

Arbitrarily Primed PCR, Ribotyping, and Plasmid Pattern Analysis Applied to Investigation of a Nosocomial Outbreak Due to *Enterobacter cloacae* in a Neonatal Intensive Care Unit

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In December 1992, *Enterobacter cloacae* was isolated from the oropharynx and respiratory tract of six ventilated neonates hospitalized in the intensive care unit (ICU) of our hospital. To establish the spread of the outbreak, 41 strains of *E. cloacae* were analyzed for genotypic markers by three methods: plasmid profile analysis, ribotyping with *EcoRI* or *PvuII* endonuclease, and arbitrarily primed (AP) PCR. The tested strains included 12 isolates from the 6 epidemic cases, 4 isolates from the respiratory tract of 4 children hospitalized in other wards during the same period, 13 isolates from 12 children hospitalized in pediatric units before or after the outbreak, and 12 epidemiologically unrelated isolates. Ribotyping and AP PCR demonstrated that each of the last 12 strains exhibited distinct genomic patterns, as did each of the strains isolated from neonates hospitalized before or after the epidemic peak. Conversely, two clones of strains were found among the isolates recovered in December, with concordant results being obtained by the three typing methods: the first clone included seven strains from five ventilated children in the ICU and two children from another ward; another clone was shared by one neonate in the ICU and an infant from another ward. These results indicate that ribotyping and AP PCR—the latter applied, to our knowledge, for the first time to the genotypic analysis of *E. cloacae*—represent very discriminatory tools for the investigation of nosocomial outbreaks caused by this species.

Enterobacter cloacae is a saprophytic member of the normal digestive flora in humans. In recent years, this species has emerged as an important pathogen in hospitalized patients (5, 8), especially when ampicillin or narrow-spectrum cephalosporins are used prophylactically, because of the intrinsic resistance of this bacterium to these antibiotics. In addition, frequent selection of mutants resistant to extended- and broad-spectrum cephalosporins or to aminoglycosides contributes to an increase in the number of *Enterobacter* infections in units in which these drugs are used extensively for curative purposes (6, 8, 16, 17). Usually, the mechanism of hospital infections caused by *E. cloacae* corresponds to endogenous translocation from the digestive tract (3, 5, 6, 8, 12, 16). The risk of translocation is increased in debilitated patients (1), a fact which explains the high frequency of *E. cloacae* infections in burn units (13, 14), in neutropenic patients (16), and in ventilated neonates (11, 19).

Surprisingly, actual outbreaks caused by *E. cloacae* have so far been reported less frequently than those caused by other members of the family *Enterobacteriaceae* (5, 6, 8, 16). However, when nosocomial epidemics caused by this bacterium occurred, they were subsequent either to the administration of contaminated pharmaceutical products or to patient-to-patient cross-infection (or cross-colonization) via hospital materials and/or the hands of personnel (reviewed in reference 8; 11, 19).

Many typing methods have been proposed for comparing strains of *E. cloacae* to distinguish between endogenous contamination and nosocomial epidemics among simultaneous or successive cases in the same area. Phenotypic markers—

including serological, phage, bacteriocin, and biotyping systems—are poorly discriminatory, except when used in combination; moreover, they cannot be applied to all strains (4, 7). Similarly, unusual antibiotic resistance patterns may contribute to the delineation of a common origin between related isolates (13, 19), but additional typing is needed. In recent years, genotypic methods have been developed to increase the discriminatory power of epidemiological investigations. Plasmid pattern analysis was found useful for the characterization of epidemic strains harboring plasmids (13, 16, 19). More recently, sensitive and reproducible molecular markers, including those used in ribotyping (3, 7, 12), small-fragment restriction endonuclease analysis, and pulsed-field gel electrophoresis (11), have been applied with success to *E. cloacae*.

Arbitrarily primed (AP) PCR (20) belongs to a new generation of typing methods in which a single short primer with arbitrarily chosen nucleotide sequences is used in a PCR to amplify genomic DNA. After separation by electrophoresis, the products of amplification generate an AP PCR type. This method has already been applied to the epidemiological analysis of *Listeria* (15) and *Legionella* (18) spp.

In December 1992, we noted a sudden increase in the isolation of *E. cloacae* strains from ventilated patients hospitalized in the neonatal intensive care unit (ICU) of our hospital. To determine whether these cases were epidemiologically related, we compared strains of *E. cloacae* isolated during, before, and after the outbreak by using three typing methods: plasmid profile analysis, ribotyping, and AP PCR. The results of this epidemiological investigation are reported here.

Epidemiological background. The neonatal ICU consists of nine beds. Most of the patients admitted to this unit need mechanical ventilation. Isolation of *E. cloacae* strains is com-

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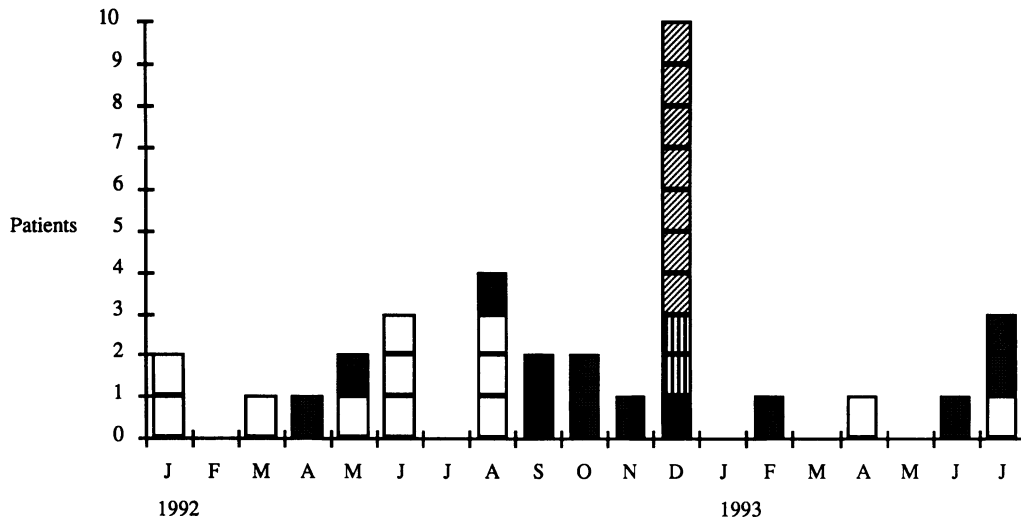


FIG. 1. Diagrammatic representation of pediatric cases of respiratory colonization or infection caused by *E. cloacae* between January 1992 and July 1993 in our hospital. Each square corresponds to a single patient. Except for patients with nontyped strains (\square), patients were the same as those belonging to groups I and II listed in Table 1. Symbols: \blacksquare , typed strains with independent profiles; \square , first epidemic clone; \boxtimes , second epidemic clone.

mon. During the first 11 months of 1992, 15 children were found colonized or infected by this species, corresponding to an overall prevalence of 7% for this period. In December 1992, six newly admitted neonates, all of them being ventilated, were shown to harbor *E. cloacae* in the oropharynx or the respiratory tract. This sudden increase in the incidence of colonization or infection in December (27% of hospitalized neonates) led us to suspect an epidemic. Therefore, an epidemiological inquiry was set up to find the origin of the outbreak.

Although no strain of *E. cloacae* could be isolated either from environmental samples or from the hands of personnel, the outbreak stopped with the reinforcement of hygienic measures, and no further strains of *E. cloacae* were isolated from specimens systematically obtained from neonates hospitalized in the ICU during the first 4 months of 1993. A diagrammatic representation of individual cases of respiratory colonization or infection caused by *E. cloacae* and observed in all the pediatric units in our hospital between January 1992 and July 1993 is shown in Fig. 1.

MATERIALS AND METHODS

Origin of bacterial strains. Forty-one strains of *E. cloacae* from 34 patients were subjected to epidemiological investigations. They included 29 strains isolated from the oropharynx or tracheal aspirates of 22 infants less than 1 year of age (designated by letters A to V representing strains isolated chronologically) and 12 epidemiologically unrelated strains isolated from 12 other patients (designated a to l) in the same hospital.

For convenience, the 34 patients were classified into three groups: (i) group I includes 10 infants colonized or infected by *E. cloacae* in December 1992, during the epidemic peak (6 correspond to the patients in the neonatal ICU mentioned above [patients I, J, L, M, N, and O] and 4 correspond to patients from other pediatric units [neonatal unit, patient K; pediatric ICU, patient R; and external sources, patients P and Q]); (ii) group II includes 12 infants colonized or infected by *E. cloacae* either before or after the epidemic period (9 were

hospitalized in the neonatal ICU [patients A, B, C, E, F, G, T, U, and V], 2 were hospitalized in the pediatric ICU [patients H and S], and 1 was hospitalized in the special baby care unit [patient D]); and (iii) group III includes 12 adults or children in the same hospital and whose strains were presumably epidemiologically unrelated (patients a to l) (isolates were recovered from urine specimens [three patients], tracheal aspirates [two patients], stool specimens [two patients], blood, peritoneal fluid, ear secretions, bronchoalveolar lavage, and whitlow suppuration [one patient each]).

For three patients (E, I, and J), successive isolates obtained during the time of hospitalization (two, three, and five strains, respectively) were included in the epidemiological study.

Identification of strains and antibiotic susceptibility. Strains were identified as *E. cloacae* by use of API 20E galleries (API System, La Balme les Grottes, France). MICs were determined by a commercial microtiter dilution method with Mueller-Hinton broth (Microscan; Baxter) by use of Neg Combo 4I panels.

Plasmid profile analysis. Extrachromosomal DNA was extracted by a rapid alkaline lysis method using protocol and reagents from a commercial kit (Quia-Prep Spin Plasmid Kit, Diagen GmbH, Düsseldorf, Germany). In brief bacterial colonies from an overnight culture grown at 35°C on brain heart agar (Biomerieux) were harvested with 200 μ l of a lysis buffer (50 mM Tris HCl, 10 mM EDTA [pH 8]) containing 100 μ g of RNase for 5 min at 4°C. After the addition of 200 μ l of 200 mM NaOH-1% sodium dodecyl sulfate (SDS) buffer, the mixture was mixed and kept in ice for a 5-min contact. Finally, 200 μ l of potassium acetate was added, and the preparation was mixed thoroughly. Plasmid DNA was recovered by centrifugation and purified on columns included in the kit. Plasmids were separated on an 0.8% agarose (Sigma) gel at 50 V for 18 h. A supercoiled DNA ladder (BRL Life Technologies, Cergy-Pontoise, France), made with 11 plasmids, provided molecular weight standards.

Extraction of total DNA. Bacterial colonies were suspended in 500 μ l of a lysis buffer (50 mM Tris HCl, 50 mM EDTA [pH 8]) containing 100 μ g of lysozyme (Sigma) and 100 μ g of

RNase (Calbiochem, Meudon, France) and incubated for 30 min at 37°C. The samples were then treated with 25 µl of 10% SDS and with 100 µg of proteinase K (Merck), each for 30 min, at 37 and 50°C, respectively. After purification by phenol-chloroform-isoamyl alcohol extractions as previously described (10), DNA was precipitated with glacial ethanol and recovered by centrifugation.

Ribotyping analysis. Ribotyping analysis was performed according to a method previously described (10). In brief, 3 µg of purified DNA from *E. cloacae* strains was digested with either *EcoRI* or *PvuII* according to the specifications of the manufacturer (Boehringer, Mannheim, Meylan, France). DNA fragments were separated on an 0.8% agarose gel at 30 V overnight. Raoul (Appligene, Illkirch, France) was used as a size marker. Restriction fragments were transferred under vacuum to nylon membranes (Nylon N+; Amersham, Les Ulis, France) by Southern blotting.

Plasmid PKK3535, a PBR322 derivative containing the *rrnB* ribosomal operon of *Escherichia coli*, was used as a probe and labelled by random priming with digoxigenin-11-dUTP (DIG DNA Labeling Kit; Boehringer Mannheim). Hybridization was performed at 42°C overnight with 5 × SSC (1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate) buffer containing 50% formamide, 0.1% *N*-lauroylsarcosine, 0.02% SDS, 4% blocking reagent, and 20 ng/ml of the probe just described. The membranes were then treated with antidigoxigenin antibodies conjugated to alkaline phosphatase, and profiles of genes coding for rRNA were revealed after the addition of substrates (nitroblue tetrazolium plus 5-bromo-4-chloro-3-indolyl phosphate) as purple-brown bands.

Each strain was tested at least twice to demonstrate the reproducibility of profiles. Ribotypes were identical, provided that they exhibited similar numbers and sizes of bands.

AP PCR analysis. AP PCR was adapted from a method described by Williams et al. (20). Amplification was performed with 50 µl of a solution containing 10 µl of 50 mM Tris HCl, 50 mM KCl, 7 mM MgCl₂ buffer (ATGC Biotechnologie, Noisy-le-Grand, France), 100 ng of template DNA (prepared as described above), 200 µM each deoxynucleoside triphosphate (Boehringer Mannheim), 400 pmol of primer, and 2.5 IU of *Taq* polymerase (ATGC Biotechnologie).

Each sample was overlaid with 50 µl of mineral oil and subjected to a first cycle of amplification (94°C, 5 min; 35°C, 5 min; 72°C, 5 min) in a Prem III thermal cycler (LEP Scientific Ltd., Flobio, France). The 28 subsequent cycles of amplification consisted of denaturation for 1 min at 94°C, low-stringency annealing for 2 min at 35°C, and extension for 2 min at 72°C, with a final extension step of 10 min at 72°C.

The primer used, HLWL74 (5'-ACGTATCTGC-3'; G+C content, 50%), which had been evaluated on *Listeria* species (15), was purchased from Genset (Paris, France). It was shown to yield informative profiles for *Enterobacter* species in preliminary experiments.

The AP PCR products were separated by electrophoresis and visualized by ethidium bromide staining. A molecular size marker (100-bp ladder; Pharmacia, St. Quentin Yvelines, France) was used for reference. A negative control, consisting of the same reaction mixture but with water instead of template DNA, was included in each reaction.

The identities of AP PCR types were ascertained on the basis of identical numbers and sizes of bands. When only small differences in the intensities of one or two bands were noted, the samples were submitted to another amplification to confirm or refute the similarity between profiles.

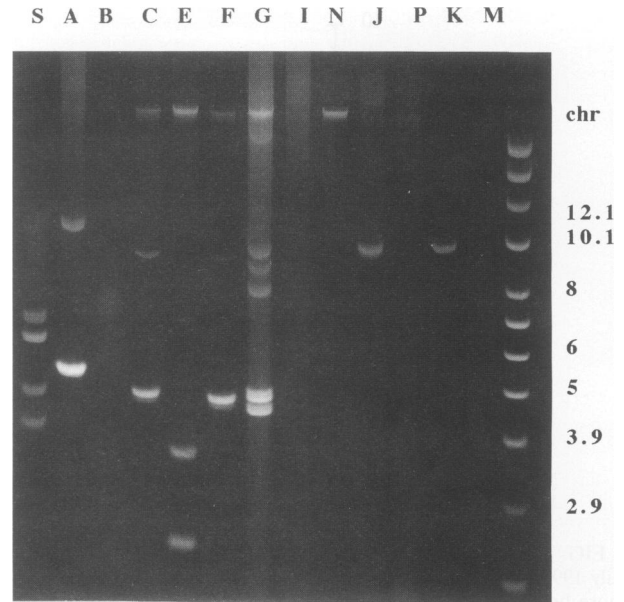


FIG. 2. Representative plasmid profiles of strains of *E. cloacae* isolated from hospitalized infants. Letters refer to patients listed in Table 1. Note the absence of a plasmid for strains from patients B, I, P, and M and the identity between profiles for patients J and K. Last lane, molecular size markers (in kilobases). chr, chromosomal band.

RESULTS

Phenotypic characteristics of strains. The 29 strains of *E. cloacae* from the patients in groups I and II exhibited four different biotypes, one of which was shared by 20 strains. With regard to antibiotic susceptibility, they were all resistant to ampicillin and cefalothin and susceptible to aminoglycosides and quinolones.

Plasmid profile analysis. Twenty-six of the 29 strains from patients in groups I and II were tested by plasmid profile analysis, as were 2 strains from group III. Some representative profiles are illustrated in Fig. 2. Successive isolates from the same patient (E, I, and J) were found identical by this technique (data not shown). Strains from 11 patients (B, D, H, I, L, M, N, O, P, Q, and R) had no plasmid. Strains from two patients (J, [five strains] and K [one strain]) shared the same pattern, with a single plasmid of 9.8 kb. All the other strains tested exhibited distinct profiles of two to five plasmids ranging from 2.2 to 9.6 kb (Fig. 2 and Table 1).

Ribotyping analysis. Except for three strains from group II and four strains from group III, all strains of *E. cloacae* were investigated by ribotyping with two different endonucleases. Both enzymes generated well-separated patterns, easy to interpret because of the small numbers of bands: 8 to 10 from 2.1 to 14 kb for *EcoRI* (Fig. 3) and 10 to 16 from 1.1 to 14 kb for *PvuII* (Fig. 4).

Successive isolates from the same patient were also found identical by this technique, as illustrated by the profiles for isolates from patients I and J after digestion with *EcoRI* (Fig. 3) or *PvuII* (Fig. 4). With both enzymes, isolates from patients J and K and isolates from patients I, L, M, N, O, P, and Q shared the same ribotypes. Each of the other isolates, including those from group III (epidemiologically unrelated isolates), had distinct profiles with the two enzymes (Fig. 3 and 4 and Table 1).

AP PCR analysis. The 41 strains were analyzed by AP PCR.

TABLE 1. Epidemiological data for strains of *E. cloacae* isolated from the oropharynx or respiratory tract of 34 infants less than 1 year of age and hospitalized in December 1992 during the outbreak period (group I) or outside this period (group II)

Patient		<i>E. cloacae</i> strain			Ribotype ^b with:		AP PCR type ^b
Designation (group)	Unit ^a	Date of isolation (day/mo/yr)	Origin	Plasmid(s) (kb)	<i>Eco</i> RI	<i>Pvu</i> II	
A (II)	1	10/04/92	Tracheal aspirate	5.5, 11.0	1	1	1
B (II)	1	13/05/92	Tracheal aspirate	0	2	2	2
C (II)	1	14/08/92	Tracheal aspirate	4.8, 9.5	3	3	3
D (II)	2	07/09/92	Throat swab	0	4	4	4
E (II)	1	09/09/92	Tracheal aspirate	2.5, 3.6	5	5	5
F (II)	1	19/10/92	Throat swab	4.8, 9.6	6	6	6
G (II)	1	02/11/92	Throat swab	4.6, 4.9, 7.8, 8.8, 9.4	7	7	7
H (II)	1	23/11/92	Nasal swab	0	8	8	8
I (I)	1	03/12/92	Throat swab	0	9	9	9
J (I)	1	04/12/92	Throat swab	9.8	10	10	10
K (I)	3	09/12/92	Nasal swab	9.8	10	10	10
L (I)	1	16/12/92	Tracheal aspirate	0	9	9	9
M (I)	1	17/12/92	Tracheal aspirate	0	9	9	9
N (I)	1	18/12/92	Tracheal aspirate	0	9	9	9
O (I)	1	19/12/92	Tracheal aspirate	0	9	9	9
P (I)	4	21/12/92	Tracheal aspirate	0	9	9	9
Q (I)	4	22/12/92	Tracheal aspirate	0	9	9	9
R (I)	5	22/12/92	Tracheal aspirate	0	11	11	11
S (II)	5	08/02/93	Tracheal aspirate	4.4, 5.5, 6.2, 7.0	12	12	12
T (II)	1	26/06/93	Tracheal aspirate	ND ^c	ND	ND	13
U (II)	1	05/07/93	Tracheal aspirate	ND	ND	ND	14
V (II)	1	05/07/93	Tracheal aspirate	ND	ND	ND	15

^a 1, neonatal ICU; 2, special baby care unit; 3, neonatal unit; 4, pediatric ICU; 5, external sources.

^b Numbers refer to different clones of *E. cloacae*. Strains belonging to the same clone are designated by the same number.

^c ND, not determined.

Each strain was tested at least twice to ascertain the reproducibility of the method. As illustrated in Fig. 5A, the patterns obtained with one strain isolated from patient I, amplified in four independent experiments, and run on the same gel in four consecutive lanes were similar, except for a fainter intensity of the band at 0.5 to 0.6 kb in lane 3. No false-positive results occurred, and water controls were always negative.

Figure 5 shows some representative AP PCR types. As with

the other techniques, successive isolates from the same patient (E [two strains] and J [five strains] in Fig. 5A and I [2 strains] in Fig. 5B) had similar AP PCR profiles. Isolates from patients J and K exhibited the same AP PCR type; a unique pattern was also shared by isolates from patients I, L, M, N, O, P, and Q. All the other strains tested were found different by AP PCR analysis, with patterns of 9 to 12 bands ranging from 0.1 to 5 kb (Fig. 5 and Table 1).

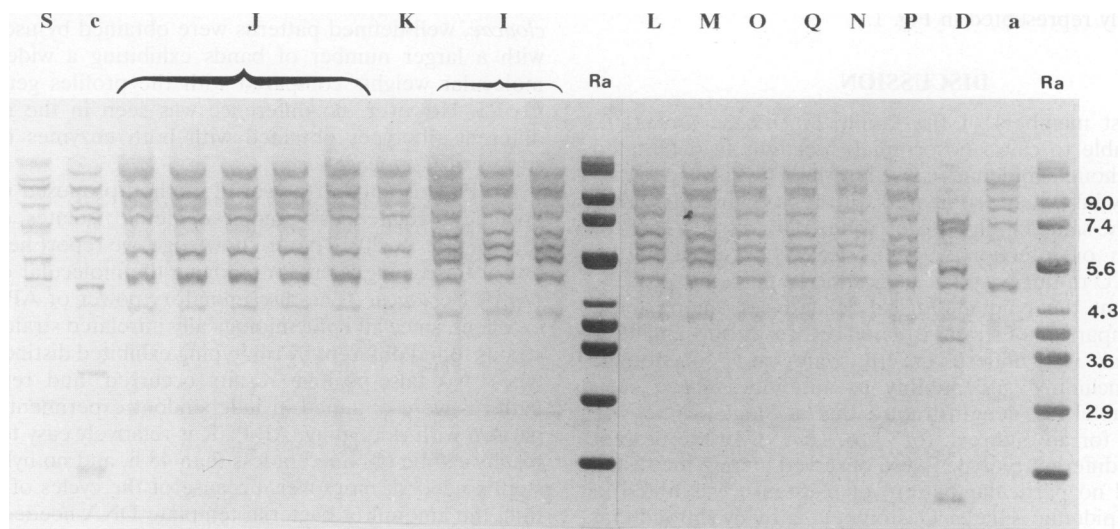


FIG. 3. Representative ribotypes generated by *Eco*RI for strains of *E. cloacae*. Uppercase letters refer to patients listed in Table 1. Lowercase letters refer to patients with epidemiologically unrelated isolates. Lanes grouped under the same letter correspond to successive isolates from the same patient. Lanes Ra, size markers (in kilobases).

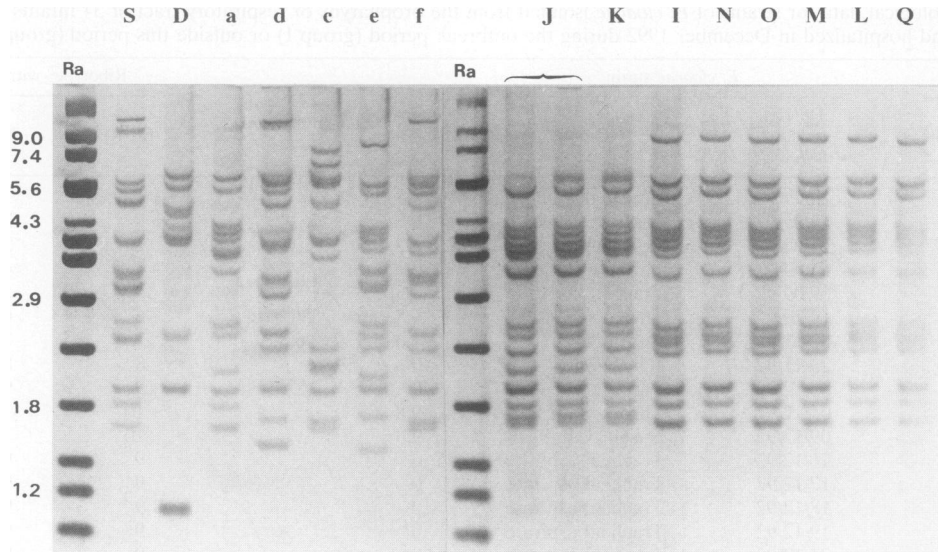


FIG. 4. Representative ribotypes generated by *PvuII* for strains of *E. cloacae*. See the legend to Fig. 3 for details.

Comprehensive results of the epidemiological study. Table 1 summarizes the results obtained for strains isolated from patients in groups I and II. All strains isolated from patients in group II, during the months before or after the epidemic peak, were found epidemiologically unrelated by the three typing methods. Conversely, two epidemic clones of *E. cloacae* circulated in group I patients hospitalized in December 1992 in pediatric units, according to the concordant results of the three typing methods. (i) Strains from patient J hospitalized in the neonatal ICU and patient K from another pediatric ward shared the same plasmid profile and similar ribotypes and AP PCR types. (ii) Strains from patients I, L, M, N, and O hospitalized in the neonatal ICU and patients P and Q from external sources harbored no plasmids and were found indistinguishable by ribotyping and AP PCR. The 10th patient from group I (R) was shown to be colonized by a different strain (Table 1). The overall results of the epidemiological study are schematically represented in Fig. 1.

DISCUSSION

Like most members of the family *Enterobacteriaceae*, *E. cloacae* is able to cause nosocomial infections in debilitated patients, although epidemics caused by this organism appear to be relatively infrequent compared with those caused by other species of the same family. In December 1992, six new cases of colonization or infection with *E. cloacae* occurred in the neonatal ICU of our hospital, suggesting an epidemic process. To document the epidemiological relatedness among these cases, a comparison of strains isolated before, during, and after the outbreak was undertaken. In some cases, phenotypic markers—including susceptibility to antibiotics—have been found suitable for demonstrating that a single clone was responsible for an outbreak (6, 7, 16, 17, 19). In the present study, four different biotypes were observed among the tested strains, and no particular pattern of resistance was noted in presumed epidemic isolates. Gaston et al. already showed that 66.6% of 790 strains of *E. cloacae* shared the same biotype (9).

When bacterial isolates are phenotypically similar, one or more genetic assays should be performed to increase the chance of finding any differences. Plasmid profile analysis has

been the most widely used molecular method for comparing isolates of *E. cloacae* (13, 16, 19). In our study, most of the epidemiologically unrelated isolates were found different by this technique. However, it could not be used for isolates without a plasmid. Instability of profiles caused by the acquisition or loss of plasmids represents another disadvantage of the method, as illustrated by Haertl and Bandlow for isolates whose plasmid profiles differed for a single band but were shown identical by two other discriminatory typing systems (11).

Ribotyping has been found very useful for discriminating among strains of *E. cloacae*, even with the use of a single restriction endonuclease, because of the natural heterogeneity of this species (3, 7). In addition to *EcoRI*, already used in the two studies just mentioned (3, 7), we tested *PvuII* in ribotyping experiments. This enzyme has been shown to provide a high discriminatory power for other bacterial species (2). For *E. cloacae*, well-defined patterns were obtained by use of *PvuII*, with a larger number of bands exhibiting a wide range of molecular weights compared with the profiles generated by *EcoRI*. However, no difference was seen in the number of different ribotypes obtained with both enzymes for the 34 strains of *E. cloacae*.

AP PCR is another powerful method proposed recently to document relatedness among epidemic isolates of various species (15, 18, 20). To our knowledge, we report here the first use of this typing assay for studying the molecular epidemiology of *E. cloacae*. The discriminatory power of AP PCR was excellent, since all epidemiologically unrelated strains and also strains found different by ribotyping exhibited distinct AP PCR types. No false-positive results occurred, and reproducible patterns were obtained in independent experiments. In comparison with ribotyping, AP PCR is relatively easy to perform: results can be obtained in less than 48 h, and no hybridization step is needed; moreover, because of the cycles of amplification, the amount of bacterial template DNA needed is greatly reduced.

From an epidemiological point of view, a glance at Fig. 1 would have been sufficient to suspect the occurrence of an outbreak in December 1992 in the neonatal ICU. However, the

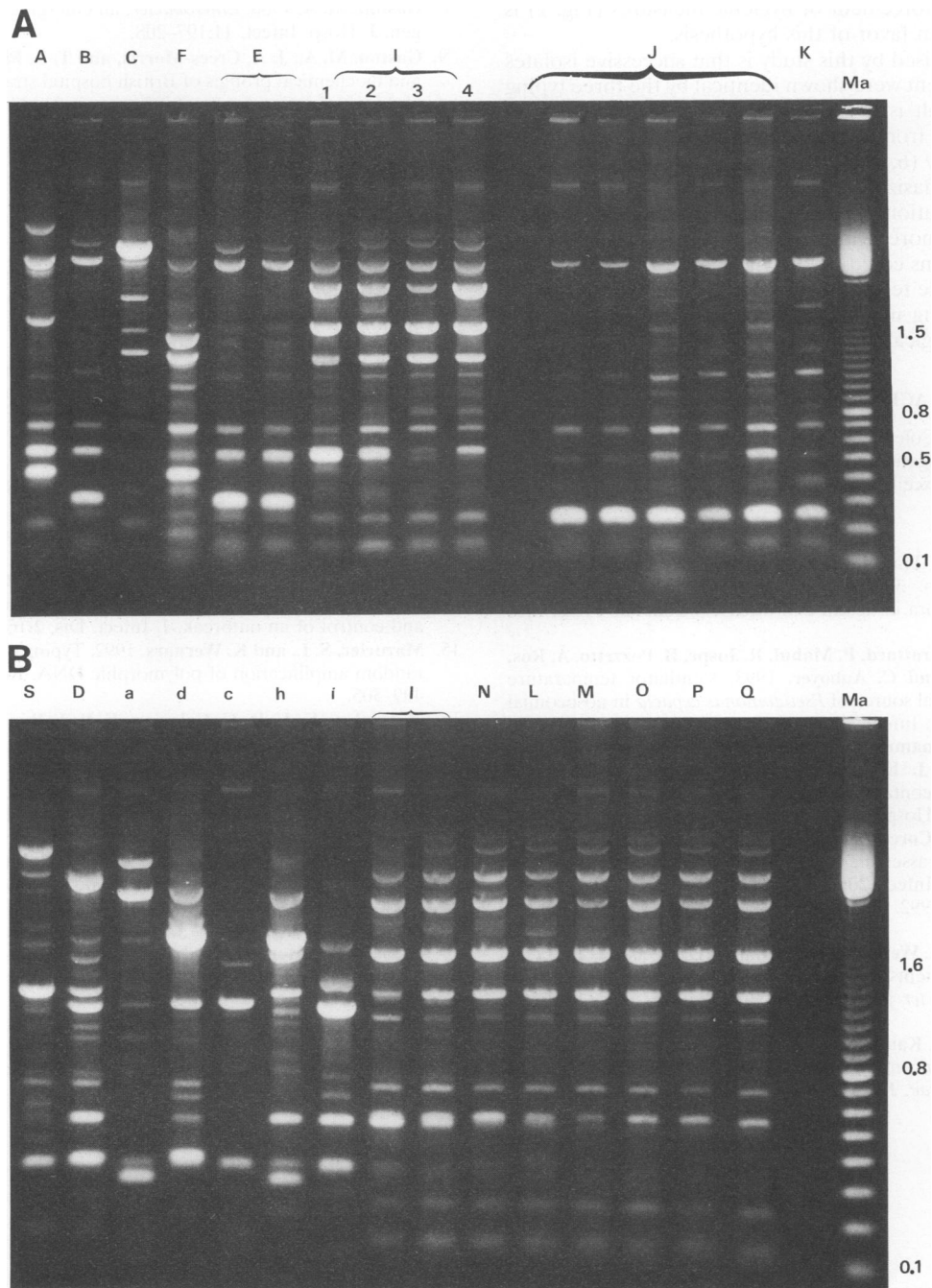


FIG. 5. Representative AP PCR types for strains of *E. cloacae*. (A and B) Independent ethidium bromide-stained gels. Uppercase letters refer to patients listed in Table 1. Lowercase letters refer to patients with epidemiologically unrelated isolates. Lanes grouped under the same letter correspond to successive isolates from the same patient (E and J in panel A and I in panel B) or to the same isolate amplified in four independent experiments (I in panel A). Unmarked lanes correspond to negative controls. Lanes Ma, size markers (in kilobases).

detection of patients colonized with *E. cloacae* during the months before the outbreak could have suggested that an epidemic strain was present in this unit some time ago. These observations led us to use a number of molecular epidemiological methods for the analysis of *E. cloacae*, all of which, ultimately, supported the same conclusions: the strains isolated before December 1992 were different, whereas two clones were responsible for 9 of the 10 cases of colonization or infection in December 1992. The first clone contaminated five infants in

the neonatal ICU and also two nonhospitalized patients who were seen regularly for chronic respiratory diseases. The other clone was shared by two infants, one from the neonatal ICU and the other from another pediatric ward. Although no strain of *E. cloacae* could be isolated from the environment or from the hands of personnel, patient-to-patient transmission is thought to have been favored by a failure in hygienic surveillance because of a lack of personnel and increased work during this period. The sudden decrease in *E. cloacae*-positive cul-

tures after the reinforcement of hygienic measures (Fig. 1) is another argument in favor of this hypothesis.

Another point raised by this study is that successive isolates from the same patient were shown identical by the three typing methods. This result is in agreement with previous observations for patients from whom *Enterobacter* spp. could be isolated persistently (6, 12).

Our results emphasize the value of molecular typing methods for the investigation of nosocomial outbreaks caused by *E. cloacae*. Although more strains would need to be tested before definitive conclusions could be drawn, AP PCR appears to be a simple and reliable technique which could be substituted for more classical typing methods for comparing *E. cloacae* isolates in epidemiological studies.

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