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Use of Low-Frequency-Cleavage Restriction Endonucleases for DNA Analysis in Epidemiological Investigations of Nosocomial Bacterial Infections

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Epidemiological investigations of bacterial infections are generally based on multiple phenotypic markers that are often difficult to verify. A more general and reliable method is genomic DNA analysis by restriction endonucleases. However, the commonly used endonucleases produce too many fragments for correct separation by agarose electrophoresis. In contrast, simple electrophoretic patterns are obtained after genomic DNA digestion by low-frequency-cleavage restriction endonucleases and pulsed-field gel electrophoresis, making it easier to compare numerous strains from the same species. This technique was used to investigate an *Acinetobacter calcoaceticus* outbreak in a urologic department and bronchial colonization of artificially ventilated patients by *Pseudomonas aeruginosa* in an intensive care unit. The method allowed a clear distinction between epidemic and self-contaminating strains in these different epidemiological situations.

Until recently, infection epidemiology has essentially been based on the study of phenotypic traits such as serotype; biotype; bacteriophage sensitivity or antimicrobial susceptibility; and production of bacteriocins, toxins, or enzymes, etc. These techniques are often very useful, but they involve several difficulties. (i) They require a wide range of methods, varying from one microorganism to another, which are difficult to perform by a single laboratory. (ii) They are not easy to apply to fastidious organisms or bacteria with poorly expressed metabolism (i.e., practically nonfermenting). (iii) Since they measure phenotypic expression, they may vary with culture conditions or subculturing. For instance, in the case of *Pseudomonas aeruginosa*, it has been shown that the pyocin type, antibiogram, biotype, bacteriophage sensitivity, or even the O serotype may change in the course of an apparent outbreak and sometimes in vitro (15).

To overcome these difficulties, molecular biological methods have been tested in bacterial infection epidemiology. The first example was a study of plasmid content by gel electrophoresis (10, 14). Subsequently, DNA analysis by restriction endonucleases was first applied to viruses and then extended to both bacterial plasmids (18) and genomic DNA. Using the last technique, many authors have reported interesting epidemiological results for human infections by microorganisms that are difficult to analyze by other methods, such as *Leptospira interrogans*, *Chlamydia psittaci*, *Neisseria* spp., etc. (3, 11, 13). Endonuclease analysis has the great advantage of being easily applicable, since it uses the same material, to nearly all cultivable microorganisms. However, since the number of fragments generated by endonuclease digestion increases with genome length, bacterial DNA fingerprints show more than 100 bands, which are not well separated by classical gel electrophoresis. Consequently, comparison of numerous strains is often difficult. To improve the method, several authors transferred the gel onto a nitrocellulose membrane and hybridized it with an isotopically labeled probe derived from a cloned toxin gene (15) or rRNA (21); the location of the probe on the electrophoretic pattern differs from strain to strain. Recently, large DNA molecules were separated by Schwartz and Cantor by using pulsed-field gel electrophoresis (19), making it possible to carry out DNA analysis with low-frequency-cleavage restriction endonucleases, such as *Not*I, *Sfi*I, *Xba*I, etc. (12). These produce fewer fragments, usually less than 40, and electrophoretic patterns can be compared more precisely and easily.

We first applied this improved technique to analyzing strains of the genus *Brucella*, a particularly homogeneous genus. We were able to distinguish strains in a given biovar that are otherwise identical (1). A related technique using field inversion gel electrophoresis has also been applied to genome fingerprinting of *P. aeruginosa* strains isolated in cystic fibrosis (5). We report here on an epidemiological investigation of two different nosocomial infections, i.e., the frequent colonization by *P. aeruginosa* of respiratory tracts of artificially ventilated patients and an outbreak of *Acinetobacter calcoaceticus* infections in a urologic department. In both cases, the use of low-frequency-cleavage restriction endonucleases coupled with pulsed-field gel electrophoresis assured easy and reproducible comparison of numerous strains.

MATERIALS AND METHODS

Bacterial strains. *A. calcoaceticus* strains were isolated from one hemoculture and from several urine specimens from patients admitted to a urologic department for surgical treatment of a variety of nephropathic diseases. Biotyping and lysotyping were performed by the Centre de Référence des Acinétobacters (E. Bergogne-Berezin, C.H.U. Xavier Bichat, Paris, France). They were compared with *Acinetobacter* strains isolated during the same period in other departments of the same hospital.

P. aeruginosa strains were isolated in an intensive care unit from sinks and from patients admitted for respiratory diseases and undergoing artificial ventilation. Bronchial aspirations were obtained on admission and once a week

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thereafter. Sink isolates were obtained at the beginning of the study, except in the case of room 9, for which cultures from the patient and sink were prepared at the same time. *P. aeruginosa* strains were isolated on cetrimide agar (Pasteur Production) and identified by conventional biochemical characters and pyocyanin production. O serotyping was performed with commercial antisera (Pasteur Production). Biotyping was done as previously described (7). Antimicrobial susceptibility was tested by the Kirby-Bauer disk method (2).

Chromosomal DNA isolation. Native DNA was prepared directly in a solid plug or insert as described by Schwartz et al. (20). The following modifications were made. Low-temperature-gelling agarose (1%; Appligène) in phosphate-buffered saline was prepared as described by Jackson and Cook (6). Equal volumes of agarose and a bacterial suspension at 10^9/ml were mixed in phosphate-buffered saline and dispensed in a slot former. Inserts were then incubated with a mixture of 0.5 M EDTA, 1% (wt/vol) sodium dodecyl sulfate, and 1 mg of pronase (Calbiochem) per ml for 48 h at 37°C. Protein digestion products were removed by washing the inserts twice for 1 h at 37°C in 10 mM Tris–0.1 mM EDTA (pH 7.5)–1 mM phenylmethylsulfonyl fluoride and then three times in Tris-EDTA alone for 1 h at room temperature.

Restriction enzyme digestion and electrophoresis. One insert of DNA was incubated overnight with 20 U of *Xba*I or *Sma*I (New England BioLabs, Inc.) in a total volume of 120 μl, following the recommendations of the manufacturer. Fragments of DNA generated by endonucleases were separated by pulsed-field gel electrophoresis, originally developed by Schwartz and Cantor (19), in a 1% agarose gel prepared and run in 0.0445 M Tris (pH 8)–0.0445 M boric acid–0.001 M EDTA on a locally constructed apparatus based on the principle of the contour-clamped homogeneous field machine. The pulse times were 5 s for 24 h at 200 V and then 10 s at 200 V for another 24 h. Molecular weight markers used were polymerized bacteriophage lambda DNA ladders produced by the following method. Lambda DNA (18 μg) (Appligène) was ligated with 24 U of T4 DNA ligase (New England BioLabs) for 48 h at 12°C. Polymerized DNA (1.4 μg) was loaded onto the gel after a 15-min incubation at 65°C. At the end of the run, gels were immersed for 45 min in running buffer containing 0.5 mg of ethidium bromide per ml and photographed under UV light with a Polaroid CU camera.

RESULTS

Choice of suitable restriction endonucleases for *P. aeruginosa* and *A. calcoaceticus* DNA digestion. The frequency of restriction endonuclease sites along a DNA molecule depends on two factors: the particular nucleotide sequence recognized and the percent G+C content of the DNA (12).

The percent G+C content of *A. calcoaceticus* DNA is variable, ranging from 38 to 47 (8). *Not*I (GCGGCCGC), *Sfi*I ($GGCCN_5GGCC$), *Sma*I (CCCGGG), and *Sac*II (CCGCGG) recognize GC-rich nucleotide sequences, which are 8 nucleotides long with the first two endonucleases. The best results were obtained with *Sma*I or *Xma*I, which are isoschizomers.

In the case of *P. aeruginosa* DNA, with a G+C content of 67.2% (15), endonucleases such as *Xba*I (TCTAGA), *Spe*I (ACTAGT), *Dra*I (TTTAAA), or *Ssp*I (AATATT) can give rise to fewer than 50 fragments. They recognize 6-nucleotide sites containing, for *Xba* and *Spe*I, a tetranucleotide (CTAG) which is rare in bacterial genomes (12), and containing only A and T nucleotides for *Dra*I and *Ssp*I. After several trials, *Xba*I was chosen because it gave good results and was far less expensive than *Spe*I.

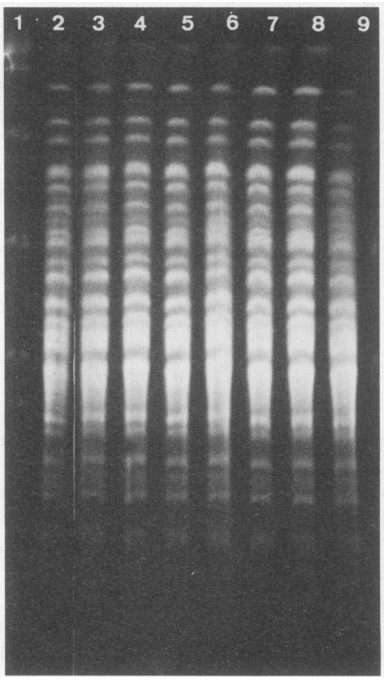

FIG. 1. *Sma*I digestion of DNA from *A. calcoaceticus* strains isolated in a urologic department. Lanes: 2, blood culture isolate; 3 to 9, urine isolates from seven patients; 1, lambda concatemers (sizes from top to bottom, 250, 200, 150, 100, and 50 kilobases).

Typing of *A. calcoaceticus* strains by *Sma*I digestion. Two series of *A. calcoaceticus* strains were tested. One corresponded to isolates obtained during 3 weeks from one blood culture or from urine cultures of patients admitted to a urologic department. The abnormally high frequency of isolation during this period and the similarities of the first antibiograms, showing only susceptibility to ticarcillin, amikacin, and imipenem, evoked the possibility of an outbreak. The second group of strains corresponded to occasional isolates from different departments of the hospital. No strains were isolated from specimens taken from the floor, wall, sink, or air in the urologic department. The urologic department isolates were typed by the Centre de Référence des Acinétobacters. The eight strains showed the same number 2 biotype and the same number 97 lysotype, but three of them differed with respect to their antibiotypes.

Figure 1 shows the electrophoretic patterns obtained after DNA digestion by *Sma*I corresponding to the possible outbreak strains. About 30 bands were observed, and all the isolates showed the same pattern. In contrast, the DNA fingerprints of the occasional isolates were all different and were also distinct from the former patterns (Fig. 2). The isolates of the first group appeared to represent a single strain or clone.

Typing of *P. aeruginosa* strains by *Xba*I digestion. *P. aeruginosa* was isolated from all the sinks of the intensive care unit and from a number of patients. Sink-derived strains were designated by the number of the room, from R-1 to R-9. Isolates from patients were identified by the number of the room in which they were harbored (P-1, P-2, etc.). If several patients subsequently occupied the same room, the strains were designated, for instance, P-2a, P-2b, etc.

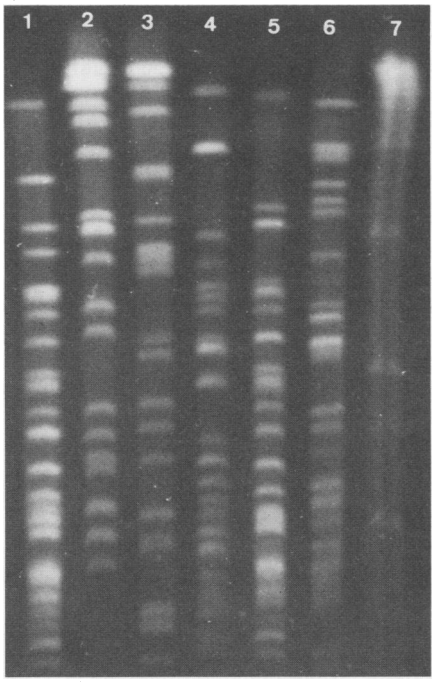

FIG. 2. *Sma*I digestion of DNA from *A. calcoaceticus* strains isolated from different departments of the same hospital. Lanes: 1, blood culture isolate (as in Fig. 1); 2 to 6, five patient isolates from five different departments; 7, lambda concatemers (sizes from top to bottom, 250, 200, 150, 100, and 50 kilobases).

The respiratory tracts of the artificially ventilated patients were found to be colonized by *P. aeruginosa* 4 to 23 days after admission (average, 14 days). No *P. aeruginosa* was isolated from respiratory equipment before use.

Typing by conventional methods was carried out. Three of the five sink-derived strains were not agglutinable by typing antisera. Most of the isolates had the same biotype, but it sometimes changed in subcultures. The same variable results were observed with antimicrobial testing, mostly in mucoid strains.

Electrophoretic patterns after DNA digestion by *Xba*I are shown in Fig. 3 and 4. The results can be summarized as follows.

(i) All strains isolated from sinks differed from one another.

(ii) Patient isolates differed from one another, even when they were subsequently harbored in the same room or used the same respiratory equipment.

(iii) Strains isolated from sinks differed from strains isolated from patients of the same room, except in the case of room 9, but this patient was infected on admission and the sink isolate was obtained during his stay.

(iv) The DNA fingerprints of patient isolate P-2c and sink isolate R-3 appeared to be identical, but electrophoresis of the former strain showed two more bands.

(v) The strains isolated from the sink of room 6 and from a patient in room 8 (R-6 and P-8b) were identical.

It can be concluded that direct transmission from patient to patient or via the respiratory equipment was quite uncommon.

DISCUSSION

Restriction endonuclease analysis of genomic DNA isolated from bacteria is now widely used. Unlike classic

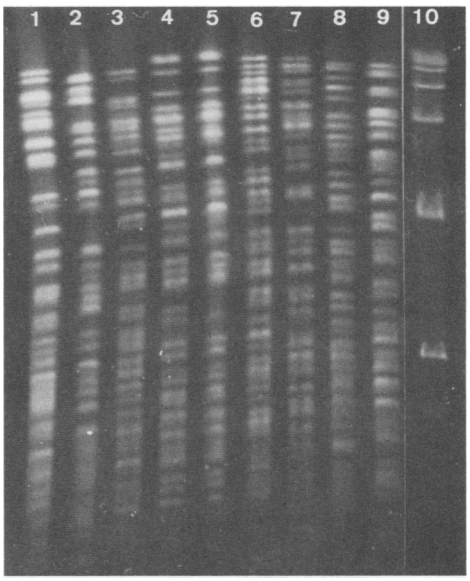

FIG. 3. *Xba*I digestion of DNA from *P. aeruginosa* strains from different patients and their rooms. Lanes: 1, strain R-2; 2, strain P-2a; 3, strain P-2b; 4, strain P-2c; 5, strain R-3; 6, strain P-3a; 7, strain P-3b; 8, strain R-5; 9, strain P-5; 10, lambda concatemers (sizes from top to bottom, 300, 250, 200, 150, 100, and 50 kilobases).

epidemiological marker investigations, the same method can be applied to all cultivable microorganisms. The technique involves restriction endonuclease digestion of the genomic DNA and separation of the generated fragments by gel electrophoresis. The reproducibility is unquestionable, since the number and locations of the restriction sites depend only on the genomic nucleotide sequence. Using the improved technique described in this paper, we were able to differentiate *Brucella* species and biovars, showing specific locations

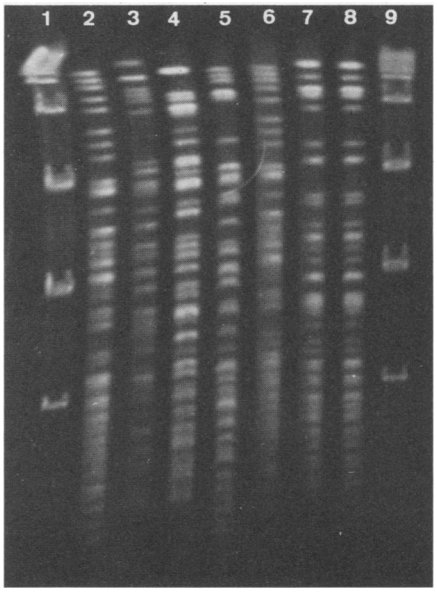

FIG. 4. *Xba*I digestion of DNA from *P. aeruginosa* strains from different patients and their rooms. Lanes: 2, strain R-6; 3, strain P-6; 4, strain R-8; 5, strain P-8a; 6, strain P-8b; 7, strain R-9; 8, strain P-9; 1 and 9, lambda concatemers (sizes from top to bottom, 300, 250, 200, 150, 100, and 50 kilobases).

of *Xba*I sites. It is highly probable that mutations or inversions are responsible for the changes in restriction site locations from strain to strain (manuscript in preparation). Thus, the electrophoretic pattern is a reflection of chromosome organization. Indeed, DNA fingerprints of *Brucella* strains subcultured for 2 years in our laboratory were remarkably stable during this period (data not shown).

The sensitivity for differentiation of distinct strains is more difficult to evaluate. Renaud et al. (17) were unable to clearly analyze *Staphylococcus epidermidis* nosocomial outbreaks by restriction endonuclease DNA digestion with *Eco*RI and recommended the use of conventional markers along with this method. However, the extremely large number of fragments generated by this enzyme produced complex electrophoretic patterns that are difficult to interpret. Hybridization with an isotopically labeled probe (15, 21) improves the sensitivity of the method but requires a different probe for each microorganism tested and includes one more step. The technique described in the present paper may be widely applied with minor modifications (choice of the suitable restriction endonuclease), and the small number of bands allows a precise comparison of electrophoretic patterns. On the other hand, the use of low-frequency-cleavage endonucleases decreases the number of sites investigated and consequently may reduce the ability to demonstrate small differences between strains. Theoretically, high-frequency-cleavage enzymes increase the number of these sites, but the electrophoretic patterns are so complex that this advantage is lost. For instance, *P. aeruginosa* P-2c and R-3, which differed by only two bands after *Xba*I digestion, were strictly identical when tested by *Hin*dIII (data not shown) or by phenotypic-typing systems. Using the same method, we were able to distinguish some strains in a *B. melitensis* biovar shown to be identical by all other typing systems (1).

It should also be mentioned that the method described here is simpler than the previously used technique. Since the DNA must be obtained in an absolutely native state, all DNA purification and digestion has to be done on bacteria embedded in agarose gel to avoid shearing forces. However, this apparent complication is actually a simplification, since DNA purification can be obtained without phenol or chloroform treatment. For gram-negative rods and cocci, treatment with proteases, such as pronase or proteinase K, in the presence of sodium dodecyl sulfate or *N*-lauroyl sarcosine is sufficient, the degradation products being eliminated by successive washes. To investigate a group G streptococcus outbreak, pretreatment by mutanolysin was required before the protease step (N. Bouziges et al., submitted for publication). Large fragments can be separated by pulsed-field gel electrophoresis (19) using one of three systems: field inversion gel electrophoresis, orthogonal field alternation gel electrophoresis, and contour-clamped homogeneous fields. Contour-clamped homogeneous field electrophoresis gave better results than field inversion gel electrophoresis, which was used by Grothues et al. (5). In the last method the bands on the gel were thick and fuzzy, so that it was often difficult to distinguish two very close bands. In contrast, orthogonal field alternation gel electrophoresis gave clear patterns, but the different lanes were so divergent that comparison of many patterns was difficult.

In the present work, results were obtained by the contour-clamped homogeneous field method in two different epidemiological situations.

In the first study, *A. calcoaceticus* strains isolated from postoperative infections in a urologic department were typed by *Sma*I DNA digestion. *Acinetobacter* species are aerobic gram-negative coccobacilli showing poor metabolic expression by conventional methods, leading to difficulties in typing based on antimicrobial susceptibility and bacteriophage sensitivity tests and biotyping. The eight strains isolated from the urologic department had the same biotype and lysotype, but three of them differed with respect to their antibiotypes. However, these three strains are not necessarily different, because the possibility of an acquired resistance during the outbreak cannot be excluded, considering that the patients were under antimicrobial treatment. The same discrepancy between antibiotic susceptibility patterns and molecular markers has also been observed in an epidemiological study of *Escherichia coli* isolated from bacteriuric women (9). Consequently, the striking homogeneity of the DNA fingerprints after *Sma*I analysis of these strains, compared with the heterogeneity shown by occasional isolates from different departments of the same hospital, suggest the occurrence of a true *A. calcoaceticus* infection outbreak resulting from cross contamination between the patients or from an environmental source which has not yet been discovered. This hypothesis is consistent with the temporary character of *A. calcoaceticus* isolation in patients of this urologic department.

The second study involved a comparison of *P. aeruginosa* strains isolated from patients and sinks in an intensive care unit. All the patient strains had different DNA fingerprints, indicating that there was no cross contamination between patients via medical care or respiratory therapy equipment. Like other authors, we found that serovar O11 was the most frequently isolated, but none of the four strains showed the same DNA fingerprint. It is clear that this most frequently used method in epidemiological studies gives questionable results. Infection of a patient from an environmental source was possible in one case (strains R-6 and P-8b) but remains debatable, since contamination of a sink by a patient was demonstrated in room 9. The most probable explanation for *P. aeruginosa* nosocomial lung infections in artificially ventilated patients is that bronchial invasion starts from resident bacteria of the oropharynx (4). Our results showing that patient isolates differed from one another seem to confirm this hypothesis. Nevertheless, considering the wide variety of environmental strains and the limited way in which the isolates were collected, contamination of a patient by an undetected strain arising from this environment cannot be excluded. Additional studies are in progress to test the hypothesis of an intrinsic origin of bronchial contamination by *P. aeruginosa* in mechanically ventilated patients.

On the basis of our results, corresponding to two different situations (i. e., a true outbreak and a probable self-contamination) it is clear that low-frequency-cleavage restriction endonuclease analysis of genomic DNA is a versatile and precise method for epidemiological investigation.

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