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Epidemiological Study by Pulsed-Field Gel Electrophoresis of an Outbreak of Extended-Spectrum β-Lactamase-Producing *Klebsiella pneumoniae* in a Geriatric Hospital

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Twelve cases of infections caused by extended-spectrum beta-lactamase (ESBla)-producing *Klebsiella pneumoniae* were reported between August 1991 and March 1993 in the Geriatric Department of the Nîmes University Hospital, where these bacteria had not been previously isolated. Restriction profiles of total genomic DNAs cleaved by *XbaI* and *SpeI* were compared by pulsed-field gel electrophoresis. The strains that were tested included the 12 isolates from *K. pneumoniae*-infected patients, strains recovered from rectal swabs of asymptomatic patients in the same ward, and strains isolated in other hospitals in Nîmes at the same time. The restriction profiles of the 12 isolates and those recovered from asymptomatic patients in the same ward were very similar. Over a period of more than 1 year, extended-spectrum beta-lactamases were not detected in *K. pneumoniae* isolates with restriction patterns different from that of the epidemic strain. It seems, therefore, that there was no transfer of a plasmid or a gene coding for ESBla to strains of *K. pneumoniae* that were different from the epidemic strain. At the same time, ESBla-producing *K. pneumoniae* isolates exhibiting restriction endonuclease profiles very different from that of the epidemic strain were isolated from other hospitals in Nîmes. None of these strains caused an outbreak. Pulsed-field gel electrophoresis, which allows precise characterization of strains beyond the species level, is a useful tool for studying the ESBla-producing *K. pneumoniae* strains involved in nosocomial outbreaks.

Members of the family *Enterobacteriaceae* that produce plasmid-mediated beta-lactamases with a high level of activity against expanded-spectrum cephalosporins have primarily been described in intensive care units (11). Widespread dissemination of such strains within hospitals is reported with increasing frequency (9, 16). Hospital colonization by extended-spectrum beta-lactamase (ESBla)-producing bacteria is usually a complex phenomenon involving many different mechanisms: dissemination of several epidemic strains and dissemination of plasmids and resistance genes (3, 4, 6, 7, 10, 13, 17, 18). We report the hospital-wide dissemination of ESBla-producing *Klebsiella pneumoniae* strains in the Geriatric Department of Nîmes University Hospital. The Geriatric Department is housed in a five-floor building that is geographically distant from the central hospital. ESBla-producing *K. pneumoniae* strains were isolated for the first time in this department in August 1991. Because the first six strains were detected in the same ward, the initial aim of the study was to determine whether it was a true epidemic as a result of the spread of a single strain, originating from a unique source, in order to eradicate a potentially dangerous nosocomial pathogen. We needed a method which allowed us to identify strains beyond the species level. We chose analysis of genomic DNA cut by low-frequency-cleavage restriction endonucleases by using pulsed-field gel electrophoresis (PFGE), a method shown to be a good epidemiological tool for several bacterial species, including members of the family *Enterobacteriaceae* (1, 8). Eight strains isolated from patients in other departments (surgery and intensive care units) during the same period were studied by the same method.

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MATERIALS AND METHODS

Bacterial strains. Twelve ESBla-producing *K. pneumoniae* strains were isolated from clinical specimens in the Geriatric Department from August 1991 to March 1993. Two strains were recovered from stools of asymptomatic contact patients in May and June 1992, respectively. During the same period, four strains were recovered from patients in other departments that were geographically distant from the Geriatric Department. These strains are described in Table 1. Three non-ESBla-producing *K. pneumoniae* strains isolated from the Geriatric Department during the same period were studied (strain 25, isolated in March 1992 from a Cliniplot mattress in ward A1; strains 26 and 27, isolated from infected patients in February 1992 in wards A3 and A2, respectively).

Biotyping. Bioprofiles were determined by using the API 20E system (API-BioMerieux) according to the manufacturer's instructions.

Antibiogram. The susceptibilities of the isolates to the following antibiotics were determined by the disk diffusion method on Mueller-Hinton agar: ampicillin (10 μg), mezlocillin (30 μg), cefazolin (30 μg), cefotaxime (30 μg), ceftazidime (30 μg), imipenem (10 μg), gentamicin (10 μg), tobramycin (10 μg), amikacin (10 μg), co-trimoxazole (25 μg), ofloxacin (5 μg), ciprofloxacin (5 μg), fosfomycin (50 μg), and amoxicillin plus clavulanic acid (20/10 μg).

Chromosomal analysis by PFGE. Genomic DNA was prepared in low-melting-point and gelling agarose (Seaplaque FMC; TEBU) plugs. Bacteria that were freshly grown in

TABLE 1. Extended-spectrum beta-lactamase-producing *K. pneumoniae* strains[a]

Strain no.	Date of isolation (day.mo.yr)	Ward	Source of isolate	pI of beta-lactamases
1	30.08.91	SCA1	Urine	6.3, 7.7
2	12.11.91	SCA1	Urine	ND
	03.12.91	SCA2	Urine	ND
3	26.11.91	SCA1	Urine	5.6, 6.3, 7.7
4	03.12.91	SCA1	Urine	ND
5	26.12.91	SCA1	Urine	ND
6	20.01.92	SCA1	Wound	5.6, 6.3, 7.7
7	27.01.92	SCA3	Urine	ND
8	28.01.92	SCA5	Urine	5.6, 6.3, 7.7
9	24.02.92	SCA1	Catheter	5.6, 6.3, 7.7
10	24.03.92	SCA3	Urine	ND
11	14.05.92	SCA3	Urine	ND
	11.06.92	SCA5	Urine	ND
12	09.10.92	SCA3	Urine	ND
13	15.05.92	SCA5	Stool	5.6, 6.3, 7.7
14	16.06.92	SCA5	Stool	5.6, 6.3, 7.7
15	03.10.91	MS2	Urine	ND
16	23.01.92	Sur-C	Urine	5.6, 6.3, 7.7
17	28.06.91	ICU-C	Urine	ND
18	25.10.91	ICU-C	Bile	5.4, 6.3, 7.7
19	07.10.92	ICU-C	Sputum	ND
20	04.05.92	Uro-C	Urine	ND
21	18.06.92	Uro-C	Urine	5.6, 6.3, 7.7
22	02.09.92	Uro-C	Urine	ND
23	20.08.92	GDR	Urine	5.4, 6.3, 7.7
24	1991	ICUM	Urine	ND

[a] Abbreviations: SC, Geriatric Department (Serre Cavalier; A1, first floor; A2, second floor; A3, third floor; A5, fifth floor); ICU-C, surgical intensive care unit, Nîmes Central Hospital; Sur-C, surgery, Nîmes Central Hospital; Uro-C, Urology Department, Nîmes Central Hospital; MS2, Chronic Care Center, Nîmes Central Hospital; GDR, Grau-du-Roi Chronic Care Center; ICUM, neurosurgery intensive care unit, Montpellier Hospital; ND, not determined.

Mueller-Hinton agar were suspended in TE buffer (10 mM Tris [pH 8], 0.1 mM EDTA) to an optical density at 650 nm of 1.250 (1×10^9 to 5×10^9 bacteria per ml). The bacterial suspensions were mixed with an equal volume of 1% agar in TE buffer. The mixture was incubated with a mixture of 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 laboratory temperature. DNA was then digested with a suitable enzyme. Three enzymes were tested. *Ssp*I cut the DNA into fragments of less than 30 kb. *Spe*I and *Xba*I gave a convenient number of fragments. DNA plugs were incubated for 5 h at 37°C with 40 U of *Xba*I (Biolabs) or 12 U of *Spe*I (Biolabs) in the manufacturer's recommended buffer. DNA fragments were separated in a 1% agarose gel (Appligene) prepared and run in 0.5× Tris-borate-EDTA (TBE) buffer on a contour-clamped homogeneous field machine (CHEF-DR2; Bio-Rad). The pulse ranges were 50 to 10 s for 35 h (Fig. 3), 40 to 5 s for 30 h (Fig. 1), and 40 to 5 s for 35 h (Fig. 2) at 180 V. The gels were then stained with ethidium bromide and photographed.

Preparation of beta-lactamase extracts and isoelectric focusing. Crude sonic extracts were prepared from the strains grown in Trypticase-soy broth (BD-Merieux) supplemented with yeast extract (5 g/liter) and glucose (10 g/liter) without inducer. Isoelectric focusing was performed on polyacrylamide gels (7% acrylamide; Kodak) containing ampholines, with a pH range of 3.5 to 10. Electrofocusing was carried out as follows: 500 V overnight with an LKB 2117 Multiphor instrument. The beta-lactamase activity was located on the gels by an iodine starch procedure. Beta-lactamases with known pIs (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) were focused in parallel with the extracts.

Epidemiological investigations. A questionnaire regarding preexisting pathology, urinary and venous catheterization, diarrhea, and previous antibiotic treatments was completed for each infected patient. No significant risk factor was identified. Environmental samples were collected in the rooms of the infected patients of ward A1: soil, sinks, beds, mattresses, and nebulizers. In ward A5, stool specimens of asymptomatic contact patients were cultured in May, June, and July 1992.

RESULTS

All the strains of ESBla-producing *K. pneumoniae* isolated from the Geriatric Department from August 1991 to March 1993 were studied. All of these strains presented indistinguishable biotype profiles. These profiles were not different from those of commonly isolated strains of *K. pneumoniae* in Nîmes University Hospital. The outbreak strains were characterized by multiresistant antibiogram profiles (resistance to cefazolin, cefotaxime, ceftazidime, tobramycin, fosfomycin, co-trimoxazole, and quinolones). Isoelectric focusing (Table 1) showed that all isolates produced three beta-lactamases with pIs of 5.6, 6.3, and 7.7, which are consistent with TEM-2, TEM-3 or CAZ-7, and SHV-1, respectively. The first strain, however, was an exception since it lacked the enzyme with a pI of 5.6 (TEM-2). The MICs of ceftazidime (64 mg/liter) and cefotaxime (8 mg/liter) are more consistent with TEM-3 than CAZ-7. Environmental samples (soil, nebulizers, mattresses, and beds) failed to reveal ESBla-producing *K. pneumoniae*.

Restriction endonuclease DNA profiles were determined by PFGE of the strains from infected and contact patients in the Geriatric Department and infected patients in the other wards of the Nîmes University Hospital. Restriction analysis by PFGE of genomic DNA from the 12 strains from patients in the Geriatric Department yielded very similar restriction patterns (Fig. 1 and 2). The patterns were indistinguishable by *Spe*I digestion. Four slightly different patterns were found by *Xba*I digestion. The pattern of the first strain (which lacked the TEM-2 beta-lactamase) differed from the patterns of the other strains by the absence of one band (strain 1; Fig. 1, lane B). The pattern of strain 7 differed by a decrease in the size of one band (Fig. 1, lane F). The pattern of strain 12 presented an additional 110-kb fragment and lacked two small fragments (Fig. 2, lane E). Strains isolated from stool specimens of asymptomatic contact patients exhibited banding patterns identical to those of their contacts (strains 13 [Fig. 2, lanes G and H] and 14 were identical to strain 11). Restriction profiles of ESBla-negative *Klebsiella* strains isolated from infected patients (strains 26 and 27) and a Cliniplot mattress (strain 25) in the Geriatric Department during the same period were completely different (Fig. 3, lanes E, F, and G). Of the ESBla-producing *K. pneumoniae* strains which were sporadically isolated in the other wards until March 1993, all but one (strain 16; Fig. 1, lane I) gave very different banding patterns that were easily distinguishable from those from the Geriatric Department

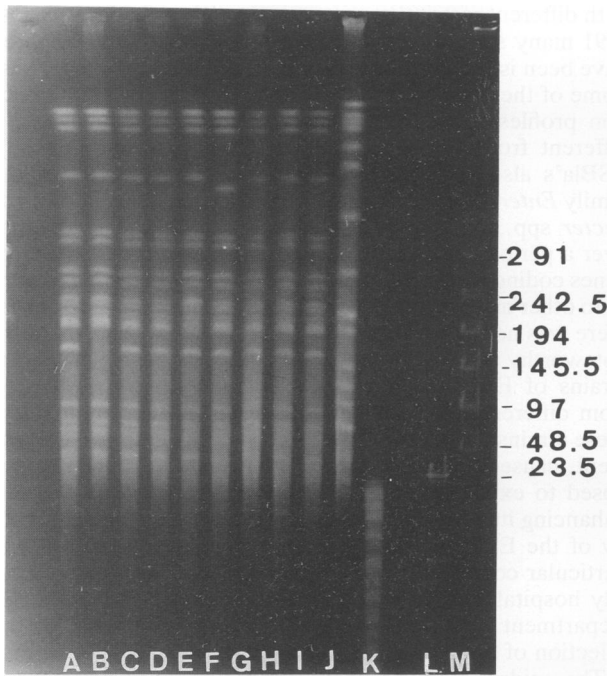

FIG. 1. DNAs of extended-spectrum beta-lactamase-producing *K. pneumoniae* from Nîmes cut by *Xba*I. Lanes A to J, DNA cut by *Xba*I; lanes A to H, strains from the Geriatric Department. Lanes: A, strain 6; B, strain 1; C, strain 9; D, strain 8; E, strain 2; F, strain 7; G, strain 3; H, strain 5; I, strain 16 (surgery); J, 18 (intensive care unit); K, 3 cut by *Ssp*I; L, lambda phage DNA cut by *Hin*dIII; M, lambda concatemer. The size of the ladder (in kilobases) is indicated on the far right.

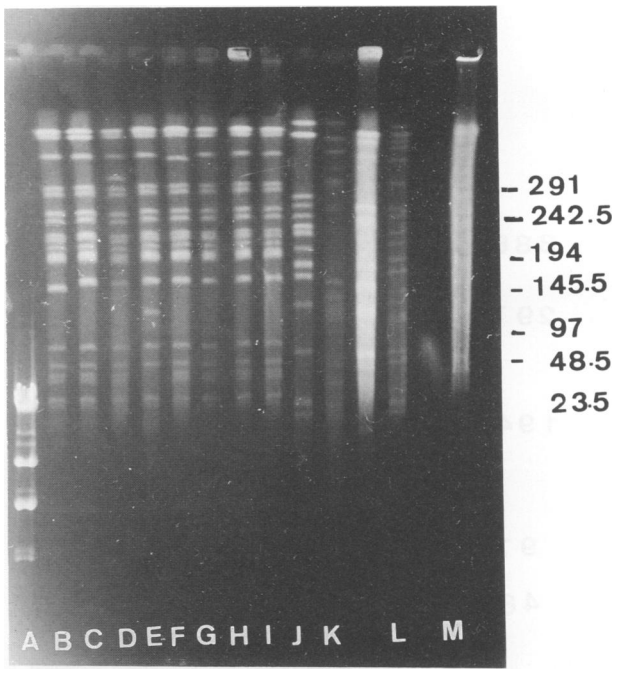

FIG. 2. DNAs of extended-spectrum beta-lactamases-producing *K. pneumoniae* cut by *Xba*I. Lanes: A, lambda phage cut by *Hin*dIII; B to D, strains from the Urology Department (B, strain 21; C, strain 22; D, strain 20); E to I, strains from the Geriatric Department (E, strain 12; F, strain 7; G, strain 11; H, strain 13, rectal swab; I, strain 2); J, strain 19 (intensive care unit, Nîmes Hospital); strain 24 (intensive care unit, Montpellier Hospital); L, strain 23 (chronic care facility); M, lambda concatemer. The size of the ladders (in kilobases) is indicated on the far right.

(strains 15 and 17 [Fig. 3, lanes C and D], strain 18 [Fig. 1, lane J], strains 19 and 23 [Fig. 2, lanes J and L]). The beta-lactamase contents of three of these strains (strains 16, 18, and 23) were determined by isoelectric focusing. The three strains produced beta-lactamases with pIs of 5.4 and 6.3. Two strains lacked the enzyme with a pI of 5.6. However, one strain, recovered from a patient in the Central Nîmes Hospital with no known relation to patients in the Geriatric Department of Nîmes University Hospital exhibited the same restriction pattern as the strains from the Geriatric Department (strain 16; Fig. 1). By isoelectric focusing, this strain was shown to express the same three beta-lactamases (pIs of 5.6, 6.3, and 7.7). Moreover, in June 1992, ward A1 of the Geriatric Department, where the first cases of *K. pneumoniae* infection had been detected, moved from the Nîmes University Hospital to the Nîmes Central Hospital. No new cases of ESBla-producing *K. pneumoniae* infection were detected in the new hospital, but three cases of infection by these bacteria occurred in the Urology Department, which was in the same building. Restriction patterns of the strains from the Urology Department were very similar to those of the strains from the Geriatric Department (both departments were at Nîmes Central Hospital), differing by only one or two bands (strains 20, 21, and 22; Fig. 2, lanes B to D). The beta-lactamase expressed by one of these strains was determined by isoelectric focusing. It was identical to those expressed by the strains from the Geriatric Department (pIs of 5.6, 6.3, and 7.7).

The DNAs of strains isolated from the intensive care unit of Montpellier Hospital were submitted to PFGE. All of the strains had the same profile (data not shown). Restriction profiles were different from those of the strains from the Geriatric Department (strain 24; Fig. 3, lane H).

DISCUSSION

PFGE has recently been shown to be useful for the study of genomic relatedness among non-ESBla-producing *K. pneumoniae* strains (12). Results of the present study confirm that it is also an excellent tool for typing ESBla-producing *K. pneumoniae* strains. A high degree of DNA polymorphism is observed among nonepidemiologically related strains of ESBla-producing *K. pneumoniae* isolated from different hospitals and from different departments of the Nîmes University Hospital, even when these strains express ESBla's with the same pI (6.3). We can therefore conclude that restriction fragment length polymorphism determination is a valuable epidemiological tool for investigating outbreaks of nosocomial infections caused by these bacteria. The purpose of the present study was to determine whether the infections caused by ESBla-producing *K. pneumoniae* strains in the Geriatric Department were caused by a single strain coming from a unique source and to determine, if possible, the mode of transmission. PFGE indicates that there is a strong relationship between the strains, with there being only three almost identical restriction patterns, and suggests a clonal origin of the strains involved in the outbreak. However, the present study raises several questions. First, because strains exhibiting patterns identical or very similar to those of the strains from the Geriatric Department

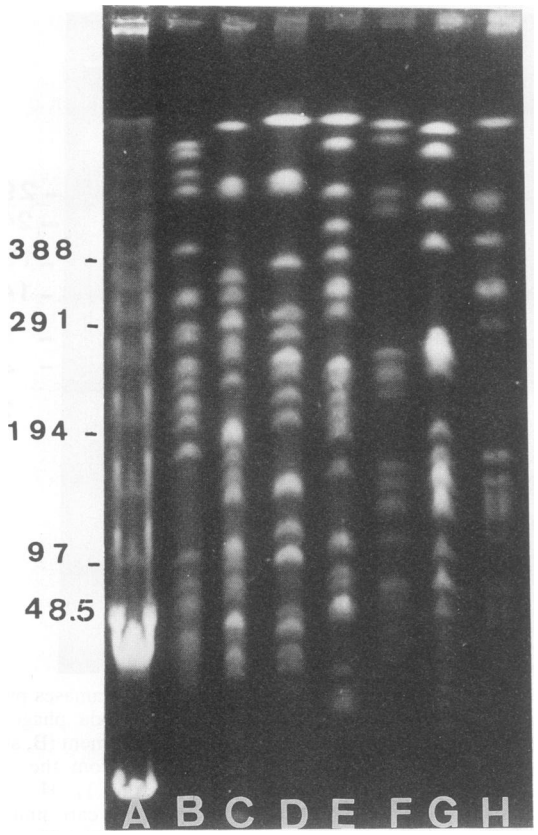

FIG. 3. DNAs of *K. pneumoniae* from Nîmes and Montpellier hospitals cut by *Xba*I. Lanes: A, lambda phage DNA cut by *Hin*dIII plus lambda concatemer; B to D, extended-spectrum beta-lactamase-producing strains from the Nîmes hospitals; B, strain 10 (SCA3); C, strain 15 (MS2); D, strain 17 (intensive care unit); E to G, non-ESBla-producing strains; E, strain 26 (SCA); F, strain 27 (SCA); G, strain 25 (mattress from SCA1); H, ESBla-producing strain from the intensive care unit, Montpellier Hospital (strain 24). The size of the ladder (in kilobases) is indicated on the left.

were isolated from patients from the Central Nîmes Hospital with no known relationship to patients in the Geriatric Department at Nîmes University Hospital, we must consider the possibility of the transport of bacterial strains between geographically distant wards of Nîmes University Hospital by unidentified animate or inanimate vectors, even by the geriatric patients themselves, because they are sometimes transported to other parts of the hospital for medical procedures such as X rays or physical therapy. We cannot discard the hypothesis, however, that there is a strain of ESBla-producing *K. pneumoniae* endemic in the population of Nîmes, France, that causes multiple sporadic cases or small outbreaks, and when a patient, already colonized at home, is admitted to a hospital ward, the selective pressure of antibiotics allows the bacteria to cause overt infections. That would explain the slight differences in the restriction profiles of the strains from the Urology and Geriatric Departments. A second interesting point in the study of the colonization of the Geriatric Department by ESBla-producing *K. pneumoniae* is the observation that all of the strains isolated up to now from infected or asymptomatic patients presented highly related restriction patterns. In the Geriatric Department, ESBla was never detected in *K. pneumoniae* isolates with different restriction patterns, even though since August 1991 many strains of non-ESBla-producing *K. pneumoniae* have been isolated from clinical and environmental samples. Some of these strains were analyzed by PFGE. The restriction profiles of these susceptible strains were completely different from those of ESBla-producing strains (Fig. 3). ESBla's also were not detected in other members of the family *Enterobacteriaceae* such as *Escherichia coli*, *Enterobacter* spp., or *Citrobacter* spp. It seems, therefore, that over a period of more than 1 year, there was no transfer of genes coding for ESBla to different strains of *K. pneumoniae* or to other members of the family *Enterobacteriaceae* or that there was no maintenance of ESBla in these strains. A third noteworthy point is the fact that, since August 1991, several strains of ESBla-producing *K. pneumoniae* were isolated from different wards of the Nîmes hospitals. Among all of these strains, only one, the strain from the Geriatric Department, caused an outbreak. Several hypotheses can be proposed to explain this fact: a character of the strain itself, enhancing its potential colonizing ability; a particular stability of the ESBla-encoding plasmid; or more probably, the particular conditions of the geriatric patients, who are usually hospitalized for long periods of time in the Geriatrics Department and receive many antibiotics, enhancing the selection of multiresistant bacteria.

The epidemiology of ESBla-producing *K. pneumoniae* is complex. Genetic determinants encoding the varied extended-spectrum beta-lactamases behave in very different ways. In some epidemics, strains harboring the extended-spectrum beta-lactamase genes are quite similar, suggesting, such as in the case of the outbreak in the Geriatric Department described here, a clonal origin of the strains and the stability of the genes encoding the resistance inside these strains. For instance, SHV-4-producing *K. pneumoniae* isolates involved in infections in five Paris hospitals belonged to the same serotype and had the same characteristic biotype, the same lysotype, and the same plasmid profile (3, 5). In other outbreaks, the spread of resistance is linked to self-transferable plasmids or to the transposition of resistance genes among different plasmids by transposable elements (16, 18). For instance, in contrast to what is observed in the Nîmes outbreak, the genetic elements encoding TEM-3 and CAZ-7 are usually reported to transfer easily from strain to strain and even from species to species (2, 7, 13, 14, 15). Bingen et al. (4) reported on an outbreak in which a surprisingly high number of genetically unrelated strains were involved. They opposed their findings to those of previous studies suggesting, on the basis of plasmid analysis, that a single strain was responsible for an outbreak. There is, in fact, no contradiction between these findings. The two epidemic modes are possible. However, for the investigation of phenomena as complex as outbreaks of ESBla-producing bacteria, plasmid analysis is certainly not sufficient to affirm the clonal origin of an outbreak and requires resolution by a method that allows for a more precise characterization of strains, that is, beyond the species level. PFGE is able to provide such confirmation.

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