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Genetic Typing in a Cluster of *Legionella pneumophila* Infections

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***Legionella pneumophila* strains isolated from six patients, three air-conditioning- and cooling tower-derived strains, and three hot water supply-derived strains were analyzed by three genetic typing methods. The results of the whole-cell DNA restriction endonuclease analysis and the restriction patterns based on genes coding for rRNA correlated with each other and demonstrated that the patient isolates were indistinguishable from the air-conditioning- and cooling tower-derived isolates but differed markedly from the hot water supply-derived isolates. The patient and air-conditioning- and cooling tower-derived strains contained plasmids of the same molecular weight; the hot water supply-derived strains were plasmidless. These results indicate that the cooling tower or the air-conditioning system was the environmental source for the examined cluster of Legionnaires disease strains.**

Nosocomial infections account for 35% of all cases of Legionnaires disease registrated in The Netherlands (8). Legionnaires disease is caused most often by *Legionella pneumophila* (29), and generally the hot water supply is the environmental source of nosocomial Legionnaires disease in The Netherlands (29). Subtyping of *L. pneumophila* has been carried out by serogrouping (5), plasmid analysis (1, 4, 15, 17, 18, 21, 23), peptide profiling (16), immunochemical methods that use absorbed antisera and monoclonal antibodies (7, 12, 13, 17, 22), alloenzyme analysis (7, 24, 27), and restriction endonuclease analysis of whole-cell DNA (27, 30).

From our work (28) and that of others (7, 17, 24, 27) it became evident that accurate subtyping of strains of *L. pneumophila* for epidemiological purposes is more reliable when more than one immunochemical or genetic typing method is used. The comprehensive study of Brenner et al. (3) demonstrated the utility of this approach for *Haemophilus aegypticus*, for example.

Plasmid analysis of legionellae revealed an extensive variety of plasmid sizes and restriction endonuclease cleavage patterns (1, 4, 18, 21). All plasmids had cryptic functions. Restriction endonuclease analysis of whole-cell DNA suffers from the large number of generated fragments, which often hinder the resolution of individual fragments. To highlight restriction length polymorphism in a more readable form, 16S and 23S rRNAs from *Escherichia coli* were used to probe the bacterial chromosomal DNA of other gram-negative and gram-positive microorganisms (9–11, 26). Genes encoding rRNA sequences are highly conserved in bacteria, and the genome of *E. coli* contains seven transcriptional units for rRNA (20). Therefore, the number of bands that would be expected to hybridize with nucleic acids that probe genes coding for rRNA (rDNA probes) would be equivalent to at least the number of transcriptional units for rRNA within the bacterial genus or species, with a greater number resulting from the presence of cleavage sites within the rRNA transcriptional units.

We were interested in a cluster of six patients with nosocomial Legionnaires disease that occurred from 1984 to 1987 in a hospital in the southern part of The Netherlands. The medical histories of all patients were highly suggestive of nosocomial infections; all patients were hospitalized during the summer. The hospital is equipped with an air-conditioning system and cooling towers on the roof of the building, but only a portion of the patient wards make use of the air-conditioning system. The hot water supply of the hospital operates on a recirculation system that includes storage tanks.

All strains (see Table 1) were isolated and identified in accordance with internationally accepted criteria (6), and serogrouping was done with immunofluorescent antisera obtained from the Centers for Disease Control, Atlanta, Ga. The six patient isolates were *L. pneumophila* serogroup 1 and were isolated from 1984 to 1987. The three hot water supply-derived strains were *L. pneumophila* serogroup 4, and the air-conditioning- and cooling tower-derived strains were *L. pneumophila* serogroup 1. All environmental isolates were isolated during a survey in 1986; the hot water supply-derived isolates were isolated from tap water, the cooling tower-derived isolate was isolated from the water on the floor of the tower, and the air-conditioning-derived isolates were isolated from condensates near the humidifier section.

Whole-cell DNA restriction endonuclease analysis was carried out as described earlier (28, 30). Approximately 2 μg of bacterial DNA was digested to completion with 10 to 20 U of *Eco*RI and *Hin*dIII, doubly digested with *Hpa*I and *Hpa*II, and electrophoresed. Plasmid analysis was conducted by the methods of Kado and Liu (14) and Birnboim and Doly (2). All strains were screened by these methods at least twice. The molecular weights of the plasmids were estimated by comparison with the plasmids of *Legionella bozemanii* (WIGA) (1). Approximately 1 μg of plasmid DNA, purified as described by Birnboim and Doly (2), was digested for restriction endonuclease analysis with 10 to 20 U of *Eco*RI and *Hin*dIII, and electrophoresis was carried out in 0.7% agarose gels containing ethidium bromide (1 μg/ml) at 4 V/cm for 4 to 6 h.

For rDNA restriction patterns, approximately 3 μg of whole-cell DNA was digested with 10 to 20 U of *Eco*RI and *Hin*dIII and, after electrophoresis, transferred to nitrocellulose membranes by the method of Southern (25). Prehybridization and hybridization were carried out in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1%

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TABLE 1. Epidemiological, serological, and genetic data for 12 strains of *L. pneumophila*

Strain	Source	Sero-group	Whole-cell DNA restriction type	Plasmid size (megadaltons)	Plasmid restriction type	rDNA restriction pattern
HG01	Patient 1	1	III	50–60	1	A
HG02	Patient 2	1	III	50–60	2	A
HG03	Patient 3	1	III	50–60	1	A
HG04	Patient 4	1	III	50–60	3	A
HG05	Patient 5	1	III	50–60	3	A
HG06	Patient 6	1	III	50–60	1	A
HG07	Air conditioning	1	III	50–60	1	A
HG08	Air conditioning	1	III	50–60	1	A
HG09	Cooling tower	1	III	50–60	1	A
HG10	Water	4	IV			B
HG11	Water	4	IV			B
HG12	Water	4	IV			B

Ficoll-polyvinylpyrrolidone-bovine serum albumin (Denhardt solution)–0.02 M Tris hydrochloride (pH 7.4)–0.1% sodium dodecyl sulfate–10% dextran sulfate–100 μg of sheared denaturated salmon sperm DNA per ml. Prehybridization was carried out at 55°C for 2 h, and ^{32}P-labeled *E. coli* rRNA (10^5 to 10^6 cpm/ml of hybridization solution) was added. 16S and 23S rRNAs from *E. coli* (Boehringer GmbH, Mannheim, Federal Republic of Germany) were end labeled with [γ-^{32}P]ATP to a specific activity of 10^7 to 10^8 cpm/μg of RNA by using T4 polynucleotide kinase after dephosphorylation with calf intestinal alkaline phosphatase (19). Incubation for hybridization was carried out for 17 h at 55°C. The blots were washed for 15 min in 0.1× SSC containing 0.1% sodium dodecyl sulfate twice at 55°C and once at room temperature. The blots were exposed for autoradiography for 1 to 3 days in the presence of an intensifying screen at −70°C. The *Hin*dIII-cleaved fragments of *Serratia fonticola*, which produces seven fragments that have known molecular weights (10) and that hybridize with the rRNA probe, were used as molecular weight markers.

The whole-cell DNA restriction endonucleases analysis revealed that the six patient isolates and the air-conditioning- and cooling tower-derived isolates (strains HG01 through HG09 in Table 1) were indistinguishable from one another but differed markedly from the hot water supply-derived isolates (strains HG10 through HG12 in Table 1) in the digestions with *Eco*RI, *Hin*dIII, and *Hpa*I-*Hpa*II. The differences in profiles were more readily detected in the double digestion with *Hpa*I and *Hpa*II, as noted earlier (28). The profiles shared by strains HG01 through HG09 were not noticed before during our epidemiological survey in The Netherlands (28); restriction endonuclease profiles resembling those of the hot water supply-derived strains were seen before.

The plasmid analysis revealed that the six patient isolates and the air-conditioning- and cooling tower-derived isolates contained plasmids, all with the same molecular mass of 50 to 60 megadaltons; no plasmids could be detected in the hot water supply-derived isolates. Surprisingly, the restriction endonuclease profiles of the plasmids revealed three types (Table 1). The plasmids isolated from strains HG01, HG03, and HG06 and the air-conditioning- and cooling tower-derived isolates shared a type 1 profile; HG02 had a unique type 2 restriction endonuclease profile, and HG04 and HG05 had a type 3 profile. A side-by-side comparison of the *Eco*RI and *Hin*dIII restriction endonuclease profiles revealed that the type 3 profile is composed of the sum of the fragments of the digests of the type 1 and type 2 plasmids. Therefore, the type 3 profiles of strains HG04 and HG05 are in fact the result of the presence of two plasmids, one with a type 1 profile and one with a type 2 profile. Hybridization experiments done for these profiles with one of the two plasmids as a probe confirmed this observation.

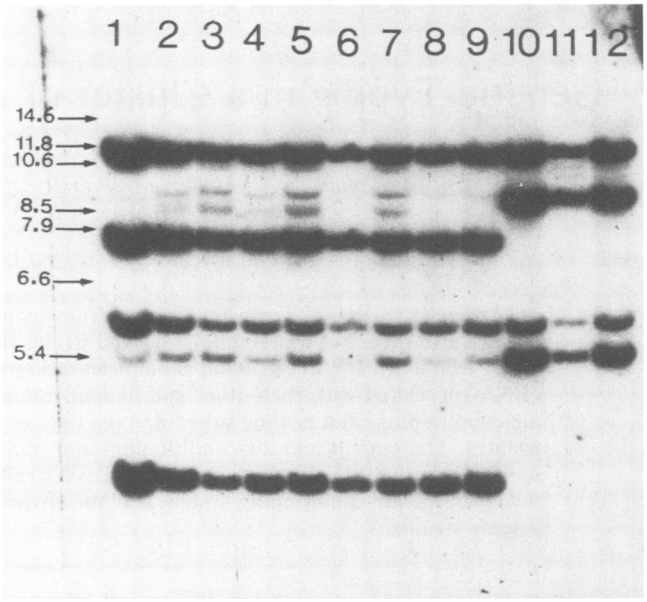

FIG. 1. Autoradiogram of *Hin*dIII digests of DNA isolated from *L. pneumophila* and hybridized with ^{32}P-labeled *E. coli* 16S and 23S rRNAs. Lanes: 1, strain HG01 (patient 1; type A); 2, strain HG02 (patient 2; type A); 3, strain HG03 (patient 3; type A); 4, strain HG04 (patient 4; type A); 5, strain HG05 (patient 5; type A); 6, strain HG06 (patient 6; type A); 7, strain HG07 (air-conditioning; type A); 8, strain HG08 (air-conditioning; type A); 9, strain HG09 (cooling tower; type A); 10, strain HG10 (water; type B); 11, strain HG11 (water, type B); and 12, strain HG12 (water; type B). Molecular weight markers (kilobases) are indicated by arrows.

The *Eco*RI and *Hin*dIII digests of the 12 strains in Table 1 were hybridized with 16S and 23S rRNAs from *E. coli*. The *Eco*RI digests produced only strongly hybridizing bands, corresponding to fragments with high molecular weights in the restriction endonuclease profile: the patient and air-conditioning- and cooling tower-derived isolates (strains HG01 through HG09 in Table 1) had three hybridizing bands, and the hot water supply-derived isolates (strains HG10 through HG12 in Table 1) had two bands. The *Hin*dIII digests are shown in Fig. 1. Strong bands and, on longer exposures of the autoradiogram, weaker bands were visible. Strains HG01 through HG09 produced five strong bands, and strains HG10 through HG12 produced four strong bands; all profiles had three strongly reacting bands of the same molecular weight. The weaker bands were less consistently discernible and differed in number depending on the exposures of the autoradiograms. The rDNA restriction patterns of the patient and air-conditioning- and cooling tower-derived isolates were indistinguishable from one another and differed markedly from the patterns of the hot water supply-derived isolates.

The results obtained with the genetic typing methods used in this study all indicated that the air-conditioning system or the cooling tower was the environmental source for this cluster of six patients with nosocomial Legionnaires disease. The cooling tower was the more likely environmental source, as most patients were not in contact with the

air-conditioning system. The drift from the cooling tower could enter opened windows and could also be responsible for contamination of the air-conditioning system after entering through the air inlets of this system. In any case the *L. pneumophila* strains isolated from the air-conditioning system were indistinguishable from the cooling tower-derived strain.

In the patient isolates we detected two plasmids with different restriction endonuclease profiles, and only one of them was detected in the isolates derived from the air-conditioning system and the cooling tower. The observation that two patient isolates had both plasmids favors the assumption that the air-conditioning system and the cooling tower also most likely harbored isolates with the undetected plasmid and with both plasmids. Plasmid exchange or deletion of one or the other plasmid from a parent strain that contained both plasmids is the most likely explanation. The three described hot water supply-derived strains and another five hot water supply-derived strains were plasmidless.

The rDNA restriction patterns revealed fewer hybridizing bands than are described for other bacteria (26), but the technique seems to be useful for *L. pneumophila*, and the results for the described strains were in accordance with the results of whole-cell DNA restriction endonuclease analysis. The weaker bands were less consistently discernible, although all weak bands in lanes 1 through 9 of Fig. 1 became visible on prolonged exposures of the autoradiogram.

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