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Species identification of *Legionella* via intergenic 16S–23S ribosomal spacer PCR analysis

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Species identification of *Legionella* in routine laboratory testing is hampered by the lack of highly discriminatory phenotypic tests. Amplification polymorphism of the intergenic 16S–23S spacer regions (ISR) has been previously developed for identification of species within the *Legionellaceae* [Hookey, J. V., Birtles, R. J. & Saunders, N. A. (1995). *J Clin Microbiol* 33, 2377–2381], but it did not provide enough resolution to distinguish all members of the bluish-white autofluorescent species and the red autofluorescent group of the *Legionellaceae*. By choosing new primers that target regions 4 (positions 1521–1541 of *Escherichia coli* 16S rRNA gene) and 6 (positions 114–132 of *E. coli* 23S rRNA gene) within the rDNA operon close to the 16S–23S intergenic spacer, 34 profiles were determined among the 79 type and reference strains representing 42 species that were tested. Analysis of the RFLP generated after *Hin*fI restriction digestion of the PCR products further improved the method, allowing complete discrimination among the species and subspecies of *Legionella* tested. Twenty-three well-identified strains from unrelated origins belonging to seven species gave amplification patterns identical to that of their type strain. The technique was also tested on 80 field isolates that could not be unequivocally assigned to groups by phenotypic methods. Seventy-two per cent (58/80) of these isolates had a profile identical to that of a type strain, while 27% (22/80) may correspond to new taxa since their ISR-PCR profiles did not match any of the known profiles.

Keywords: *Legionellaceae*, 16S–23S intergenic spacer, ISR-PCR

INTRODUCTION

The genus *Legionella* is represented at the present time by 42 species (2, 10). The recognition of *Legionella* species is based on the study of classical phenotypic traits including growth characteristics, biochemical reactivity and direct fluorescent antibodies typing (DFA) (19). However, these methods do not allow differentiation of all species and 16S rDNA sequencing (10) or DNA–DNA hybridization (3) are sometimes the sole methods allowing the recognition of species. Other genotypic methods such as ribotyping (5), arbitrarily primed PCR (AP-PCR) and random amplified polymorphic DNA (RAPD) analysis have been proposed for differentiation of *Legionella* at the species level (13). However, ribotyping is a time-consuming method and is not suitable for routine identification of *Legionella* (5), while AP-PCR and RAPD are hampered by their lack of reproducibility (15). Amplification polymorphism of the intergenic 16S–23S spacer regions (ISR) have been used for identification at the species level in various genera including *Legionella* (6, 8, 9, 12, 16, 17). However, in the case of *Legionella*, the primers used by Hookey *et al*. for the ISR-PCR were targeting regions situated further away from the ISR itself, thus amplifying 400 nt of the 16S gene, resulting in larger amplicons that were harder to

Abbreviations: AP-PCR, arbitrarily primed PCR; DFA, direct fluorescent antibodies; ISR, intergenic 16S–23S spacer region; RAPD, random amplified polymorphic DNA.

The GenBank accession numbers for the sequences or partial sequences of Philadelphia-1, Los Angeles-1 and Micu-B3 strains of *L. pneumophila* are AF000654, AF000655 and AF000656, respectively.

Table 1. *Legionella* strains tested for the 16S–23S rDNA ISR-PCR assay

Clustering as determined by ISR-PCR (after restriction when necessary). ATCC, American Type Culture Collection; NCTC, National Collection Type Culture; ^{T}, type strains; ^{L}, well-identified strains described in the literature. Other strains are field isolates obtained from the French *Legionella* Reference Centre.

Species	Cluster	No. strains	Reference strains and isolates tested
L. adelaidensis	1	1	ATCC 49625^{T}
L. anisa	2	23	ATCC 35292^{T}, CH47-C3^{L}, CH47-C1^{L}, Card76A1, FoMeI-3, Gene11-1, Golf1-1, Golf1-2, HD10K2, HEH15D3, PaDebIH3, Stra14-14, Stra14-17, StraIX-1, StraIX-2, Stra17-5, Stra18-1, StSimI-7, Tour97I-1, Tour97I-2, Tour97I-3, Tour97I-4, Cob1
L. birminghamensis	3	3	ATCC 43702^{T}, Aix12C15, Greo8P2
L. bozemaniae serogroup 1	4	6	ATCC 33217^{T}, Arizona-1^{L}, GAPH^{L}, BruxellesD^{L}, DignC61, PortII-18
L. bozemaniae serogroup 2		4	ATCC 35545^{T}, Paris-96010250, Paris-96010251, UL-7T/2
L. brunensis	5	1	ATCC 43878^{T}
L. cherrii	6	1	ATCC 35252^{T}
L. cincinnatiensis	7	1	ATCC 43753^{T}
'*L. donaldsonii*'*	8	1	LC878
L. dumoffii	9	8	ATCC 33279^{T}, ATCC 35850^{L}, Aix80B3, Aix96VII2-4, Aix96VII7-1, Lech2F10, Stra-92101226, Toul23-6
L. erythra serogroup 1	10	5	ATCC 35303^{T}, Aix96VII2-1, AngeIVLevant, CEP3F29, Toul26-20
L. erythra serogroup 2		5	NCTC 11987^{T}, Aix96VII2-6, Madr12-6, Stra96XXII-5, UL-13
L. fairfieldensis	11	1	ATCC 49588^{T}
L. feeleii serogroup 1	12	4	ATCC 35072^{T}, Ly126.92a, Ly126.92b, Ly166.96
L. feeleii serogroup 2		1	ATCC 35849
L. geestiana	13	2	ATCC 49504^{T}, ParDid16-1
Legionella genomospecies 1	14	1	ATCC 51913^{T}
L. gormanii	15	4	ATCC 33297^{T}, Greo9C3, Greo9C5, Toul29-16
L. gratiana	16	1	ATCC 49413^{T}
L. hackeliae serogroup 1	17	1	ATCC 35250^{T}
L. hackeliae serogroup 2	18	1	ATCC 35999
L. israelensis	18	1	ATCC 43119^{T}
L. jamestowniensis	20	1	ATCC 35298^{T}
L. jordanis	21	3	ATCC 33623^{T}, BesacII-1, Ly95.96
L. lansingensis	22	1	ATCC 49751^{T}
L. londiniensis serogroup 1	23	7	ATCC 49505^{T}, CF97I-1, Frankfurt3, IP8-1, IB35-1, Gre5-1, Mul12A1
L. londiniensis serogroup 2		1	MulhB26
L. longbeachae serogroup 1	24	1	ATCC 33462^{T}
L. longbeachae serogroup 2		1	ATCC 33484
L. maceachernii†	25	2	ATCC 35300^{T}, Toul20-5
L. micdadei	26	10	ATCC 33218^{T}, CF97I-2, PPA^{L}, Virginia-1^{L}, PortIII10, Toul29-9, Aix73G9, ToulonVI-5, Toul16-5, LugII124
L. moravica	27	3	ATCC 43877^{T}, CoimbS20, NancII-1
L. nautarum	28	1	ATCC 49506^{T}
L. oakridgensis	29	13	ATCC 33761^{T}, Aix31H1, Dign2C34, Dijo6-2, Greo8E18, LillA1, Nant3, NantIV-1, NantV-2, NantV-6, Nant-930101937, Nant-93101868, PortII-16
L. parisiensis	30	2	ATCC 35299^{T}, ATCC 700174^{L}
L. quateirensis	31	1	ATCC 49507^{T}

Table 1. (*cont.*)

Species	Cluster	No. strains	Reference strains and isolates tested
L. pneumophila	32	75	ATCC 33823, subsp. *pneumophila* (ATCC 33152^{T}, ATCC 35096, ATCC 33154, ATCC 33155, ATCC 33215, ATCC 35289, ATCC 43130, ATCC 43283, ATCC 43290, ATCC 43703, ATCC 43736), subsp. *fraseri* (ATCC 33156^{T}, ATCC 33216, ATCC 35251), subsp. *pascullei* (ATCC 33735), Bellingham-1^{L}, Benidorm 030E^{L}, Bon, Brag97I, Cambridge-2^{L}, Detroit-1^{L}, CFIII1C, IP23-1, IP23-2, Jac, Knoxville-1^{L}, Lisb95-1, Lisb95-2, Lisb95-3, Lisb95-4, Lisb95-5, Lisb95-6, Lisb95-7, Lisb95-8, Mars96II1, Mars96II2, NevIN1, NevIN2, NevIIIA1, NevIIIA2, OLDA^{L}, Oxford-1^{L}, Pontiac^{L}, Portland^{L}, Rab, NantL3, PauL12, ToulL13, ColmL23, ReimL27, StOmL47, NeufL48, StBriL51, CretL52, MarsL54, MorsbL31, HEH96IV2-1, HEH96IV1-3, Laus96II3, NancL392, Stra96XIII16, Poi96III2, Stra96XIV4, Golf96I, NiceL403, NanL215, StEt92I1, StEt95VII2, StEt95VII4, StEt95VIII3, StEt95VIII5, LisbIVA10, StEtVII1, StEtVIII4
L. quinlivanii serogroup 1	33	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
L. quinlivanii serogroup 2		1	NCTC 12434
L. rubrilucens	34	48	ATCC 35304^{T}, Aix56, Ango6-1, Dijo45-1, Dijo48-2, Gene2, Lech2E58, VillefA1, TuriI-1, TuriI-2, TuriII-180, TuriII-195, Laus15-6, Gene10-1, Laus96V-1, HoDi2B3, HoDi2B4, HoDi2B12, Toul26-14, C/SE1C25, C/SE1C26, GapIB41, GapIB42, MoulIC1, MoulID1, MoulID6, IB39-6, Toul26-17, Toul28-7, Merc96I8-8, Merc96I8-7, HeGaIe1C7, HeGae3C7, HeGae3C10, CrRo2C9, CrRo2C13, LisbIIIA5, PortA12, PortA13, PortA14, PortA16, PortA17, PortA19, PortA20, MadrI-4, MadrIV-1, BM-750, Toul28-8
L. sainthelensi serogroup 1	35	1	ATCC 35248^{T}
L. sainthelensi serogroup 2		1	ATCC 49322
L. santicrucis	36	1	ATCC 35301^{T}
L. shakespearei	37	1	ATCC 49655^{T}
L. spiritensis serogroup 1	38	1	ATCC 35249^{T}
L. spiritensis serogroup 2	39	1	NCTC 12082
L. steigerwaltii	40	1	ATCC 35302^{T}
L. tucsonensis	41	1	ATCC 49180^{T}
L. wadsworthii	42	1	ATCC 33877^{T}
L. waltersii	43	1	ATCC 51914^{T}
L. worsleiensis	44	3	ATCC 49508, SBPIB1, SBPIB2
Legionella sp. type 1	45	3	Greo11D13, Mulh12A22, Mulh12A23
Legionella sp. type 2	46	3	IBV-2, IBV-3, VeniIA1
Legionella sp. type 3	47	1	DignB112
Legionella sp. type 4	48	1	RochA21
Legionella sp. type 5	49	1	HEH11G3
Legionella sp. type 6	50	1	Francf3
Legionella sp. type 7	51	1	Toul24-16
Legionella sp. type 8	52	1	Corse13B8
Legionella sp. type 9	53	1	Toul28-5, CFVII-1
Legionella sp. type 10	54	1	IP8-1
Legionella sp. type 11	55	2	MadVII-1, Mad12-2
Legionella sp. type 12	56	1	Nevers1O1
Legionella sp. type 13	57	5	MontA1, MontA2, MontA3, MontA4, MontA5

* Kindly provided by T. G. Harrison (*Legionella* Reference Unit, Respiratory and Systematic Infection Laboratory, Central Public Health Laboratory, Colindale, London, UK).

† Validated as *Tatlockia maceachernii* comb. nov. [Validation List no. 38; IJSB **41**, 456–457 (1991)].

resolve and did not provide enough discrimination to distinguish members of the bluish-white autofluorescent species of the *Legionellaceae* (9). In the present study, consensus primers designed from multiple alignment of 16S and 23S genes of diverse bacteria (17, 18) were tested for their ability to differentiate all *Legionella* species including the bluish-white autofluorescent species.

METHODS

Bacterial strains. Seventy-nine reference strains representing 42 species (including 15 *Legionella pneumophila*) were tested together with 212 clinical and environmental isolates obtained from the French *Legionella* Reference Centre (Table 1). Among these, 189 field isolates were previously tested by conventional methods (growth characteristics, biochemical reactivity and DFA with specific antisera prepared by rabbit hyperimmunization at the French *Legionella* Reference Centre, Lyon). The remaining 23 strains correspond to well identified strains from previous studies (Table 1). All strains were stored in liquid nitrogen until used and then cultured on BCYE agar plates (4).

Preparation of DNA templates for PCR. Chromosomal DNA templates were prepared from 72 h cultures on BCYE following a thermal lysis procedure (11). Nucleic acids were purified by the addition of phenol/chloroform/isoamyl alcohol (14) and precipitated by the addition of absolute ethanol. The pellet was vacuum-dried, resuspended in 200 µl sterile distilled water and stored at −20 °C until used.

PCR amplification of intergenic 16S–23S rDNA sequences. Primers FGPS1490-72 (5′-TGCGGCTGGATCCCCTCC-TT-3′) (located at nucleotides 1521–1541 of the 16S rRNA gene sequence of *Escherichia coli* – GenBank accession no. J01695) and FGPL132′-38 (5′-CCGGGTTTCCCCATTC-GG-3′) (located at nucleotides 114–132 of the 23S rRNA gene sequence of *E. coli* – accession no. J01695) were designed previously from multiple alignments of 16S and 23S genes of diverse bacteria (18). Primer FGPS1490-72 is located three bases away from the 3′ end of the 16S gene, and primer FGPL132′-38 is located 114 bases away from the 5′ end of the 23S gene. These primers are complementary to the conserved regions 4 (positions 1521–1541 for 16S rRNA gene) and 6 (positions 114–132 for the 23S rRNA gene) within the rDNA operon and match the recommended positions to detect spacer variation at the species level (7). DNA templates (5 µg, 10 µl) were amplified in a 100 µl reaction volume that contained 2·5 U *Taq* polymerase (Perkin Elmer Cetus), 1 mM each primer, 0·2 mM of each of the four dNTPs (Pharmacia Biotech), 10 mM Tris/HCl (pH 8·3), 50 mM KCl, 1·5 mM $MgCl_2$ and 0·1 mg gelatin ml^{-1} (Perkin Elmer). Reaction mixtures were overlaid with 50 µl mineral oil and heated to 95 °C for 2 min prior to amplification. Amplification was carried out in a PHC-3 Dri-Block cycler (Techne, Cambridge, UK) for 35 cycles. Each amplification cycle was as follows: 1 min (denaturation) at 95 °C, 1 min (annealing) at 55 °C, and 2 min (extension) at 72 °C. Controls were included in each set of amplifications, namely, a reaction mixture with no DNA added.

Detection of the amplification products. Following amplification, 20 µl each sample was electrophoresed through 1·5 % agarose gel (FMC BioProducts) in 0·5 × TBE buffer as described (11). Gels were then stained with ethidium bromide and photographed under UV light. DNA fragment sizes were compared with the DNA Molecular Weight Marker VI (Boehringer Mannheim). The sizing of fragments of each PCR product and the schematic representation of ISR-PCR profiles were done using the Taxotron software package (Institut Pasteur, Paris, France).

To assess PCR reproducibility, the 16S–23S rDNA intergenic spacer regions were amplified more than twice for each strain. The two separate PCR amplification mixtures prepared for each strain were run side by side as well as on different gels. To calculate the lengths of amplicons, a DNA molecular size marker was included. The gels were photographed and scanned. The lengths of PCR products were calculated automatically by using the RestrictoScan and RestrictoTyper programs (P. A. D. Grimont, Taxolab, Institut Pasteur, Paris, France), and mean sizes (standard deviations) were calculated for each strain.

PCR-RFLP analysis. When necessary, amplicon DNA without any further purification step was restricted with *Hin*fI (Appligene) for 1 h at 37 °C. The digested products were then resolved on a 3 % (w/v) agarose gel.

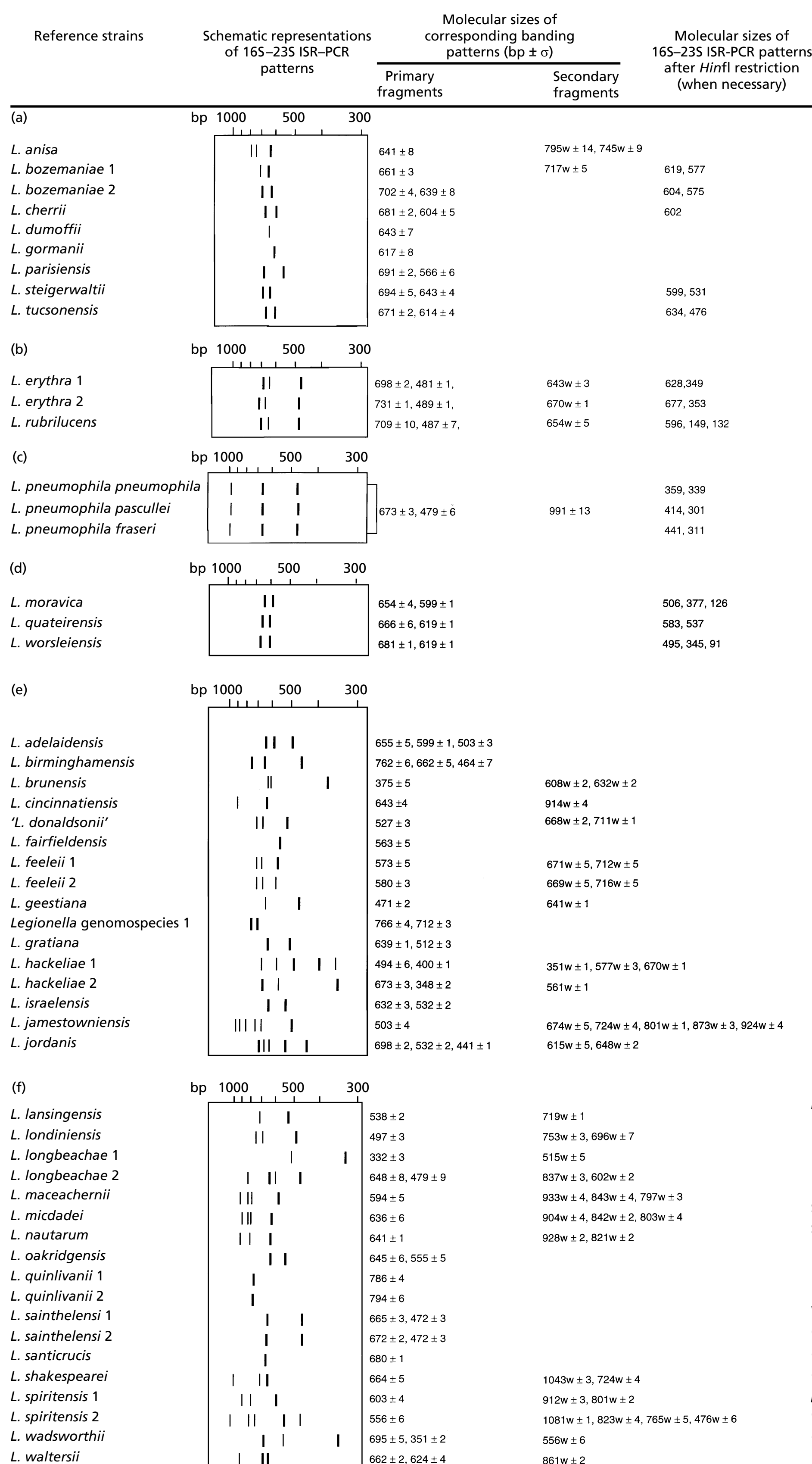
DNA sequencing of the 16S–23S spacers regions. DNA sequencing was performed on the 450 bp purified PCR products from *L. pneumophila* subsp. *pneumophila* strain Philadelphia-1, *L. pneumophila* subsp. *fraseri* strain Los Angeles-1 and *L. pneumophila* subsp. *pascullei* strain Micu-B3. After agarose gel electrophoresis, DNA bands were excised from the gel and purified using the Geneclean Kit II (Bio101). Automated DNA sequencing of the purified DNA was performed on an Applied Biosystems 373A sequencer by a commercial company (Genome Express)

DNA sequences were analysed and compared in the DDBJ/EMBL/GenBank databases with the BLAST program (1).

RESULTS

The strategy of evaluation of the ISR-PCR method as a taxonomic tool for the *Legionellaceae* was as follows: (i) use on reference strains; (ii) use on isolates identified by DFA typing; and (iii) use on atypical strains not clearly assigned to a known taxon.

Legionella reference strains displayed various PCR profiles (Fig. 1) made of 1–6 bands with sizes ranging from 330 to 1080 bp, which is consistent with the expected length of the 16S–23S intergenic region of members of the *Proteobacteria* (18). However, to eliminate the possibility that the shortest PCR products, due to their small size, could correspond to intragenic rDNA, these products were sequenced for three strains of *L. pneumophila*, namely *L. pneumophila* subsp. *pneumophila* Philadelphia-1 strain (ATCC 33152^{T}), *L. pneumophila* subsp. *fraseri* Los Angeles-1 (ATCC 33156^{T}) and *L. pneumophila* subsp. *pascullei* Micu-B3 (ATCC 33735^{T}). No significant differences in composition were seen between the three sequences that were highly homologous to the ISR of numerous members of the alpha and gamma subclasses of the *Proteobacteria*. For instance, the sequence of the *L. pneumophila* subsp. *fraseri* 16S–23S spacer region had 86 % homology with that of *Pseudomonas stutzeri* (GenBank accession no. U65012). Additionally, as expected for ISR DNA, some of these sequences contained highly conserved $tRNA^{ala}$ gene.



Reference strains	Primary fragments (bp ± σ)	Secondary fragments (bp ± σ)	Molecular sizes of 16S–23S ISR-PCR patterns after *Hin*fI restriction (when necessary)
(a)			
L. anisa	641 ± 8	795w ± 14, 745w ± 9	
L. bozemaniae 1	661 ± 3	717w ± 5	619, 577
L. bozemaniae 2	702 ± 4, 639 ± 8		604, 575
L. cherrii	681 ± 2, 604 ± 5		602
L. dumoffii	643 ± 7		
L. gormanii	617 ± 8		
L. parisiensis	691 ± 2, 566 ± 6		
L. steigerwaltii	694 ± 5, 643 ± 4		599, 531
L. tucsonensis	671 ± 2, 614 ± 4		634, 476
(b)			
L. erythra 1	698 ± 2, 481 ± 1,	643w ± 3	628,349
L. erythra 2	731 ± 1, 489 ± 1,	670w ± 1	677, 353
L. rubrilucens	709 ± 10, 487 ± 7,	654w ± 5	596, 149, 132
(c)			
L. pneumophila pneumophila	673 ± 3, 479 ± 6	991 ± 13	359, 339
L. pneumophila pascullei			414, 301
L. pneumophila fraseri			441, 311
(d)			
L. moravica	654 ± 4, 599 ± 1		506, 377, 126
L. quateirensis	666 ± 6, 619 ± 1		583, 537
L. worsleiensis	681 ± 1, 619 ± 1		495, 345, 91
(e)			
L. adelaidensis	655 ± 5, 599 ± 1, 503 ± 3		
L. birminghamensis	762 ± 6, 662 ± 5, 464 ± 7		
L. brunensis	375 ± 5	608w ± 2, 632w ± 2	
L. cincinnatiensis	643 ±4	914w ± 4	
'*L. donaldsonii*'	527 ± 3	668w ± 2, 711w ± 1	
L. fairfieldensis	563 ± 5		
L. feeleii 1	573 ± 5	671w ± 5, 712w ± 5	
L. feeleii 2	580 ± 3	669w ± 5, 716w ± 5	
L. geestiana	471 ± 2	641w ± 1	
Legionella genomospecies 1	766 ± 4, 712 ± 3		
L. gratiana	639 ± 1, 512 ± 3		
L. hackeliae 1	494 ± 6, 400 ± 1	351w ± 1, 577w ± 3, 670w ± 1	
L. hackeliae 2	673 ± 3, 348 ± 2	561w ± 1	
L. israelensis	632 ± 3, 532 ± 2		
L. jamestowniensis	503 ± 4	674w ± 5, 724w ± 4, 801w ± 1, 873w ± 3, 924w ± 4	
L. jordanis	698 ± 2, 532 ± 2, 441 ± 1	615w ± 5, 648w ± 2	
(f)			
L. lansingensis	538 ± 2	719w ± 1	
L. londiniensis	497 ± 3	753w ± 3, 696w ± 7	
L. longbeachae 1	332 ± 3	515w ± 5	
L. longbeachae 2	648 ± 8, 479 ± 9	837w ± 3, 602w ± 2	
L. maceachernii	594 ± 5	933w ± 4, 843w ± 4, 797w ± 3	
L. micdadei	636 ± 6	904w ± 4, 842w ± 2, 803w ± 4	
L. nautarum	641 ± 1	928w ± 2, 821w ± 2	
L. oakridgensis	645 ± 6, 555 ± 5		
L. quinlivanii 1	786 ± 4		
L. quinlivanii 2	794 ± 6		
L. sainthelensi 1	665 ± 3, 472 ± 3		
L. sainthelensi 2	672 ± 2, 472 ± 3		
L. santicrucis	680 ± 1		
L. shakespearei	664 ± 5	1043w ± 3, 724w ± 4	
L. spiritensis 1	603 ± 4	912w ± 3, 801w ± 2	
L. spiritensis 2	556 ± 6	1081w ± 1, 823w ± 4, 765w ± 5, 476w ± 6	
L. wadsworthii	695 ± 5, 351 ± 2	556w ± 6	
L. waltersii	662 ± 2, 624 ± 4	861w ± 2	

Fig. 1. Intergenic 16S–23S amplification patterns of *Legionella* strains. Left, reference strains and schematic representations of 16S–23S ISR-PCR patterns drawn with the Taxotron software package; middle, molecular sizes and inter-gel standard errors (σ) of primary and secondary fragments of ISR-PCR; right, molecular sizes of ISR-PCR products after *Hin*fI restriction. Strains were disposed according to phylogenic groups for the bluish-white autofluorescent species (a), the red autofluorescent species (b), the *L. pneumophila* group (c), or on the basis of closely related ISR-PCR profiles (d), and by alphabetical order for the remaining species (e, f).

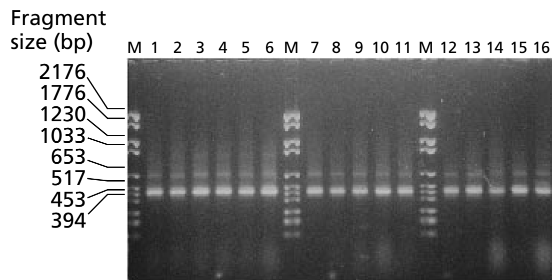


Fig. 2. Agarose gel electrophoresis of PCR-amplified 16S–23S rDNA spacer regions from *Legionella pneumophila* strains. Lanes: M, molecular size markers; 1, subsp. *pneumophila* ATCC 43130; 2, subsp. *pneumophila* ATCC 43290; 3, subsp. *pneumophila* ATCC 43736; 4, subsp. *pneumophila* ATCC 43703; 5, subsp. *fraseri* ATCC 35251; 6, subsp. *pascullei* ATCC 33735; 7, subsp. *pneumophila* ATCC 33215; 8, ATCC 33823; 9, subsp. *pneumophila* ATCC 35096; 10, subsp. *pneumophila* ATCC 35289; 11, subsp. *pneumophila* ATCC 43283; 12, subsp. *pneumophila* ATCC 33152^T; 13, subsp. *pneumophila* ATCC 33154; 14, subsp. *pneumophila* ATCC 33155; 15, subsp. *fraseri* ATCC 33156^T; 16, subsp. *fraseri* ATCC 33216.

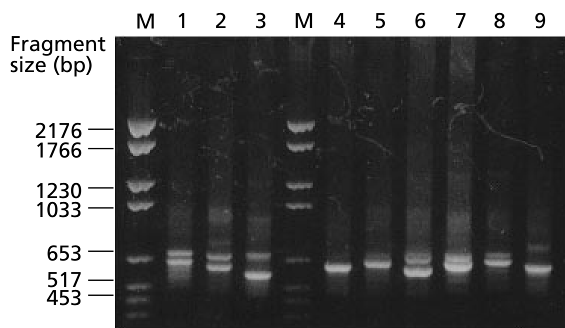


Fig. 3. Agarose gel electrophoresis of PCR-amplified 16S–23S rDNA spacer regions from type and reference strains of bluish-white autofluorescent species. Lanes: M, molecular size marker; 1, *L. anisa* ATCC 35292^T; 2, *L. bozemaniae* serogroup 1 ATCC 33217^T; 3, *L. bozemaniae* serogroup 2 ATCC 35545; 4, *L. cherrii* ATCC 35252^T; 5, *L. dumoffii* ATCC 33279^T; 6, *L. gormanii* ATCC 33297^T; 7, *L. parisiensis* ATCC 35299^T; 8, *L. tucsonensis* ATCC 49180^T; 9, *L. steigerwaltii* ATCC 35302^T.

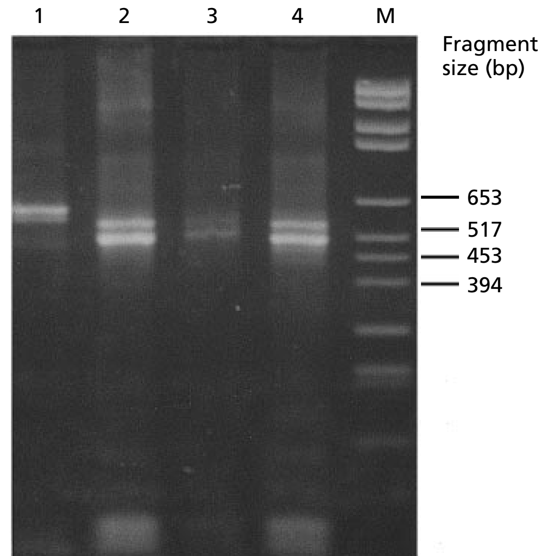


Fig. 4. Agarose gel electrophoresis of PCR-amplified 16S–23S rDNA spacer regions from *Legionella bozemaniae* and *Legionella steigerwaltii* strains after *Hin*fI digestion. Lanes: M, molecular size marker; 1, *L. bozemaniae* serogroup 2 ATCC 35545; 2–4, multiple amplicons of *L. steigerwaltii* ATCC 35302^T.

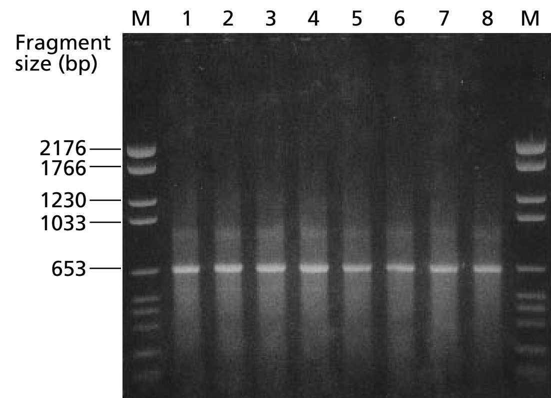


Fig. 5. Agarose gel electrophoresis of PCR-amplified 16S–23S rDNA spacer regions from non-epidemiologically related *Legionella dumoffii* strains. Lanes: M, molecular size marker; 1, ATCC 33279^T; 2, ATCC 35850; 3, Aix80B3; 4, Aix96VII2-4; 5, Aix96VII7-1; 6, Lech2F10; 7, Stra-92101226; 8, Toul23-6.

The patterns of the reference strains were compared visually and also by calculating the molecular size of each band. The abundant fragments are referred to as primary products, and the weaker ones are referred to as secondary products (Fig. 1). The mean estimated molecular sizes and standard errors were calculated from repeated inter-gel ISR profiles of each reference strain. A maximum difference of 14 bp (*L. anisa* secondary fragment) leads us to conclude that the level of uncertainty in the calculated sizes of amplification products did not exceed 2–2·5%.

Thirty-four type and reference strains showed distinctive ISR-PCR profiles. *L. pneumophila* showed a species-specific profile that was identical for the 15 serogroups (Fig. 2). In several cases, such as the bluish-white autofluorescent group, ISR-PCR profiles corresponding to different species or subspecies were either strictly identical or closely related, i.e. within the range of standard error of fragment size (Fig. 3, lanes 1 and 7). In such cases, the profiles were differentiated after *Hin*fI restriction digest of the PCR products (Figs 1 and 4).

One hundred and thirty-two isolates identified previously by DFA (including 23 strains from the literature) belonging to 18 species were tested. ISR-PCR profiles obtained for each of these isolates were either

strictly identical or closely related, i.e. within the range of standard error of fragment size, to those obtained for the type strains (see Fig. 5, which shows the example of *Legionella dumoffii*). Although *Legionella longbeachae* and *Legionella spiritensis* ISR-PCR patterns are not, strictly speaking, species-specific (Fig. 1), the majority of the other species tested showed no strain-to-strain variations (Figs 2 and 5). However, intraspecies variations cannot be excluded for the species for which only the type strains were available.

The ability of ISR-PCR to identify phenotypically aberrant strains of *Legionella* was tested on 80 field isolates which were not formally identified at the species level on the basis of major cross-reactions using the DFA assay and/or atypical biochemical characteristics. These isolates mostly belonged to the bluish-white autofluorescent group. Fifty-seven per cent (46/80) of these isolates showed profiles identical to that of a type or reference strain for which a cross-reaction was observed by DFA. In 42% of cases (34/80), results of ISR-PCR differed from those of DFA. Of these, 12 strains showed ISR profiles that did not correspond to those of the type strains which gave cross-reaction by DFA, and 22 strains showed ISR profiles unrelated to any of the known profiles. These unidentified strains may correspond either to new species or subspecies or to a subtype within a given species.

DISCUSSION

Species identification of *Legionella* based on phenotypic traits is time-consuming and restricted to specialized laboratories. In the present study, we show that intergenic ribosomal PCR is a rapid (less than 2 d) and efficient tool for identification of the *Legionella* at the species and subspecies levels. Our results differed from a comparable study conducted by Hookey *et al.* (9) by the sizes of the amplified products and in some cases by the number of bands in the profiles. The primers choosen in the present study from regions 4 (positions 1521–1541 for the 16S rRNA gene) and 6 (positions 114–132 for the 23S rRNA gene) within the rDNA operon (7) were much closer to the ISR itself, and not surprisingly were successful in detecting spacer variation even between the bluish-white autofluorescent species (Fig. 1a) and the red autofluorescent species (Fig. 1b) which are otherwise difficult to identify. By designing primers that match nucleotides 1113–1130 of the 16S rDNA, Hookey and co-workers have amplified fragments that were longer by nearly 400 bp, resulting in decreased efficiency to detect spacer variation by standard agarose gel electrophoresis. For instance, we were able to reveal subtle differences between size fragments of *Legionella cherrii* (681 ± 2 and 604 ± 2 bp) and *Legionella tucsonensis* (671 ± 2 and 614 ± 4 bp) (Fig. 1a), which were not resolved between fragments in the size range of 1100 and 1200 bp in Hookey's study (9). Analysis of the RFLP generated after *Hin*fI restriction digestion of the PCR products further improved the discrimination between species or subspecies (Figs 1 and 4). Hence, *L. pneumophila* subsp. *pneumophila* showed profiles that were distinct from those of *L. pneumophila* subsp. *fraseri* and *L. pneumophila* subsp. *pascullei* (Fig. 1c). Such discrimination can otherwise be only obtained by time-consuming methods such as DNA–DNA hybridization (3), or whole-cell protein SDS-PAGE (20).

Importantly, intraspecies stability of ISR-PCR profile was demonstrated for a number of species by showing that multiple well-identified isolates of a given species showed an identical pattern to that of the reference and type strains (shown in Fig. 2 for *L. pneumophila* and in Fig. 5 for *L. dumoffii*). In this matter, determination of fragment sizes and standard errors for each ISR-PCR pattern provided a convenient way of comparing patterns, since the level of uncertainty in the calculated sizes of amplification products did not exceed 2–2·5%. ISR-PCR was also able to identify a collection of phenotypically aberrant *Legionella* isolates at the species level. In most cases, the species assignment by ISR-PCR was within the group of species for which cross-reactions were observed by DFA. This confirms the usefulness of the method in cases where definite identification cannot be easily obtained by conventional methods. However, a number of strains remained unidentified by this approach and may correspond to new taxa; these strains will be further characterized by DNA–DNA hybridization and 16S rRNA gene sequencing.

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REFERENCES

1. **Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990).** Basic local alignment search tool. *J Mol Biol* **215**, 404–410.
2. **Benson, R. F., Thacker, W. L., Daneshvar, M. I. & Brenner, D. J. (1996).** *Legionella waltersii* sp. nov. and an unnamed *Legionella* genomospecies isolated from water in Australia. *Int J Syst Bacteriol* **46**, 631–634.
3. **Brenner, D. J. (1986).** Classification of *Legionellaceae*. Current status and remaining questions. *Isr J Med Sci* **22**, 620–632.
4. **Edelstein, P. H. (1981).** Improved semiselective medium for isolation of *Legionella pneumophila* from contaminated clinical and environmental specimens. *J Clin Microbiol* **14**, 294–303.
5. **Grimont, F., Lefèvre, M., Ageron, E. & Grimont, P. A. D. (1989).** rRNA gene restriction patterns of *Legionella* species: a molecular identification system. *Res Microbiol* **140**, 615–626.
6. **Gürtler, V. & Barrie, H. D. (1995).** Typing of *Staphylococcus aureus* strains by PCR-amplification of variable-length 16S–23S rDNA spacer regions: characterization of spacer sequences. *Microbiology* **141**, 1255–1265.

7. **Gürtler, V. & Stanisich, V. A. (1996).** New approaches to typing and identification of bacteria using the 16S–23S rDNA spacer region. *Microbiology* **142**, 3–16.
8. **Harrell, L. J., Andersen, G. L. & Wilson, K. H. (1995).** Genetic variability of *Bacillus anthracis* and related species. *J Clin Microbiol* **33**, 1847–1850.
9. **Hookey, J. V., Birtles, R. J. & Saunders, N. A. (1995).** Intergenic 16S rRNA gene (rDNA)-23S rDNA sequence length polymorphisms in members of the family *Legionellaceae*. *J Clin Microbiol* **33**, 2377–2381.
10. **Hookey, J. V., Saunders, N. A., Fry, N. K., Birtles, R. J. & Harrison, T. G. (1996).** Phylogeny of *Legionellaceae* based on small-subunit ribosomal DNA sequences and proposal of *Legionella lytica* comb. nov. for *Legionella*-like amoebal pathogens. *Int J Syst Bacteriol* **46**, 526–531.
11. **Jaulhac, B., Nowicki, M., Bornstein, N., Meunier, O., Prevost, G., Piemont, Y., Fleurette, J. & Monteil, H. (1992).** Detection of *Legionella* spp in bronchoalveolar lavage fluids by DNA amplification. *J Clin Microbiol* **30**, 920–924.
12. **Jensen, M. A., Webster, J. A. & Straus, N. (1993).** Rapid identification of bacteria on the basis of polymerase chain reaction-amplified ribosomal DNA spacer polymorphisms. *Appl Environ Microbiol* **59**, 945–952.
13. **Lo Presti, F., Riffard, S., Vandenesch, F., Reyrolle, M., Ronco, E., Ichai, P. & Etienne, J. (1997).** The first clinical isolate of *Legionella parisiensis*, from a liver transplant patient with pneumonia. *J Clin Microbiol* **35**, 1706–1709.
14. **Marmur, J. (1961).** A procedure for the isolation of deoxyribonucleic acid from microorganisms. *J Mol Biol* **3**, 208–218.
15. **Meunier, J. R. & Grimont, P. A. D. (1993).** Factors affecting reproducibility of random amplified polymorphic DNA fingerprinting. *Res Microbiol* **144**, 373–379.
16. **Navarro, E., Simonet, P., Normand, P. & Bardin, R. (1992).** Characterization of natural populations of *Nitrobacter* spp. using PCR/RFLP analysis of the ribosomal intergenic spacer. *Arch Microbiol* **157**, 107–115.
17. **Normand, P., Cournoyer, B., Nazaret, S. & Simonet, P. (1992).** Analysis of a ribosomal RNA operon in the actinomycete *Frankia*. *Gene* **111**, 119–124.
18. **Normand, P., Ponsonnet, C., Nesme, X., Neyra, M. & Simonet, P. (1996).** ITS analysis of prokaryotes. In *Molecular Microbial Ecology Manual*, pp. 1–12. Edited by A. D. L. Akkermans, J. D. van Elsas & F. J. De Bruijn. Dordrecht, The Netherlands: Kluwer.
19. **Rodgers, F. G. & Pasculle, A. W. (1991).** *Legionella*. In *Manual of Clinical Microbiology*, 5th edn, pp. 442–453. Edited by A. Balows, W. J. Hausler, Jr, K. L. Herrmann, H. D. Isenberg & H. J. Shadomy. Washington, DC: American Society for Microbiology.
20. **Verissimo, A., Morais, P. V., Diogo, A., Gomes, C. & Costa, M. S. D. (1996).** Characterization of *Legionella* species by numerical analysis of whole-cell protein electrophoresis. *Int J Syst Bacteriol* **46**, 41–49.