

Identification of *Legionella* Species by Random Amplified Polymorphic DNA Profiles

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Random amplified polymorphic DNA (RAPD) was used for the identification of *Legionella* species. Primer SK2 (5'-CGGCGGCGGCGG-3') and standardized RAPD conditions gave the technique a reproducibility of 93 to 100%, depending on the species tested. Species-specific patterns corresponding to the 42 *Legionella* species were consequently defined by this method; the patterns were dependent on the recognition of a core of common bands for each species. This specificity was demonstrated by testing 65 type strains and 265 environmental and clinical isolates. No serogroup-specific profiles were obtained. A number of unidentified *Legionella* isolates potentially corresponding to new species were clustered in four groups. RAPD analysis appears to be a rapid and reproducible technique for identification of *Legionella* isolates to the species level without further restriction or hybridization.

The Philadelphia outbreak of pneumonia in 1976 led to recognition of a new genus, *Legionella* (6), responsible for both legionellosis and Pontiac fever. In 1996, the family *Legionellaceae* contained 42 described species (2) and three subspecies (5). Usually, the bases for the identification of *Legionella* species are cultural characteristics, biochemical reactions, and serotyping by slide agglutination test or direct immunofluorescence assay (7, 15, 16, 26). Additionally, analysis of whole-cell fatty acids, ubiquinone contents, and whole-cell protein profiles can be useful to achieve identification of *Legionella* isolates at the species level (3, 17, 29). However, in several instances, as for the bluish white autofluorescent species and all of the cross-reacting serogroups, these methods do not discriminate to a sufficient extent for definitive species identification. In these cases, genotypic methods such as DNA-DNA hybridization (4) and ribotyping (13) are the only means for identification at the species level.

One of the new genotypic methods, known as random amplified polymorphic DNA (RAPD) analysis (30, 31), has been used mostly for intraspecies discrimination in epidemiological studies (12, 22, 27). However, for some other taxa, such as *Enterococcus*, *Candida*, and *Leptospira* species, this method has been successfully applied for species identification (9, 18, 23). At first, RAPD analysis was used to differentiate the bluish white autofluorescent *Legionella* species, allowing identification for the first time of a clinical isolate of *L. parisiensis* (19). Use of RAPD analysis for identification of *Legionella* species has recently been reported by Bansal and McDonell, but that investigation was limited by the use of type strains of *Legionella* species and included nonreference isolates only for *Legionella pneumophila* (1). In the present study, we have evaluated and validated RAPD analysis for the identification of *Legionella* species by using a large number of strains representing 42 species. Importantly, this method has been optimized for our laboratory conditions, as exemplified by its nearly complete reproducibility.

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MATERIALS AND METHODS

Bacterial strains. The strains used in this study included 65 type strains of 42 species (plus three *L. pneumophila* subspecies) and 64 serogroups, 28 well-defined strains described in the literature, and 186 environmental and clinical isolates from patients with pneumonias that were collected by the French National Reference Center for *Legionella* (Table 1). Fifty-one strains belonging to four potentially new species were also tested (Table 1). All of the strains were tested after being conserved in liquid nitrogen. Some isolates were also tested as fresh isolates or after lyophilization.

Bacteriologic methods. Bacteria were grown on buffered charcoal yeast extract (BCYE) agar supplemented with 0.1% α -ketoglutarate in 2.5% CO₂ (11) and, when required, on glycine-vancomycin-polymyxin B-colistin BCYE agar plates. Controls were prepared with unsupplemented BCYE agar and blood agar plates (3). Environmental and clinical isolates were identified by combinations of biochemical reactions (16), direct immunofluorescence assay with unadsorbed and adsorbed sera (15), ubiquinone content and whole-cell fatty acid composition analysis (3), and whole-cell protein profiling (3). Bacteria were lysed by the boiling water method as previously described (19), and DNA extracts were quantified spectrophotometrically.

Choice of primer. Three primers were tested for the ability to differentiate *Legionella* species: SK2 (5'-CGGCGGCGGCGG-3') (21), 910-05 (5'-CCGGCGGCG-3') (20), and BG2 (5'-TACATTCGAGGACCCTAAGTG-3') (27).

Optimization of PCR conditions. Optimal conditions for each primer were achieved by using the Taguchi method as modified by Cobb and Clarkson (8). The development of the required conditions was at first attained with *L. anisa* ATCC 35292, *L. bozemanii* serogroup 1 strain ATCC 33217, *L. micdadei* ATCC 33218, *L. pneumophila* serogroup 1 strain ATCC 33152, and *L. pneumophila* serogroup 3 strain ATCC 33155.

Optimized RAPD technique. RAPD was performed with primer SK2 as follows. A 5- μ g sample of DNA, 3 mM MgCl₂, 0.4 mM each primer, 2.5 U of *Taq* DNA polymerase (Amplitaq; Perkin-Elmer Cetus, Branchburg, N.J.), and 0.1 mM each deoxynucleoside triphosphate (Pharmacia Biotech, Uppsala, Sweden) were mixed for each sample reaction in 1 \times PCR buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 0.001% gelatin; Perkin-Elmer Cetus). PCR cycles were as follows: 4 min of denaturation at 94°C followed by 42 cycles of 1 min at 94°C, 1 min at 30°C, and 2 min at 72°C and a final extension step of 72°C for 3 min. PCR products were then separated on standard 1.5% agarose gel (SeaKem GTG; FMC BioProducts, Rockland, Maine), stained with ethidium bromide, and photographed under UV light with an MP-4 Camera (Polaroid, Cambridge, Mass.). The profiles were analyzed by the Taxotron package (Institut Pasteur, Paris, France) using the Dice coefficient (10); clustering was based on the single-linkage method. A linear error of 2% at 100 bp to 3% at 3,500 bp was tolerated.

Reproducibility of the RAPD assay. The reproducibility of the method was assessed by 20 independent PCR assays with primer SK2 for each reference strain with different DNA extracts. A control strain, *L. pneumophila* serogroup 1 strain Philadelphia-1, was included in each RAPD assay.

RESULTS

Optimization. The conditions were optimized for DNA, MgCl, and primer concentrations, template annealing temper-

TABLE 1. *Legionella* strains used in this study

Species (serogroup)	No. of strains	Strain designation(s) ^a
<i>L. adelaidensis</i>	1	ATCC 49625 ^T
<i>L. anisa</i>	24	ATCC 35292 ^T , CH47-C1 ^L , CH47-C3 ^L , Card76A1, Cobac, FMI-3, G11-1, Go1-1, Go1-2, HD10K2, HEH15D3, PaDebIH3, S14-14, S14-17, SIX-1, SIX-2, S17-5, S18-1, S97XXIII1, StSil-7, Tour97I-1, Tour97I-2, Tour97I-3, Tour97I-4
<i>L. birminghamensis</i>	4	ATCC 43702 ^T , A12C15, CFVII3A, Greo8P2
<i>L. bozemanii</i> (1)	8	ATCC 33217 ^T , Arizona-1 ^L , GAPH ^L , Bruxelles D ^L , DignB111, DignC61, Ly86.88, PortII-18
<i>L. bozemanii</i> (2)	4	ATCC 35545 ^S , Paris-96010250, Paris-96010251, UL-7T/2
<i>L. brunensis</i>	4	ATCC 43878 ^T , FS-60, Ji-642, OV-182
<i>L. cherii</i>	1	ATCC 35252 ^T
<i>L. cincinnatiensis</i>	2	ATCC 43753 ^T , IB97III3
" <i>L. donaldsonii</i> "	1	LC 878
<i>L. dunoffii</i>	9	ATCC 33279 ^T , ATCC 35850 ^L , A80B3, A97I-1, A97I-2, L2F10, S-92101226, T23-3, T23-6
<i>L. erythra</i> (1)	5	ATCC 35303 ^T , A96VII2-1, AngIVLev, CEP3F29, T26-20
<i>L. erythra</i> (2)	5	LC217 ^S , A96VII2-6, M12-6, S96XXII-5, UL-13
<i>L. fairfieldensis</i>	1	ATCC 49588 ^T
<i>L. feeleeii</i> (1)	4	ATCC 35072 ^T , Ly126.92a, Ly126.92b (ATCC 700513), Ly166.96 (ATCC 700514)
<i>L. feeleeii</i> (2)	1	ATCC 35849 ^S
<i>L. geestiana</i>	2	ATCC 49504 ^T , ParDid16-1
<i>Legionella</i> genomospecies 1	1	ATCC 51913 ^T
<i>L. gormanii</i>	4	ATCC 33297 ^T , Greo9C3, Greo9C5, Toul29-16
<i>L. gratiana</i>	1	ATCC 49413 ^T
<i>L. hackeliae</i> (1)	1	ATCC 35250 ^T
<i>L. hackeliae</i> (2)	1	ATCC 35299 ^S
<i>L. israelensis</i>	1	ATCC 43119 ^T
<i>L. jamestowniensis</i>	1	ATCC 35298 ^T
<i>L. jordanis</i>	3	ATCC 33623 ^T , BesacII-1, Ly95.96
<i>L. lansingensis</i>	1	ATCC 49751 ^T
<i>L. londiniensis</i>	16	ATCC 49505 ^T , A52F94, CF17-1, CF97I-1, Co9C3, Dij13-1, Frankfurt 3, Grèce 5-1, IB35-1, IP8-1, IP13-1, IZ-200 ^L , Mulhouse B26 (ATCC 700510), Mulh12A1, NancV-4, Port5
<i>L. longbeachae</i> (1)	3	ATCC 33462 ^T , BruxIII-3, 88010337
<i>L. longbeachae</i> (2)	1	ATCC 33484 ^S
<i>L. maceachernii</i>	2	ATCC 35300 ^T , Toul20-5
<i>L. micdadei</i>	12	ATCC 33218 ^T , A73G9, CF97I-2, LugII-124, Lyon-91103670, Nantes-93101936, PortIII-10, PPA ^L , T16-5, T29-9, Toulon VI-5, Virginia-1 ^L
<i>L. moravica</i>	1	ATCC 43877 ^T
<i>L. nautarum</i>	1	ATCC 49506 ^T
<i>L. oakridgensis</i>	15	ATCC 33761 ^T , A31H1, Dign2C34, Dij6-2, Greo8E18, Greo8P12, Lille A1, N3, NIV-1, NIV-2, NV-2, NV-6, Nantes-930101937 (ATCC 700516), Nantes-93101868 (ATCC 700515), PortII-16
<i>L. parisiensis</i>	3	ATCC 35299 ^T , FLP1 ^L , FLP2 (ATCC 700174) ^L
<i>L. pneumophila</i>	96	ATCC 33152 ^T , ATCC 33154 ^S , ATCC 33155 ^S , ATCC 33156 ^T , ATCC 33216 ^S , ATCC 33215 ^S , ATCC 33823 ^S , ATCC 35096 ^S , ATCC 35289 ^S , ATCC 43283 ^S , ATCC 43130 ^S , ATCC 43290 ^S , ATCC 43736 ^S , ATCC 43703 ^S , ATCC 35251 ^S , ATCC 33735 ^L , ATCC 33737 ^T , Bellingham-1 ^L , Benidorm 030E ^L , Cambridge-2 ^L , Detroit-1 ^L , CFIIIIC, Golf7-9, HEH96IVe1c3, HEH96IVe2c3, IP23-1, IP23-2, Knoxville-1 ^L , L3, L12, L13, L23, L27, L31, L32, L33, L34, L35, L36, L37, L38, L39, L40, L41, L42, L43, L44, L45, L46, L47, L48, L51, L52, L54, L59, L60, L61, L62, L64, L65, L66, L67, L68, L71, L211, L212, L214, L215, LisbIII A10, Lisb95-1, Lisb95-2, Lisb95-3, Lisb95-4, Lisb95-5, Lisb95-6, Lisb95-7, Lisb95-8, Lyon-96010018, Ly154.95, Mars96II1, Mars96II2, Mars96II3, MI97122245, Nancy-96010154, NevIN1, NevIN2, NevIIIA1, NevIIIA2, Nevers-950102440, Nice-96010131, OLDA ^L , Oxford-1 ^L , Pontiac ^L , Portland-1 ^L , S96XIII16, S96XIV4
<i>L. quateirensis</i>	1	ATCC 49507 ^T
<i>L. quinlivanii</i> (1)	8	ATCC 43830 ^T , 1448-AUS-E ^L , 1449-AUS-E ^L , 1451-AUS-E ^L , 1452-AUS-E ^L , 2359-AUS-E ^L , Lech2A10, Lech2A47
<i>L. quinlivanii</i> (2)	1	NCTC 12434 ^S
<i>L. rubrilucens</i>	8	ATCC 35304 ^T , A56, Ang6-1, Dij45-1, Dij48-2, Gen2, Lech2E58, VilleA1
<i>L. sainthelensi</i> (1)	1	ATCC 35248 ^T
<i>L. sainthelensi</i> (2)	5	ATCC 49322 ^S , Bord-97010302, ED-4a ^L , IZ-86 ^L , Ly176.97 (ATCC 700517)
<i>L. santicrucis</i>	2	ATCC 35301 ^T , A9C27
<i>L. shakespearei</i>	1	ATCC 49655 ^T
<i>L. spiritensis</i> (1)	2	ATCC 35249 ^T , IB96VI-1
<i>L. spiritensis</i> (2)	2	NCTC 12082 ^S , Lnetti
<i>L. steigerwaltii</i>	1	ATCC 35302 ^T
<i>L. tucsonensis</i>	1	ATCC 49180 ^T
<i>L. wadsworthii</i>	2	ATCC 33877 ^T , Dign2C67
<i>L. waltersii</i>	1	ATCC 51914 ^T
<i>L. worsleiensis</i>	3	ATCC 49508 ^T , SBPIB1, SBPIB2
<i>Legionella</i> sp. 1	3	Greo11D13 (ATCC 700509), Mulh12A22, Mulh12A23 1
<i>Legionella</i> sp. 2	3	IBV-2, IBV-3 (ATCC 700511), VeniIA
<i>Legionella</i> sp. 3	5	MontA1 (ATCC 700512), MontA2, MontA3, MontA4, MontA5
<i>Legionella</i> sp. 4	40	Turin I no. 1 (ATCC 700508), Turin I-2, Turin II-180, Turin II-195, BM-750, C/SE1C25, C/SE1C26, CrRo2C9, CrRo2C13, GapIB41, GapIB42, Gen10-1, HD2B3, HD2B4, HD2B12, HGe1C7, HGe13C7, HGe13C10, IB39-6, Laus15-6, Laus96V-1, LisbIIIA5, MI-4, MIV-1, MoulIC1, MoulID5, MoulID6, Merc96I8-7, Merc96I8-8, PortA12, PortA13, PortA14, PortA16, PortA17, PortA19, PortA20, T26-14, T26-17, T28-7, T28-8

^a Superscripts: L, strain described in the literature; S, serogroup type strain; T, species type strain.

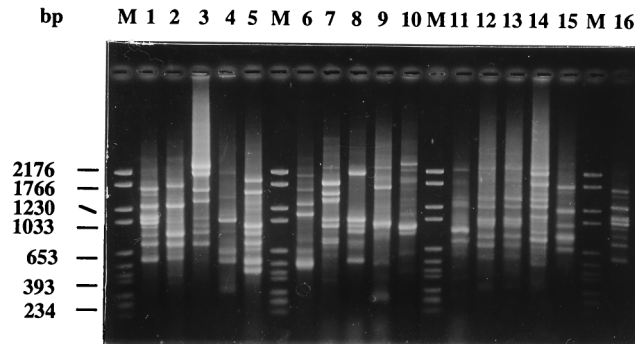


FIG. 1. RAPD amplification patterns of *Legionella* type strains. Lanes: M, molecular weight marker VI (Boehringer Mannheim); 1 and 16, *L. pneumophila* serogroup 1 strain ATCC 33152; 2, *L. pneumophila* serogroup 3 strain ATCC 33155; 3, *L. micdadei* ATCC 33218; 4, *L. maceachernii* ATCC 35300; 5, *L. anisa* ATCC 35292; 6, *L. bozemanii* serogroup 1 strain ATCC 33217; 7, *L. parisiensis* ATCC 35299; 8, *L. gratiana* ATCC 49413; 9, *L. tucsonensis* ATCC 49180; 10, *L. longbeachae* serogroup 1 strain ATCC 33462; 11, *L. longbeachae* serogroup 2 strain ATCC 33484; 12, *L. erythra* serogroup 1 strain ATCC 35303; 13, *L. erythra* serogroup 2 strain LC217; 14, *L. rubrilucens* ATCC 35304; 15, *L. spiritensis* serogroup 1 strain ATCC 49655.

ature, and number of PCR cycles by using the modified Taguchi method. Conditions allowing the best readable patterns were therefore chosen for further experiments. The RAPD fingerprints obtained with primer SK2 gave 2 (i.e., for *L. steigerwaltii*) to 11 (i.e., for *L. micdadei*) DNA bands with various intensities ranging in size from 200 bp to approximately 3 kbp. Within a species, primer SK2 was found to generate more fragments common to nonepidemiologically related isolates, whereas primer BG2 generated very diverse patterns (data not shown).

Reproducibility. For all of the reference strains tested under these optimized RAPD assay conditions, over 20 independent determinations, the reproducibility of the patterns ranged from 93 to 100%, depending on the species tested. For instance, 51 of the 55 profiles obtained for strain Philadelphia-1 (*L. pneumophila* serogroup 1) were strictly identical, whereas 4 profiles differed by only 1 or 2 minor bands over a total of 14 bands.

Differentiation of *Legionella* type strains. A type strain-specific pattern was obtained for the 42 species. Species with more than one serogroup, except *L. pneumophila*, *L. bozemanii*, *L. longbeachae*, and *L. sainthelensi*, did not present additional profiles corresponding to each serogroup and showed only a species-specific profile. For *L. pneumophila*, minor variations were detected among the 15 serogroups tested, but a core of three common bands (750, 1,080, and 1,675 bp) deduced from a total number of bands varying from 5 to 14 and corresponding to a species-specific pattern was observed in all strains (Fig. 1, lanes 1 and 2). *L. bozemanii* serogroups 1 and 2 had five DNA fragments in common and were differentiated by four (putatively) serogroup 1-specific bands and two (putatively) serogroup 2-specific bands. The two serogroup type strains of *L. longbeachae* had seven fragments in common (Fig. 1, lanes 10 and 11), the same as for *L. sainthelensi*. Each serogroup type strain of these two species also contained (putatively) strain-specific fragments.

Identification of *L. pneumophila* isolates. For the 96 *L. pneumophila* strains tested (16 serogroup type strains, 10 strains described in the literature, and 70 environmental and clinical isolates), 50 profiles were produced. The species-specific pattern made by three bands (described above) was always present, whatever the serogroups or the subspecies tested (Fig. 2). These three bands were simultaneously present only in the case of *L. pneumophila* strains. The following additional DNA

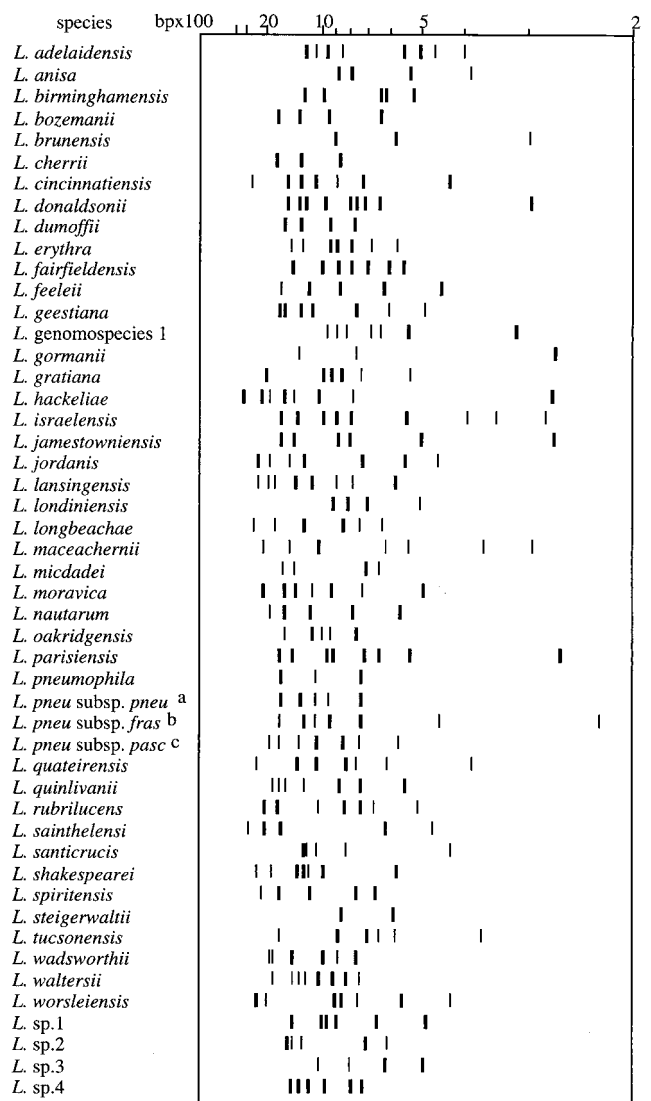


FIG. 2. Schematic core of species- and subspecies-specific patterns of *Legionella* species. Schematic of RAPD patterns constructed with Taxotron software. The bands represented are those always present in a given species; bands are represented according to their intensities. a, *L. pneumophila* subsp. *pneumophila*; b, *L. pneumophila* subsp. *fraseri*; c, *L. pneumophila* subsp. *pascullei*.

fragments were frequently found: 1,296 bp (95% of the strains), 966 bp (82%), and 575 bp (79%). Neither serogroup nor monoclonal subtype patterns were observed. However, the four available strains of *L. pneumophila* subsp. *fraseri* exhibited three other bands simultaneously in their patterns (1,247, 455, and 227 bp). The latter three bands were never found in the other subspecies of *L. pneumophila*. At the time of the study, the two available strains of *L. pneumophila* subsp. *pascullei* showed the same profile, while their subspecies-specific bands (1,950 and 860 bp) were never present in the other two subspecies. According to these results, strains belonging to *L. pneumophila* subsp. *pneumophila* could be differentiated from the subspecies *pascullei* by the presence of a 966-bp DNA fragment and the absence of the two bands specific to *L. pneumophila* subsp. *pascullei* (Fig. 2).

Identification of *Legionella* species. Of the 234 non-*L. pneumophila* strains studied (49 serogroup and species type strains,

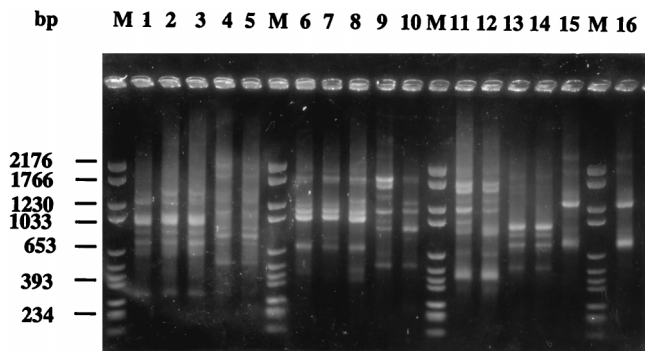


FIG. 3. Visual identification of *Legionella* isolates by RAPD patterns. Lanes: M, molecular weight marker VI (Boehringer Mannheim); 1, *L. erythra* serogroup 1 strain ATCC 35302; 2, *L. erythra* serogroup 2 strain LC217; 3, *L. erythra* serogroup 2 strain Aix96VII2-1; 4, *L. rubrilucens* aix56; 5, *L. rubrilucens* ATCC 35304; 6, *L. bozemanii* serogroup 1 strain GAPH; 7, *L. bozemanii* serogroup 1 strain Bruxelles D; 8, *L. bozemanii* serogroup 1 strain DignB111; 9, *L. bozemanii* serogroup 2 strain Paris-96010250; 10, *L. bozemanii* serogroup 2 strain ATCC 35545; 11, *L. feeleii* serogroup 1 strain ATCC 35072; 12, *L. feeleii* serogroup 1 strain Ly126.92a; 13, *L. londiniensis* ATCC 49505; 14, *L. londiniensis* IP8-1; 15, *L. jordanis* ATCC 33623; 16, *L. jordanis* Ly95.96.

18 strains described in the literature, 116 environmental and clinical isolates of known species, and 51 isolates unidentified at the species level), 135 profiles were obtained. Species-specific fragments were found when multiple isolates of a species were analyzed (Fig. 3), supporting the existence of type strain-specific patterns, as noted above. For these, the number of common fragments varied among the species from three (*L. gormanii*) to eight (*L. parisiensis*). Again, no serogroup-specific patterns were found (Fig. 3, lanes 1 to 3). For *L. bozemanii*, a species-specific pattern was confirmed but not the serogroup-specific patterns proposed for the type strains. For the species *L. longbeachae* and *L. sainthelensi*, no serogroup-specific patterns were found either.

After numerical comparisons with the Taxotron package, the 330 strains (*L. pneumophila* included) were grouped into species-specific clusters. The 51 unidentified isolates were clustered into four groups corresponding to four potentially new genomic species (data not shown).

DISCUSSION

In the present study, we have evaluated the RAPD assay technique for the identification of *Legionella* species. Since preliminary experiments revealed that the method suffered from low reproducibility, our efforts were applied to optimization of this technique. Therefore, we examined conditions that would allow maximum reproducibility and reliable typeability for *Legionella*. The modified Taguchi method (8) enabled us to attain PCR conditions that generated reproducible profiles for all of the species tested. Primer SK2 proved to be an efficient tool for taxonomic purposes, whereas primer BG2 confirmed its usefulness for the previously demonstrated intraspecies differentiation of *Legionella* (27). It should be noted that the RAPD assay method using various other primers has also been applied for this purpose in other studies (12, 22, 27).

The realization of type strain-specific patterns corresponding to the 42 *Legionella* species led us to evaluate the intraspecies stability of RAPD profiles by testing 265 environmental and clinical isolates. By observing that multiple well-identified strains of a given species had a core of bands in common, we demonstrated that minimal profiles can be proposed for each species. In fact, this was found even for the bluish white

autofluorescent species and the red autofluorescent species, which are otherwise difficult to identify. For *L. pneumophila*, we were able to obtain a minimal profile for each of the subspecies, thus allowing them to be differentiated from each other. However, in most cases, the level of taxonomic resolution did not reach the serogroup level, in that no serogroup-specific profile was identified when multiple strains were tested. This observation is in agreement with previous findings which show that isolates from different serogroups or different monoclonal antibody subtypes can have identical genotypes (14, 22, 25, 28) and that strains from different *L. pneumophila* subspecies can belong to a unique serogroup (5, 24). Hence, the claim of others that serogroup-specific profiles can be obtained by RAPD analysis was probably too optimistic and could well be due to the fact that a limited number of strains were tested (type strains only) (1).

Several species were tested with only one isolate because no other isolates were available, and the number of bands forming these species-specific profiles will probably decrease when more strains are tested. This point reinforces the need for exchanges of rarely isolated bacterial species between microbiologists.

Analysis of RAPD profiles with the Taxotron package confirmed the visual identification and allowed isolates of each species to be clearly clustered together. The standardization of the method provided a convenient way to compare patterns obtained from independent gel runs. This method of analysis has proved to be extremely useful for determining the most probable species assignment of a given isolate. The entire procedure can be done in 10 h (2 h for DNA extraction, 5 h for the PCR procedure, and 3 h for electrophoresis) but remains practicable for laboratories having all of the *Legionella* reference strains. Moreover, it contributes to the identification of potentially new species by grouping a number of unidentified isolates into four additional clusters which were unrelated to those corresponding to the type strains.

In conclusion, it appears that once optimized, the RAPD technique is a rapid, reproducible, and powerful method for identification of *Legionella* isolates to the species level without further restriction or hybridization.

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