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Comparison of Ribotyping and Restriction Enzyme Analysis Using Pulsed-Field Gel Electrophoresis for Distinguishing *Legionella pneumophila* Isolates Obtained during a Nosocomial Outbreak

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Because of the ubiquity of *Legionella* isolates in aquatic habitats, epidemiologic evaluation of *Legionella pneumophila* strains is important in the investigation and subsequent control of nosocomial outbreaks of legionellosis. In this study, ribotyping and restriction enzyme analysis by pulsed-field gel electrophoresis (PFGE) were used to compare isolates of *L. pneumophila* obtained from patients and the environment during a nosocomial outbreak with unrelated control strains. Restriction enzyme analysis by PFGE resolved 14 different patterns among the *L. pneumophila* serogroup 1 and *L. pneumophila* serogroup 6 isolates involved in the study. Two of the patterns were observed in the three *L. pneumophila* serogroup 6 isolates from patients with confirmed nosocomial infections and environmental isolates from the potable water supply, which was, therefore, believed to be the source of the patients' infections. Three more patterns that were not present in isolates from patients with legionellosis were seen in isolates from the hospital environment, demonstrating the presence of multiple strains in the hospital environment. In the outbreak, one distinct pattern occurred among the *L. pneumophila* serogroup 1 isolates from patients with nosocomial infections, suggesting a common source; however, the source could not be determined. By comparison, ribotyping generated five patterns. However, some control strains of both *L. pneumophila* serogroups 1 and 6 possessed the same ribotypes as were present in the outbreak isolates. Both techniques were used successfully to subtype the isolates obtained during the investigation of the outbreak. Furthermore, restriction enzyme analysis by PFGE was useful for subdividing ribotypes and for distinguishing strains involved in the outbreak from epidemiologically unrelated strains.

Legionella pneumophila has been recognized as an important cause of nosocomial pneumonia, particularly among patients with impaired host defenses. Potable water systems (4, 11, 14, 26), cooling towers (7, 8), and respiratory devices (3) are among the sources implicated in outbreaks of Legionnaires' disease.

The ubiquity of *Legionella* spp. in natural and artificial aquatic environments has complicated the establishment of an epidemiologic link between isolates from the environment and those from patients. Recently, several techniques, including monoclonal antibody typing (17), plasmid analysis (20), restriction enzyme analysis (33), and chromosomal probe fingerprinting (31), have been used to fingerprint *L. pneumophila* isolates. In order to increase the ability to discriminate between strains of *L. pneumophila* in investigations of outbreaks, numerous researchers have combined two or more techniques, such as restriction enzyme and alloenzyme analyses (30); monoclonal antibody subtyping and restriction fragment length polymorphism analysis (25); analysis of whole-cell DNA, genes coding for rRNA, and plasmids (32); monoclonal antibody subtyping and plasmid analysis (16, 18, 22); and monoclonal antibody subtyping, plasmid analysis, and outer membrane profiling (28).

Various subtyping and marker systems have been applied to *L. pneumophila* serogroup 1 because this serogroup is responsible for the majority of legionellosis cases. However, *L. pneumophila* serogroup 6, which is second in prevalence to *L. pneumophila* serogroup 1 (23), is also a frequent cause of legionellosis and, likewise, requires subtyping systems that can discriminate between isolates of the two serogroups. Many of the current subtyping systems cannot reliably differentiate between organisms of the same serogroup.

During the summer and fall of 1989, an outbreak of nosocomial pneumonia caused by *L. pneumophila* serogroup 1 and *L. pneumophila* serogroup 6 occurred in the renal transplant unit of an upstate New York hospital. Because genetic polymorphism among various bacteria has been successfully detected by using pulsed-field gel electrophoresis (PFGE) to separate large chromosomal endonuclease fragments (1, 2, 19, 21), a study was initiated to determine whether this technique could be applied to differentiate *L. pneumophila* isolates from this outbreak. In addition, ribotyping of *L. pneumophila* isolates from the patient and environmental sources implicated in this outbreak was performed. The results indicate that both techniques were useful for subtyping strains isolated during the investigation of the legionellosis outbreak. Furthermore, restriction enzyme analysis by PFGE was useful for subdividing ribotypes and further distinguishing strains involved in the outbreak from epidemiologically unrelated strains.

MATERIALS AND METHODS

Epidemiologic investigation. A confirmed case of Legionnaires' disease was defined as a patient who had radiographic evidence of pneumonia in addition to either isolation of *L. pneumophila* from respiratory secretions or a greater than or equal to fourfold rise of serum antibodies to *L.*

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pneumophila, as measured by indirect immunofluorescence, to a titer of >1:128 (34). In order to be classified as a nosocomial case, a patient had to have been hospitalized for at least 48 h before the onset of respiratory symptoms or to have been readmitted to the hospital within 10 days of a previous discharge. The charts of patients with culture-confirmed legionellosis were reviewed and the patients were interviewed, if possible. Also, the chart of the only patient diagnosed with legionellosis in 1988 was reviewed.

To identify additional cases of nosocomial legionellosis, the charts of patients admitted between 1 May and 28 July 1989 were reviewed if any of the following criteria were met: the patient had a discharge diagnosis of pneumonia; the patient had received intravenous erythromycin; *Legionella* testing had been done; or the patient had a renal transplant, chronic obstructive pulmonary disease, or AIDS or was receiving treatment for a malignancy.

Bacterial strains and growth conditions. Bronchial washings or sputa from patients suspected of having Legionnaires' disease were plated onto buffered charcoal-yeast extract agar containing 0.1% alpha-ketoglutarate (BCYEα) and BCYEα containing cephalothin, colistin, vancomycin, and cycloheximide (CCVC) (BBL, Cockeysville, Md.) (5, 9) with and without an acid wash (5). Environmental samples were concentrated or diluted when needed and then plated onto BCYEα, BMPAα, and MWY media (9, 10) with an acid wash, if needed (5). Plates were incubated at 37°C and were examined for 10 days. Six isolates (LB308, LB309, LB310, LB312, LB412, LB469) were recovered from respiratory secretions of patients with confirmed cases of nosocomial legionellosis. Environmental isolates were obtained from 7 of 22 environmental sources tested during the initial investigation and follow-up sampling in August 1989. One to 10 colonies, which were designated by a numeral following the specimen number (e.g., LB290-1), were randomly selected from each environmental source. The following tests were used for identification: L-cysteine requirement; autofluorescence; catalase, oxidase, and gelatin hydrolysis; and the presence of β-lactamase (12). Serogroup determination was by direct fluorescent-antibody tests (SciMedX, Denville, N.J.). Control cultures for *L. pneumophila* serogroup 1 were Philadelphia 1 (ATCC 33152 [American Type Culture Collection Rockville, Md.]), Knoxville 1 (ATCC 33153), LB311 (from a patient with a community-acquired case of legionellosis who was treated in the same hospital where the outbreak occurred), LB246 (from a patient with legionellosis involved in an outbreak at a different geographic location), and LB394 (an environmental isolate that was epidemiologically unrelated to the outbreak isolates). Control cultures for *L. pneumophila* serogroup 6 included Chicago 2 (ATCC 33215), as well as LB104 and LB635, which were environmental isolates from two separate, unrelated outbreaks. The source and date of isolation of outbreak strains are given in Table 1.

Chromosomal analysis by PFGE. Genomic DNA was prepared by a modification of the procedure of Smith and Cantor (27). *Legionella* cultures were grown for 48 h on BCYEα agar plates at 37°C. The cells were harvested and washed and were then resuspended in Pett IV buffer (1.0 M NaCl, 10 mM Tris-HCl [pH 7.6]). A portion of this suspension was mixed with an equal volume of 1.5% Incert Agarose (FMC Bioproducts, Rockland, Maine), pipetted into a plug mold (Bio-Rad Laboratories, Richmond, Calif.), and then allowed to solidify on ice. For lysis, the plugs were incubated at 37°C with gentle shaking in 2 volumes of EC lysis buffer (6 mM Tris-HCl, 1 M NaCl, 100 mM EDTA, 0.5% Brij 58, 0.2% deoxycholate, 0.5% *N*-lauroylsarcosine, 1 mg of lysozyme per ml, 20 μg of RNase per ml [pH 7.6]) per volume of plugs. Following overnight incubation, the plugs were transferred to ESP (0.5 M EDTA [pH 8.0], 1.0% *N*-lauroylsarcosine, 1 mg of proteinase K [Boehringer Mannheim Biochemicals, Indianapolis, Ind.] per ml) and were incubated for 48 h at 50°C with one change of ESP at 24 h. To inactivate the proteinase K, the plugs were incubated in 10 mM Tris–0.1 mM EDTA–1.0 mM phenylmethylsulfonyl fluoride (pH 7.5) overnight at 37°C. The plugs were then washed twice for 2 h each time with TE (10 mM Tris, 0.1 M EDTA [pH 7.5]) and stored at 4°C.

For restriction endonuclease digestion, a 2-mm square of the plug was placed in a microfuge tube with 100 μl of 1× restriction buffer supplied by the enzyme manufacturer. After 1 h for equilibration at 4°C, 5 U of *Sfi*I (New England Biolabs, Beverly, Mass.) was added and the tubes were incubated at 50°C overnight. The plugs were then loaded into 1.0% Seakem LE agarose gels (FMC Bioproducts) that were prepared in the running buffer, 0.5× TBE (45 mM Tris, 45 mM boric acid, 1.0 mM EDTA [pH 8.0]). Bacteriophage lambda concatemers (FMC Bioproducts) were used as size standards. The fragments were separated by PFGE using a contour-clamped homogeneous electric field apparatus (CHEF-DR II; Bio-Rad). The initial pulse time of 7 s was increased linearly to 74 s over 24 h at 200 V at 16°C. Gels were then stained with ethidium bromide, destained in water, and photographed under UV illumination.

Ribotyping. The bacterial growth from one BCYEα plate that was incubated at 37°C for 48 h was used to isolate chromosomal DNA by a miniprep procedure (6). Briefly, bacteria were lysed at 37°C in 900 μl of lysis buffer (25% sucrose, 0.05 M Tris [pH 8.0]), to which lysozyme (90 μl, 10 mg/ml), pronase (90 μl, 10 mg/ml), and sodium dodecyl sulfate (SDS; 54 μl, 20%) were added. The DNA was extracted first with phenol-chloroform-isoamyl alcohol (25:24:1) and then with chloroform; finally, it was precipitated with 95% ethanol after the addition of sodium acetate (24). The precipitated DNA was resuspended in 400 μl of sterile, distilled water, treated with RNase (5 μl, 2 mg/ml), extracted with phenol-chloroform-isoamyl alcohol, reprecipitated as described above, and then resuspended in 200 μl of water. Restriction enzyme digestions with 3 μg of DNA and 10 U of enzyme *Hpa*I or *Eco*RI (New England Biolabs) were incubated under conditions specified by the manufacturer for 5 h at 37°C and electrophoresed at 30 V for 18 h.

Ribosomal 16S plus 23S RNA from *Escherichia coli* (Boehringer), which was dephosphorylated with calf intestinal alkaline phosphatase, was end-labeled with [γ-^{32}P]ATP (Amersham, Arlington Heights, Ill.) by using a 5′ DNA terminus-labeling kit (Bethesda Research Laboratories, Gaithersburg, Md.). Unincorporated [γ-^{32}P]ATP was separated from the labeled probe with a G-50 Quick-Spin Column (Boehringer).

Southern transfer of DNA fragments to nylon filters (Hybond N; Amersham) was accomplished with a Vacu-blot apparatus (Pharmacia LKB, Piscataway, N.J.) by using 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (24) as the transfer buffer. The DNA was cross-linked (UV, 3 min), and the filters were baked for 1 h at 80°C in a vacuum oven. Southern blots were prehybridized for 3 h and were then hybridized for 18 h at 50°C in a hybridization solution consisting of 5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM $NaPO_4$, and 1 mM EDTA [pH 7.7]), 5× Denhardt's solution, 0.1% SDS, and 0.1 mg of denatured salmon sperm per ml (24) and containing the denatured (100°C for 5 min) *E. coli*

TABLE 1. Ribotype and chromosomal restriction enzyme patterns of the *L. pneumophila* isolates used in this study

Serogroup and isolate	Source[a]	Date of isolation (mo/yr)	Ribotype pattern (R)		Chromosome restriction pattern (P) (*Sfi*I)
			*Hpa*I	*Eco*RI	
Serogroup 1					
LB309	P, O, BW (case 2)	7/1989	1	4	1
LB310	P, O, BW (case 3)	7/1989	1	4	1
LB469	P, O, SP (case 6)	9/1989	1	4	1
LB311	P, C		1	4	11
LB394	E, C		1	4	2
LB246	P, C		1	1	5
Knoxville 1	P, C		2	3	3
Philadelphia 1	P, C		1	1	4
Serogroup 6					
LB308	P, O, SP (case 1)	6/1989	2	2	6
LB312	P, O, SP (case 4)	6/1988	1	1	7
LB412	P, O, BW (case 5)	11/1989	2	2	6
LB286	E, O, shower	8/1989	2	2	6
LB290-1	E, O, patient room sink	8/1989	1	1	7
LB290-2	E, O, patient room sink	8/1989	1	1	7
LB290-3	E, O, patient room sink	8/1989	1	1	7
LB292	E, O, room sink	8/1989	1	1	7
LB299-1	E, O, hot water tank	8/1989	1	1	7
LB299-2	E, O, hot water tank	8/1989	2	2	13
LB299-3	E, O, hot water tank	8/1989	2	2	13
LB299-4	E, O, hot water tank	8/1989	1	1	7
LB299-5	E, O, hot water tank	8/1989	ND[b]		13
LB299-6	E, O, hot water tank	8/1989	ND		7
LB299-7	E, O, hot water tank	8/1989	ND		13
LB299-8	E, O, hot water tank	8/1989	ND		7
LB299-9	E, O, hot water tank	8/1989	ND		13
LB299-10	E, O, hot water tank	8/1989	ND		13
LB303	E, O, cooling tower 1	8/1989	1	3	9
LB329	E, O, cooling tower 2	8/1989	1	1	14
LB352	E, O, ultrasound room sink	8/1989	ND		7
LB104	E, C		2	2	10
LB635	E, C		1	1	12
Chicago 2	P, C		1	1	8

[a] P, isolate from a patient; O, outbreak-associated isolate; BW, brochial washing; SP, sputum; C, control isolate unrelated to outbreak; E, isolate from hospital environment.
[b] ND, not done.

rRNA probe (10^5 cpm/ml). Following incubation, the filters were washed at 50°C with 2× SSC–0.1% SDS (four times, 30 min each time), dried, and exposed to Kodak X-Omat AR film at −70°C with an intensifying screen.

RESULTS

Epidemiologic investigation. In the epidemiologic investigation, 274 (92.3%) of the 295 charts to be reviewed were located. Ten patients who had nosocomial pneumonia of unexplained etiology were identified, and their charts were reviewed further for evidence of legionellosis. Of those 10 patients, testing for *L. pneumophila* was done on 4 patients; 3 of the 4 patients had nonreactive titers, and 1 patient had a positive urine antigen test and a convalescent-phase *L. pneumophila* titer of 1:1,024 and was therefore classified as a probable case. Legionnaire's disease status could not be determined for six patients.

Six cases of confirmed nosocomial legionellosis were identified during the investigation and follow-up. A cluster of three cases occurred in the renal transplant unit during June and July 1989 in renal transplant patients who had been admitted to the hospital because of graft rejection. A retrospective review of cases of pneumonia during the previous year revealed a case in a renal transplant patient who had stayed in the same room as one of the patients with pneumonia in July 1989. After the initial cluster of cases and the finding of positive cultures of environmental samples, hyperchlorination and superheating of the hot water system were done; however, regrowth in the hot water system occurred within 2 months and two more cases of legionellosis occurred (13). One of the two cases occurred in a patient with AIDS who was housed on the floor directly above the renal transplant unit, and the final case occurred 4 days postoperatively in a renal transplant patient.

The data on potential water exposures were incomplete, but they were provided by nursing staff and interviews with patients, when possible. Five patients used the sinks in their rooms and four were known to have used the patient showers. The three patients whose pneumonia occurred in June and July 1989 used the window air-conditioners in their rooms rather than opening the windows, and there were no reports of stagnant water or poorly operating air-conditioning units. The renal transplant patients also walked in the hall, but they wore masks, which was required when they were outside of their rooms. The ages, sexes, symptoms, immunosuppressive treatments, and possible exposures of patients with pneumonia are given in Table 2.

TABLE 2. Epidemiologic data for case patients[a]

	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6
Age (yr)	45	25	35	51	35	41
Sex	M	F	M	F	M	F
Symptoms						
Fever, >38.2°C	Y	Y	Y	Y	Y	Y
Cough	N	Y	Y	Y	Y	U
Shortness of breath	Y	Y	Y	Y	U	Y
Pleuritic chest pain	Y	Y	Y	N	U	Y
High-dose steroids	Y	Y	Y	Y	N	Y
Graft radiation	N	Y	N	N	N	U
Water exposure						
Patient showers	Y	Y	N	U	Y	Y
Room sinks	Y	Y	Y	Y	Y	U
Open windows	N	N	N	U	U	U
Window air-conditioners	Y	Y	Y	U	U	U

[a] M, male; F, female; Y, yes; N, no; U, unknown.

Bacterial strains. During the investigation of the outbreak described here, six isolates (three *L. pneumophila* serogroup 1 [LB309, LB310, LB469] and three *L. pneumophila* serogroup 6 [LB308, LB312, LB412]) were obtained from respiratory secretions of patients with nosocomial legionellosis. *L. pneumophila* serogroup 6 was isolated from 7 of 22 environmental sources examined, including the hospital's hot water tank (isolate LB299), the patient shower (isolate LB286), the sink in the room occupied by two of the *L. pneumophila*-infected patients (isolate LB290), the sink in another patient's room (isolate LB292), a sink in the ultrasound room (isolate LB352), and two hospital cooling towers (isolates LB303 and LB329). The numbers of *L. pneumophila* serogroup 6 isolates ranged from approximately 10^3 CFU/liter in the patients' rooms to 10^7 CFU/liter in the hospital's hot water tank. Two other *Legionella* species were isolated from one source each. *L. pneumophila* serogroup 1 was not isolated, despite an extensive search.

Ribotyping and restriction enzyme analysis by PFGE. The ribotype and genomic restriction endonuclease digestion patterns of the isolates examined in this study (Table 1) were designated P for the DNA digested with *Sfi*I and by R for ribotype patterns. The two numerals for the ribotype pattern designate the pattern that was found after digestion with each of the enzymes (*Hpa*I, *Eco*RI). As shown in Table 1 and Fig. 1, *L. pneumophila* serogroup 1 chromosomal fragments separated by PFGE revealed six patterns. The three *L. pneumophila* serogroup 1 outbreak isolates (LB309, LB310, LB469) had the same pattern, while each of the control strains exhibited a pattern different from those of the outbreak strains and different from each other.

Ribotyping revealed that the *L. pneumophila* serogroup 1 outbreak isolates (LB309, LB310, LB469) and two control isolates (LB311, LB394) shared the same ribotype R pattern (pattern 1,4) (Table 1; Fig. 2). Isolates with ribotype R pattern 1,4 were subdivided into three subtypes (P1, P2, P11) by PFGE. Two different ribotypes were seen in the other three control strains (LB246, Knoxville 1, Philadelphia 1). The two control strains (LB246 and Philadelphia 1) that belonged to ribotype R pattern 1,1 had two separate patterns (patterns P4 and P5) by PFGE.

L. pneumophila serogroup 6 isolates analyzed by PFGE of *Sfi*I-digested genomic DNAs from the isolates revealed eight patterns (Table 1). Examples of each pattern are shown in Fig. 3. Five patterns were seen in the outbreak strains. One isolate from a patient (isolate LB312) and isolates from four environmental sources (the hot water tank [LB299-1, LB299-4, LB299-6, LB299-8], the sink in the room occupied by the patients from whom LB308 and LB312 were isolated [isolate LB290], a sink in another patient's room [isolate LB292], and a sink in the ultrasound room [isolate LB352]) had identical patterns (P7). A second pattern was observed among isolates from the two other patients (isolates LB308 and LB412) and the patient's shower (isolate LB286) (P6). A third pattern not noted in isolates from the patients was also present in isolates from the hospital's hot water tank (isolates LB299-2, LB299-3, LB299-5, LB299-7, LB299-9, and

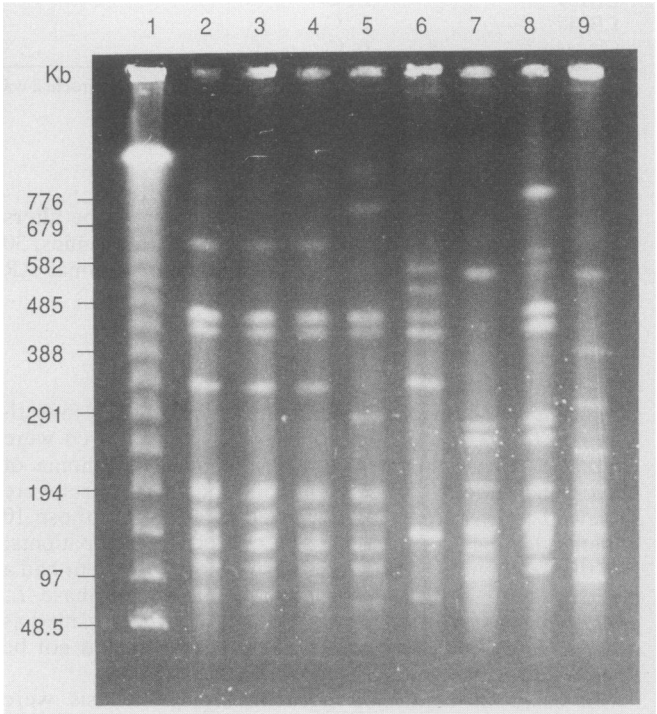

FIG. 1. PFGE of *Sfi*I-cleaved genomic DNAs from *L. pneumophila* serogroup 1 isolates. Lanes: 2, LB309; 3, LB310; 4, LB469; 5, LB311; 6, LB394; 7, LB246; 8, Knoxville 1; 9, Philadelphia 1. Bacteriophage lambda concatemers (lane 1) were used as molecular size standards (48.5 kb).

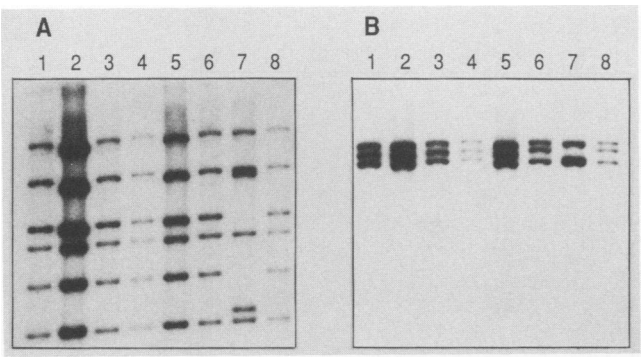

FIG. 2. Autoradiogram of *Hpa*I (A) and *Eco*RI (B) digests of DNA isolated from *L. pneumophila* serogroup 1 and hybridized with ^{32}P-labeled *E. coli* 16S and 23S rRNAs. Lanes: 1, LB309; 2, LB310; 3, LB469; 4, LB311; 5, LB394; 6, LB246; 7, Knoxville 1; 8, Philadelphia 1.

LB299-10) (P13). A total of 10 *L. pneumophila* serogroup 6 colonies from the hospital's hot water tank were analyzed by PFGE of large genomic fragments (isolates LB299-1 to LB299-10) to determine whether the three patterns present among the isolates recovered from the potable water system were also present among isolates derived from the hot water tank. All 10 isolates demonstrated either pattern P7 or P13; pattern P6 was not found. The *L. pneumophila* serogroup 6 isolates from the cooling towers (isolates LB303 and LB329) and the control isolates demonstrated patterns different from each other and different from those of the outbreak isolates (P9, P14).

Ribotyping revealed three different patterns among the *L. pneumophila* serogroup 6 outbreak isolates, R patterns 1,1, 1,3, and 2,2 (Table 1; Fig. 4). The three isolates from patients showed two ribotypes that were also present among the isolates from the hospital's hot water tank, R patterns 1,1 and 2,2. One of these ribotypes, R pattern 1,1, was demonstrated in isolates from the hot water tank (isolates LB299-1 and LB299-4), the isolate from the sink in the room used by two of the patients (isolate LB290), as well as an isolate from a cooling tower (isolate LB329). However, the control strains *L. pneumophila* Chicago 2 and LB635 also shared this ribotype. *L. pneumophila* serogroup 6 isolates with ribotype R pattern 1,1 were further divided into four subtypes by PFGE. One PFGE pattern (P7) was seen in outbreak isolates from a patient (isolate LB312) and water samples (isolates LB290, LB292, LB299-1, and LB290-4), one pattern (P14) was seen in a cooling tower sample (isolate LB329), and two patterns (P8, P12) were seen in control isolates (isolates LB365 and Chicago 2). The two other *L. pneumophila* serogroup 6 isolates from patients (isolates LB308 and LB412), the hot water tank (isolates LB299-2 and LB299-3), a patient shower (isolate LB286), and control strain LB104 possessed a single ribotype, R pattern 2,2. By PFGE, the pattern of the isolate from the patient's shower (isolate LB286) matched the pattern seen in isolates from the two patients (pattern P6). The hot water tank isolates (LB299-2, LB299-3) demonstrated a second pattern (P13) and control isolate LB104 demonstrated a third pattern (P10) within this ribotype. A third ribotype, R pattern 1,3, was found in an isolate from a cooling tower (isolate LB303), which also demonstrated a PFGE pattern different from those of the other outbreak isolates and controls (pattern P9).

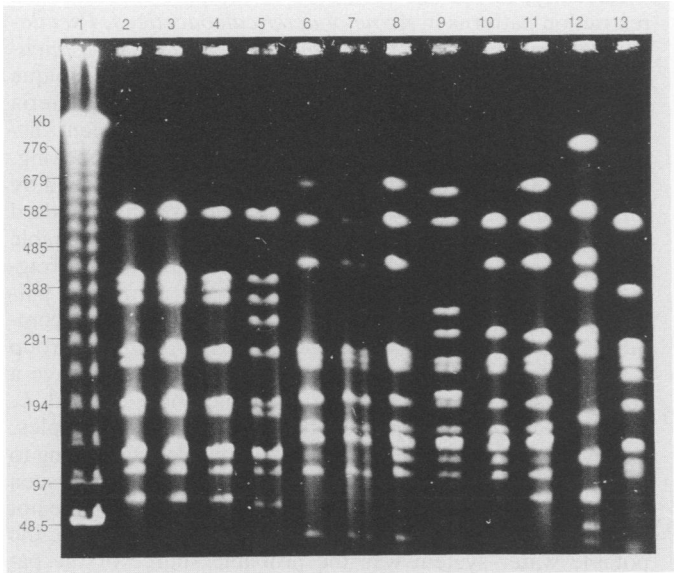

FIG. 3. PFGE of *Sfi*I-cleaved genomic DNA from *L. pneumophila* serogroup 6. Lanes: 2, LB308; 3, LB412; 4, LB286; 5, LB299-2; 6, LB312; 7, LB292; 8, LB299-1; 9, LB303; 10, LB329; 11, Chicago 2; 12, LB104; 13, LB635. Bacteriophage lambda concatemers (lane 1) were used as molecular size standards (48.5 kb).

There were instances in which isolates with the same ribotype demonstrated PFGE patterns that were quite different from each other. For example, *L. pneumophila* serogroup 1 strains LB309, LB310, and LB469 (pattern P1) (Fig. 1, lanes 2 to 4, respectively) looked different from LB394 (pattern P2) (Fig. 1, lane 6) when they were examined by restriction enzyme analysis by using PFGE and all had ribotype R pattern 1,4. Among the *L. pneumophila* serogroup 6 isolates, isolates LB308, LB412, and LB286 (P6) (Fig. 3, lanes 2 to 4, respectively) demonstrated numerous differences in PFGE bands when they were compared with those of LB104 (P10) (Fig. 3, lane 12), which has the same ribotype.

There were also several instances in which isolates with common ribotypes showed similar PFGE banding patterns. *L. pneumophila* serogroup 1 strain LB311 (Fig. 1, lane 5) and outbreak strains LB309, LB310, and LB469 (Fig. 1, lanes 2 to 4, respectively) had ribotype R pattern 1,4 and showed only minor differences in PFGE banding patterns. Among the *L. pneumophila* serogroup 6 isolates, LB299-2 (Fig. 3, lane 5) differed from outbreak isolates LB308, LB412, and LB286 by the placement of one band (Fig. 3, lanes 2 to 4, respectively) and all had ribotype R pattern 2,2. Control isolate *L. pneumophila* serogroup 6 Chicago 2 (Fig. 3, lane 11) and outbreak isolates LB312, LB292, and LB299-1 (Fig. 3, lanes 6 to 8, respectively) had ribotype R pattern 1,1 and very similar PFGE patterns. Fragment sizes in the DNAs separated by PFGE ranged from <50 to approximately 800 kb, as determined by comparison of the fragments with the mobilities of lambda concatemers. When this comparison was used as an approximation for the fragment sizes, the sum of the fragments for the strains ranged from 2.6×10^6 to 3.3×10^6 bp. Chromosomal restriction with enzymes *Not*I, *Sal*I, *Spe*I, *Dra*I, *Pac*I, *Xba*I, *Sma*I, and *Xho*I was also performed on some strains but generated a large number of fragments or did not yield evaluable patterns.

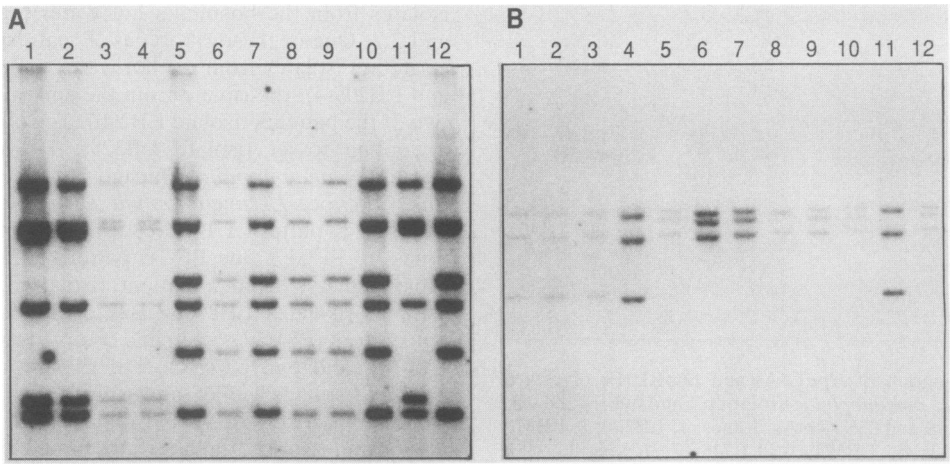

FIG. 4. Autoradiogram of *Hpa*I (A) and *Eco*RI (B) digests of DNA isolated from *L. pneumophila* serogroup 6 and hybridized with ^{32}P-labeled *E. coli* 16S and 23S rRNAs. Lanes: 1, LB308; 2, LB412; 3, LB286; 4, LB299-2; 5, LB312; 6, LB292; 7, LB299-1; 8, LB303; 9, LB329; 10, Chicago 2; 11, LB104; 12, LB635.

DISCUSSION

Evaluation of *Legionella* strains for epidemiologic tracking has been approached by using various techniques. Monoclonal antibody subtyping, especially for *L. pneumophila* serogroup 1 strains, has been successfully used in investigations of nosocomial legionellosis (18, 22, 28). Although a monoclonal antibody subtyping scheme for *L. pneumophila* serogroup 1 has been developed (17), the development of subtyping systems for serogroups other than serogroup 1 has not been thoroughly addressed. In addition, the number of subgroups for *L. pneumophila* serogroup 1 is relatively small. In this study, the three outbreak isolates of *L. pneumophila* serogroup 1 (LB309, LB310, LB469) and three of the unrelated isolates (LB311, LB246, Philadelphia 1) that were sent to the Centers for Disease Control for monoclonal antibody subtyping belonged to the same monoclonal antibody subtype: 1,2,5,6. The rest of the outbreak isolates were *L. pneumophila* serogroup 6, for which a standardized subtyping system has not been established. Monoclonal antibody subtyping was, therefore, not particularly useful in the investigation of the outbreak described here.

Plasmid analysis has been used to subtype *L. pneumophila* serogroup 1 isolates (16, 18, 20, 22, 28). Some of these studies have reported that 40 to 50% of the isolates are plasmid free (22, 28). In addition, isolates frequently contain only one plasmid, and restriction endonuclease digestion is necessary to fully characterize these isolates. Bacteria that consistently contain plasmids, preferably more than one, are better suited for plasmid analysis. The *L. pneumophila* serogroup 1 isolates from patients in the outbreak described in this report contained a 77-kb plasmid. However, the *L. pneumophila* serogroup 6 isolates were plasmid free (data not shown), so plasmid analysis was not pursued.

Comparison of chromosomal digestion patterns (30, 33) has been used in epidemiologic studies. However, digestion of chromosomal DNA with restriction endonucleases followed by conventional electrophoresis generally yields a large number of fragments that are difficult to analyze. Probing of these chromosomal digests with *E. coli* rRNA (31, 32) or specific *L. pneumophila* DNA (25, 31) simplifies the analysis, but it complicates the procedure and, especially in the case of ribotyping with *E. coli* rRNA, may limit the discriminatory power of the assay. Determination of the restriction pattern after hybridization with *E. coli* rRNA was proposed as a general method for typing bacterial strains (29). This method has the advantage that a single, commercially available probe can be used to type many bacterial species. Successful application of this technique has been described for a number of bacteria (15, 29, 32), and we found that it produced five distinguishable patterns among the strains involved in the outbreak. However, unrelated isolates, in some instances, had the same ribotype as the outbreak strains. Ribotyping with other enzymes (*Pst*I, *Bgl*II, *Cla*I, *Cfo*I, *Pvu*II) was performed on some strains and did not further differentiate among strains (data not shown). However, use of additional enzymes might allow differentiation of strains.

Recently, PFGE has been used to compare chromosomal restriction patterns in *Acinetobacter calcoaceticus*, *Pseudomonas aeruginosa* (1), *Pseudomonas cepacia* (2), *L. pneumophila* (21), and *Enterococcus faecalis* (19). This technique is relatively simple, is applicable to a wide range of bacteria with little modification, and appears to have sufficient discriminatory power to be useful for epidemiologic tracking. We found that *Legionella* isolates were readily analyzed by this technique without major modification of the standard protocol described by Smith and Cantor (27). Evaluable patterns were generated, and considerable restriction fragment polymorphism was present. Identical restriction patterns were interpreted as representing isolates with a common source. Therefore, the three *L. pneumophila* serogroup 1 strains from patients in the outbreak appeared to have a common source, although we were not able to find *L. pneumophila* serogroup 1 in the environmental samples. Likewise, the pattern seen in one patient isolate belonging to *L. pneumophila* serogroup 6 was identical to the pattern seen in isolates from four potable water sources, including the hot water tank and the sink in the patient's room; therefore, the potable water system was the probable source of the patient's infection. The other two isolates from patients demonstrated the same pattern as the isolate from a shower. Although information on possible water exposure was incomplete, both of these patients stated that they had used the patient's shower; the identities of the restriction endo-

nuclease patterns indicate that the shower was a likely source of the patients' infections. The multiple patterns present in the *L. pneumophila* serogroup 6 isolates raise the question of whether isolates with each of the patterns seen were present throughout the hot water system or whether areas existed where one subpopulation predominated. It is possible that a small subpopulation with pattern P6 existed in the hot water tank. PFGE of large restricted fragments appeared to be able to distinguish these subpopulations, but the number of isolates from each source was insufficient to resolve this question. The two patterns of large genomic fragments seen in the isolates from the cooling towers, the latter located upwind and at a distance from the renal transplant unit, differed from the patterns seen in the patient and the potable water isolates and were, therefore, probably not the source of the contamination of the potable water system.

Ott et al. (21) reported, in a study in which they typed 10 *Legionella* strains, that *Sfi*I restriction cleavage generated a high number of fragments, which led to a "crowded arrangement of bands" and therefore limited precise typing. In our experience, digestions with the optimal concentration and total amount of DNA in the *Sfi*I digestion produced 8 to 13 bands that were easily distinguishable. Larger-molecular-size bands that were seen on digests containing a larger amount of DNA disappeared under these conditions, suggesting that they were partial digestion products (data not shown).

A comparison of ribotyping and restriction enzyme analysis by PFGE in this study demonstrated that both techniques are useful for subtyping *L. pneumophila*. PFGE was useful for subdividing ribotypes; ribotype R pattern 1,1 could be subdivided into two patterns (P4 and P5) among the *L. pneumophila* serogroup 1 isolates and into four patterns (P7, P8, P12, and P14) among the *L. pneumophila* serogroup 6 isolates, ribotype R pattern 1,4 could be subdivided into three patterns (P1, P2, and P11), and ribotype R pattern 2,2 could be subdivided into three patterns (P6, P10, and P13). In no instance was ribotyping successful in subdividing a PFGE pattern. The greater heterogeneity detected by PFGE raises the question of genetic instability. The significance of minor changes in PFGE banding patterns and the changes that occur over time in response to host defenses or eradication measures in a dynamic population must be considered. It is interesting that the PFGE pattern of the isolate (LB312) from the case of legionellosis found in 1988 was identical to the pattern seen in isolates from the hot water supply collected over 1 year later. The interpretation of subtyping data is most useful when multiple techniques are used and epidemiologic information is carefully examined.

In summary, comparison of ribotyping and restriction endonuclease analysis by PFGE in this study indicates that both techniques are useful in subtyping strains of *L. pneumophila* that are obtained during an outbreak. These two methods of examining chromosomal DNA showed close agreement; however, the use of restriction enzyme analysis by PFGE allowed the subdivision of ribotypes. Restriction enzyme analysis by PFGE proved to be useful in the investigation of this outbreak, although a small number of cases occurred, to differentiate among subtypes of *L. pneumophila* serogroup 6 in the hospital environment and to implicate the potable water system as the source of the patients' infections. The evaluation of large genomic fragments by PFGE is easily performed, is reliable, and detects considerable polymorphism. The clarity of interpretation suggests that it will be useful in the investigation of *Legionella* outbreaks.

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