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Molecular Typing of Nosocomial Strains of *Legionella pneumophila* by Arbitrarily Primed PCR

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Arbitrarily primed PCR with two different primers was compared with ribotyping and monoclonal antibody analysis for typing *Legionella* strains. Applied to 11 epidemiologically unrelated strains, arbitrarily primed PCR resulted in an index of discrimination of 100% with both primers. It was found able to identify an epidemic clone of *Legionella pneumophila* serogroup 1 that was isolated from both patients and a hot water circuit of the same hospital.

Nosocomial outbreaks of Legionnaires' disease are observed in immunocompromised patients, especially those hospitalized in intensive care and transplantation units and those receiving corticosteroid therapy (10, 12). Specific markers are needed to trace the transmission of legionellae from environmental reservoirs to patients in order to determine whether strains are clonally related and therefore responsible for the outbreak.

A first case of nosocomial infection due to *Legionella pneumophila* serogroup 1 occurred in our hospital in 1992. At this time, the hot water system was found to be contaminated with *L. pneumophila* serogroup 1. Despite repetitive attempts to eliminate the bacteria from the water system, five additional cases of infection with *L. pneumophila* serogroup 1 were noted over a 3-year period. All patients were immunocompromised. Clinical and environmental strains were retrospectively compared by three methods: monoclonal antibody (MAb) analysis, ribotyping, and PCR with arbitrarily chosen primers (AP-PCR). The determination of the environmental reservoir of the epidemic strain led to an optimization of the disinfection procedures of the water distribution system in the hospital, resulting in a cessation of the outbreak. The results of the molecular investigations are presented herein.

Legionella strains were cultured for 72 h on BCYE agar (buffered charcoal yeast extract supplemented with α-ketoglutarate). MAb subtyping was performed on cultures by immunofluorescence with a panel of 11 subgroup-specific MAbs to *L. pneumophila* serogroup 1 (9). For molecular analysis, bacterial cells were lysed with lysozyme, sodium dodecyl sulfate, and proteinase K, and DNA was purified by phenol-chloroform-isoamyl alcohol extractions (4). Ribotyping was performed with a digoxigenin-labelled rRNA-encoding gene probe after digestion with three different endonucleases, *Hin*dIII, *Cla*I, and *Pvu*II (Boehringer Mannheim), as described previously (4–6). AP-PCR analysis was done on 100 ng of template DNA in a mixture containing 6 μM a 10-mer primer, 200 μM (each) deoxynucleoside triphosphate, and 1.25 IU of *Taq* DNA polymerase (ATGC Biotechnologie) in 10 mM Tris HCl–50 mM KCl–1.5 mM $MgCl_2$ buffer. Two primers were used alternately, 5′-AACGCGCAAC-3′ (primer 1) (6) and 5′-GGTGGTGGCT-3′ (primer 2) (16). Each sample was submitted to a first cycle of denaturation, annealing, and hybridization for 5 min each at 94, 35, and 72°C, respectively, followed by 28 cycles of consecutive denaturation, annealing, and hybridization (94°C, 1 min; 35°C, 2 min; 72°C, 2 min) and a final extension step of 10 min at 72°C (4, 6). Amplimers were separated in a 0.8% agarose gel. A negative control without DNA was included in each reaction mixture. Reproducibility of the profiles was tested in at least two independent experiments. Strains were considered to be linked if they showed identical profiles or if minor differences in the intensity of one of two bands were not confirmed in repetitive experiments or with the use of another primer. The discriminatory power of each typing method was evaluated by the index of discrimination (ID) proposed by Hunter and Gaston (8).

Twenty-seven *Legionella* strains—including 11 epidemiologically unrelated strains, 4 strains of *L. pneumophila* serogroup 1 isolated from four of the six patients mentioned above, and 12 environmental strains of *L. pneumophila* serogroup 1 isolated from different water circuits of the hospital—were analyzed by AP-PCR (Fig. 1) and by ribotyping with *Pvu*II (Fig. 2), *Hin*dIII, or *Cla*I (data not shown). Results are summarized in Table 1. All strains of serogroup 1 were also submitted to MAb subtyping.

AP-PCR—performed with any of the two primers chosen—was found to be much more discriminatory than the two other methods for distinguishing epidemiologically unrelated isolates: the 11 unrelated strains exhibited distinct profiles (strains a to k in Fig. 1), resulting in an ID of 100% with both primers. In comparison, ribotyping with *Cla*I, *Hin*dIII, and *Pvu*II generated six profiles for 10 strains (ID, 78%), seven profiles for 10 strains (ID, 87%), and eight profiles for 11 strains (ID, 93%), respectively. The analysis of the six unrelated strains of *L. pneumophila* serogroup 1 by 11 subspecific MAbs generated five different patterns of recognition (data not shown). Of interest is the fact that two strains of *L. pneumophila* serogroup 1 (f and h) which shared the same ribotypes were differentiated both by AP-PCR and by MAb analysis, pleading for the lower discriminatory power of ribotyping rather than for an excessive sensitivity of AP-PCR (Table 1).

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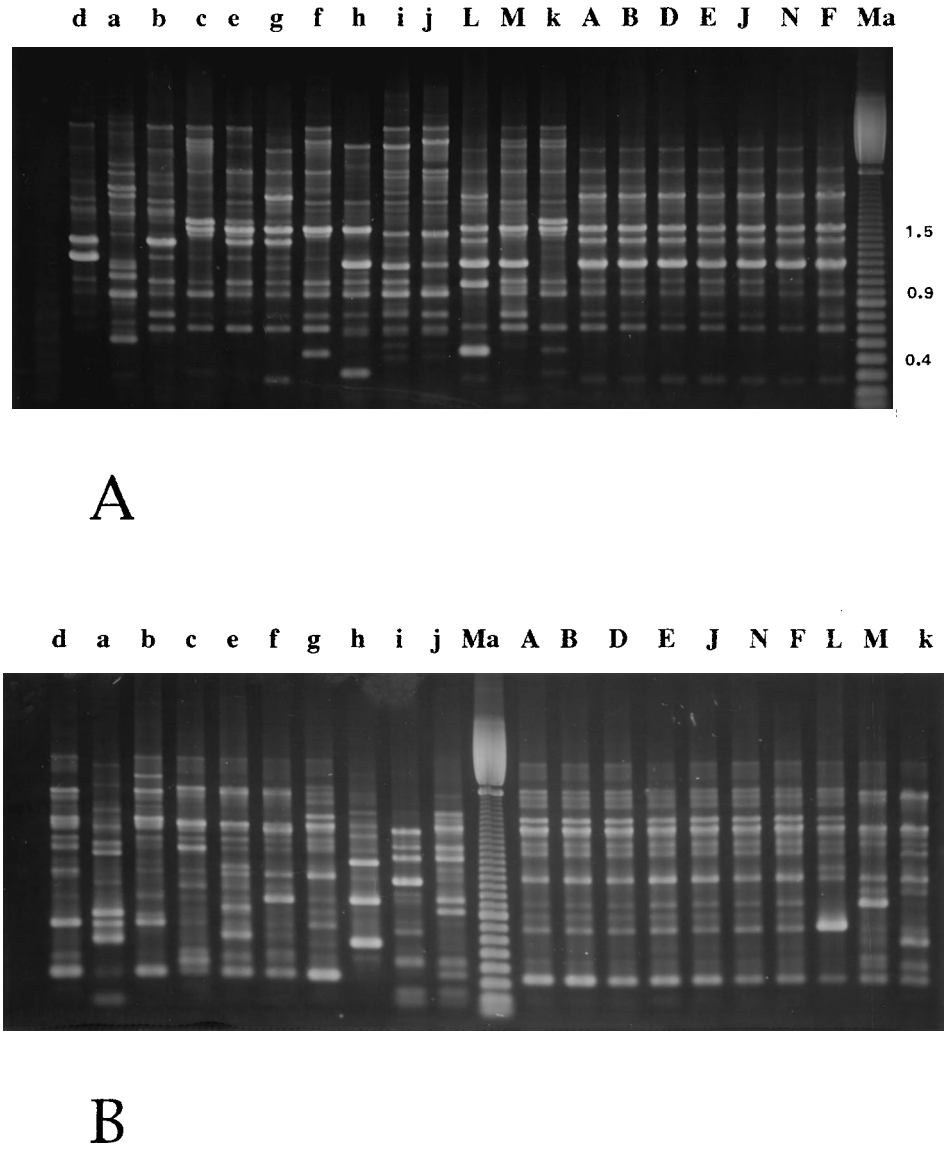

FIG. 1. Representative AP-PCR types of *Legionella* strains, obtained with primer 1 (A) and primer 2 (B). Letters refer to strains described in Table 1. Unmarked lanes correspond to negative controls. Lanes Ma, size markers (in kilobases).

The three typing methods gave concordant results for the epidemic strains of *L. pneumophila* serogroup 1 isolated in Saint-Etienne from patients and from the hot water circuit in relation with their unit of hospitalization (strains A to K in Table 1). All the strains shared a genotypic pattern by AP-PCR and ribotyping, as illustrated in Fig. 1 and 2. All the strains had an identical MAb profile (data not shown) with the exception of strain G, which exhibited a slight phenotypic difference with regard to the epidemic pattern (1 MAb of 14). Antigenic diversity of strains closely related by genomic characters has been already reported (7, 13). Two strains of *L. pneumophila* serogroup 1 (L and M) isolated from other water circuits showed independent AP-PCR profiles (Fig. 1, strains L and M); one of these strains (strain M) was also differentiated from the epidemic strain by ribotyping with *Hin*dIII (data not shown).

Many typing systems have been used to trace the relationship between *L. pneumophila* serogroup 1 isolates (1–3, 7, 11–15). MAb subtyping allows the differentiation of strains belonging to serogroup 1 into a limited number of subgroups. The discriminatory power of this technique was found unsatisfactory since the epidemic pattern was also shared by unrelated isolates from other French hospitals. With the use of three different enzymes, ribotyping provided an ID varying from 78 to 93%. Interestingly, *Pvu*II was the most powerful endonuclease for distinguishing unrelated isolates, a fact we had previously described with other bacterial species (4, 5). However, *Hin*dIII was found able to differentiate one of the two environmental strains isolated from water circuits different from the one implicated in nosocomial cases. These results support the use of a combination of enzymes to increase the discriminatory power of ribotyping, as recommended by Bangsborg et al. (1).

With an ID of 100%, AP-PCR was undoubtedly more discriminative than the two other typing methods. DNA fingerprinting by AP-PCR or by repetitive element PCR has been

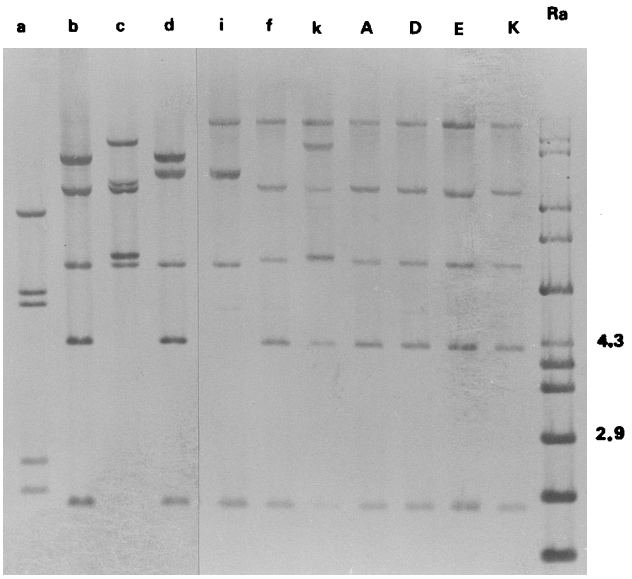

FIG. 2. Representative ribotypes of *Legionella* strains after digestion with *Pvu*II. Letters refer to strains described in Table 1. Lane Ra, size markers (in kilobases).

already applied successfully to the characterization of outbreak-related strains or epidemiologically unrelated strains of *L. pneumophila* serogroup 1 (2, 14, 15) and to the discrimination between *Legionella* isolates within the same serogroup and between different species (2). Furthermore, AP-PCR may distinguish strains sharing the same MAb profile (3, 15). However, since the reproducibility of AP-PCR patterns may vary depending upon the procedure of DNA extraction (3), we recommend use of purified DNA preparations (4, 6) and suggest combination of the results obtained with two different primers before drawing any conclusion about the relatedness between strains. The two primers proposed here were shown to be informative for typing *Enterobacter aerogenes* (6) and *Pseudomonas aeruginosa* (16) species. In this study, we were able to differentiate between the epidemic clone isolated both from case patients and from their water circuit and environmental isolates recovered from other circuits of the same hospital. In the same way, Van Belkum et al. noted that the use of different primers in repetitive element PCR enabled detection of minor genomic variation among *L. pneumophila* strains and that these variations could be correlated with differences detected by pulsed-field gel electrophoresis (15). A recent study demonstrated that the latter method could be more efficient than AP-PCR for typing *Legionella* isolates (11). However, AP-PCR and related techniques are simple, cost-effective, and

TABLE 1. Epidemiological data and molecular characterization of 16 strains of *L. pneumophila* serogroup 1 isolated in Saint-Etienne and of 11 epidemiologically unrelated *Legionella* isolates

Strain designation, sp.[a]	Geographic origin	Source[b]	Yr of isolation	Water circuit[c]	AP-PCR type[d]		Ribotype[d]		
					Primer 1	Primer 2	*Pvu*II	*Hin*dIII	*Cla*I
A, Lp 1	St-Etienne	EI, case I	1992	1	1	1	1	1	1
B, Lp 1	St-Etienne	CI, case III	1993		1	1	1	1	1
C, Lp 1	St-Etienne	EI, case III	1993	1	1	1	1	1	1
D, Lp 1	St-Etienne	CI, case II	1992		1	1	1	1	1
E, Lp 1	St-Etienne	CI, case IV	1993		1	1	1	1	1
F, Lp 1	St-Etienne	CI, case VI	1994		1	1	1	1	1
G, Lp 1	St-Etienne	EI, case IV	1994	1	1	1	1	1	1
H, Lp 1	St-Etienne	EI, case VI	1994	1	1	1	1	1	1
I, Lp 1	St-Etienne	EI, case V	1994	1	1	1	1	1	1
J, Lp 1	St-Etienne	EI, case V	1994	1	1	1	1	1	1
K, Lp 1	St-Etienne	EI	1994	1	1	1	1	1	1
L, Lp 1	St-Etienne	EI	1994	2	2	2	1	1	ND
M, Lp 1	St-Etienne	EI	1994	3	3	3	1	2	ND
N, Lp 1	St-Etienne	EI	1994	3	1	1	1	1	ND
O, Lp 1	St-Etienne	EI	1994	3	1	1	1	1	ND
P, Lp 1	St-Etienne	EI	1995	3	1	1	1	1	ND
a, Lm	ATCC 33218[e]	RS *L. micdadei*			4	4	2	3	2
b, Lp 1	ATCC 33152[e]	RS Philadelphia 1			5	5	3	4	3
c, Lp 1	CDC[f]	RS Pontiac			6	6	4	5	1
e, Lp 1	Grenoble	EI	1991		7	7	1	1	1
f, Lp 1	Grenoble	CI	1990		8	8	1	1	1
g, Lp 1	Briançon	CI	1993		9	9	1	1	1
h, Lp 1	Grenoble	EI	1993		10	10	5	ND	1
d, Lp 3	Lille	EI	NA[g]		11	11	6	6	4
i, Lp 3	Grenoble	EI	1993		12	12	7	6	5
j, Lp 6	Grenoble	EI	1993		13	13	7	6	6
k, Lp 5,10	St-Etienne	EI	1995	4	14	14	8	7	ND

[a] Lp 1, *L. pneumophila* serogroup 1; Lm, *Legionella micdadei.*
[b] EI, environmental isolate; CI, clinical isolate; RS, reference strain.
[c] Numbers refer to different water circuits in Saint-Etienne's hospital.
[d] Numbers refer to different *Legionella* clones. Strains belonging to the same clone are designated by the same number. ND, not determined.
[e] American Type Culture Collection, Rockville, Md.
[f] CDC, Centers for Disease Control and Prevention, Atlanta, Ga.
[g] NA, not available.

time-saving and may be very discriminative when two different primers are used in parallel.

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