic soaps or alcohol solutions), wearing of disposable gloves and gowns when caring for carriers, and carrier identification with a "wash your hands" sign during hospitalization and at the time of patient transfer. Other reports have described the efficacy of such a program in decreasing the incidence of multiresistant bacteria (28) such as that which we observed from February to May 1999 in our hospital. The new increase observed during the summer months can be explained by understaffing of the hospital wards, which decreases compliance with isolation precautions and increases the risk of cross-transmission (36). At the end of the study, in August 1999, the incidence had just been stabilized at 0.25/

1,000 days of hospitalization, which was above the incidence recorded 3 years before in October 1996.

The increasing number of TEM 24-producing *E. aerogenes* outbreaks is a threat to hospital ecology in France. Our study sheds new light on the epidemic behavior of this strain described by Bosi et al. in 1999 (8). Reinforcement of infection control measures, including the use of disposable gloves and the spatial segregation of patients infected or colonized with ESBL-EA, can prevent outbreaks but fails to eliminate the presence of the endemic clone.

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