MOLECULAR IDENTIFICATION AND EPIDEMIOLOGY

An evaluation of intergenic rRNA gene sequence length polymorphism analysis for the identification of *Legionella* species

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There are currently more than 40 species of *Legionella* and the identification of most of these by standard methods is technically difficult. The aim of this study was to assess the suitability of a previously published PCR-based method of identifying *Legionella* spp. Intergenic 16S–23S rDNA spacer regions were amplified with primers complementary to conserved regions of the rRNA genes. Following electrophoretic separation of the products, data analyses were performed with the Taxotron[®] software package. Computer-assisted analysis (with an empirically derived error tolerance of 3%) could differentiate only 26 of the 43 strains (representing 43 species), with the remaining 17 species clustering into four groups (group I, comprising 10 species; group II, three species; group III, two species and group IV, two species). Analysis of well-characterised ‘non-type’ strains of some *Legionella* spp. (e.g., from type culture collections) resulted in patterns distinct from the corresponding type strain in most cases. Furthermore, recent isolates (identified by conventional methods) were identified by this PCR method to the presumed correct species (or species group) in only a minority of cases. Well characterised strains and recent isolates of *Legionella* showed heterogeneity within many species. This intra-species variation severely limits the usefulness of the method for the identification of isolates. However, this property may be useful for epidemiological typing within such species.

Introduction

The family Legionellaceae was initially proposed for a single genus, *Legionella*, and one species, *L. pneumophila* [1]. Currently, there are 42 validly described species [2–4] and several others awaiting formal description. To date, 18 of these species have been shown to be pathogenic for man [5]. The identification of the most common causative agent of legionellosis (*L. pneumophila*) by standard microbiological methods is straightforward [6, 7], but the lack of discriminatory phenotypic tests for *Legionella* spp. other than *L. pneumophila* makes their current identification difficult, costly and time-consuming. Several molecular methods of differentiation and identification of bacteria to the level of genus, species and strain have been applied to *Legionella* [8–13], including a method for the differentiation of *Legionella* spp. based on PCR-amplification of the intergenic spacer region (ISR) of

the 16S and 23S rRNA genes [14]. However, this latter study was based on patterns obtained with type strains of *Legionella* and only two recent isolates were examined. The aim of the present study was to fully evaluate this ISR method of identifying legionellae by compiling a database of patterns from the type strain of each species, and then to compare sets comprising (1) well-characterised strains (e.g., those available from type culture collections or those with 16S rRNA gene sequence or DNA:DNA homology data available) and (2) recent isolates (identified by standard methods) against this database. The Taxotron[®] software package was used to assist in the analysis of the patterns.

Materials and methods

Bacterial strains and culture conditions

Type strains and other representatives of the 42 published species of *Legionella* and one proposed new species, ‘*L. donaldsonii*’ (R. J. Fallon and personal unpublished observations), were obtained from the culture collection of the Respiratory and Systemic

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Infection Laboratory (RSIL), the National Collection of Type Cultures (NCTC), Central Public Health Laboratory (CPHL), London and the American Type Culture Collection (ATCC), MD, USA. These are listed in Table 1 [2, 15–18]. Recent isolates, including clinical and environmental strains, were obtained from the RSIL, CPHL. Strains of legionellae were cultured on buffered charcoal yeast extract agar [19] at 30° or 37°C in a moist chamber or, in the case of legionella-like amoebal pathogens (LLAPs), their amoebal host [16, 17, 20].

Non-type strains of legionellae used in this study were divided into two sets: dataset I (52 strains) comprising well characterised strains and dataset II (46 strains) comprising recent clinical and environmental isolates. Strains assigned to dataset I were available either from type culture collections (NCTC or ATCC), or had been the subject of phylogenetic studies, such as 16S rRNA (gene) sequence analysis [16, 17, 20, 21], DNA restriction fragment length polymorphism (RFLP)

analysis [11], or DNA:DNA homology [18]; the identity of these strains was taken to be ‘true’. Strains assigned to dataset II were identified in the RSIL by standard phenotypic techniques, including growth requirements, biochemical characteristics and serological tests with hyperimmune rabbit antisera [6, 7], or genotypic RFLP analysis [11]. Strains identified as *L. pneumophila* were confirmed by fluorescence microscopy with a *L. pneumophila* species-specific monoclonal antibody (Genetic Systems Corporation, Redmond, WA, USA). Consequently, confidence in the accuracy of the identity of these recent isolates ranged from ‘strong’ for *L. pneumophila* to ‘poor’ for the individual species within the blue-white autofluorescent group.

Extraction of genomic DNA

Genomic DNA suitable as template in the PCRs was extracted with the Nucleon® genomic DNA extraction kit (GeneSys Biotech Ltd., Coatbridge, Strathclyde).

Table 1. Members of the family Legionellaceae showing the type strains of species used to establish the identification database designated ‘type strain database’

	<i>Legionella</i> species	NCTC no.	ATCC no.	Original designation	Clinical isolate
1.	<i>L. pneumophila</i> ^{T*}	11192	33152	Philadelphia-1	Yes
2.	<i>L. adelaidensis</i> ^T	12735	49625	1762-AUS-E	No
3.	<i>L. anisa</i> ^T	11974	35292	WA-316-C3	Yes
4.	<i>L. birminghamensis</i> ^T	12437	43702	1407-AL-H	No
5.	<i>L. bozemanii</i> ^T	11368	33217	WIGA	Yes
6.	<i>L. brunensis</i> ^T	12240	43878	441-1	No
7.	<i>L. cherrii</i> ^T	11976	35252	ORW	No
8.	<i>L. cincinnatiensis</i> ^T	12438	43753	72-OH-H	Yes
9.	‘ <i>L. donaldsonii</i> ’	ND	ND	86/35784	No
10.	<i>L. dumoffii</i> ^T	11370	33279	NY-23	Yes
11.	<i>L. erythra</i> ^T	11977	35303	SE-32A-C8	No
12.	<i>L. fairfieldensis</i> ^T	12488	49588	1725-AUS-E	No
13.	<i>L. feeleyi</i> ^T	12022	35072	WO-44C	Yes
14.	<i>L. geestiana</i> ^T	12373	49504	51/83-1308	No
15.	<i>L. genomospecies 1</i> ^T	13018	51913	2055-AUS-E	No
16.	<i>L. gormanii</i> ^T	11401	33297	LS-13	Yes
17.	<i>L. gratiana</i> ^T	12388	49413	Lyon 8420412	No
18.	<i>L. hackeliae</i> ^T	11979	35250	Lansing-2	Yes
19.	<i>L. israelensis</i> ^T	12010	43119	Bercovier-4	No
20.	<i>L. jamestowniensis</i> ^T	11981	35298	JA-26-GI-E2	No
21.	<i>L. jordanis</i> ^T	11533	33623	BL-540	Yes
22.	<i>L. lansingensis</i> ^T	12830	49751	1677-MI-H	Yes
23.	<i>L. londiniensis</i> ^T	12931	49505	59/84-1477	No
24.	<i>L. longbeachae</i> ^T	11477	33462	Long Beach-4	Yes
25.	<i>L. lytica</i> ^T	ND	ND	L2	Yes
26.	<i>L. maceachernii</i> ^T	11982	35300	PX-1-G2-E2	Yes
27.	<i>L. micdadei</i> ^T	11371	33218	TATLOCK	Yes
28.	<i>L. moravica</i> ^T	12239	43877	316-36	No
29.	<i>L. nautarum</i> ^T	12932	49506	199/52-1224	No
30.	<i>L. oakridgensis</i> ^T	11531	33761	Oak Ridge-10	No
31.	<i>L. parisiensis</i> ^T	11983	35299	PF-209C-C2	Yes
32.	<i>L. quateirensis</i> ^T	12376	49507	200/83-1335	No
33.	<i>L. quinlivanii</i> ^T	12433	43830	1442-AUS-E	No
34.	<i>L. rubrilucens</i> ^T	11987	35304	WA-270A-C2	No
35.	<i>L. sainthelensi</i> ^T	11988	35248	Mt St. Helens-4	Yes
36.	<i>L. santicrucis</i> ^T	11989	35301	SC-63-C7	No
37.	<i>L. shakespearei</i> ^T	12829	49655	214	No
38.	<i>L. spiritensis</i> ^T	11990	35349	Mt St. Helens-9	No
39.	<i>L. steigerwaltii</i> ^T	11991	35302	SC-18-C9	No
40.	<i>L. tucsonensis</i> ^T	12439	49180	1087-AZ-H	Yes
41.	<i>L. wadsworthii</i> ^T	11532	33877	81-716A	Yes
42.	<i>L. waltersii</i> ^T	13017	51914	2074-AUS-E	No
43.	<i>L. worsleiensis</i> ^T	12377	49508	95/83-1347	No

NCTC, National Collection of Type Cultures; ATCC, American Type Culture Collection; ND, not yet deposited; ^Ttype strain of species.
* *L. pneumophila* comprises three subspecies: subsp. *pneumophila*, *fraseri* and *pascullei* [2].

Template DNA from the LLAPs was kindly provided by R. Birtles, prepared by the Chelex (BioRad) extraction method [22], or by boiling a suspension of the organisms.

PCR amplification of 16S rDNA–23S rDNA intergenic spacer region

This was performed essentially according to the method of Hookey *et al.* [14]. Genomic DNA was amplified by the PCR with primers complementary to regions on the 16S rRNA (*Escherichia coli* numbering [23], 1113–1130 [24]) and 23S rRNA (115–130 [25]) genes. The sequences of the primers were as follows: 5' CGC AAC GAG CGC AAC CCT 3' (primer 16S-f-1130) and 5' GGG TTB CCC CAT TCG G 3' (B = C/G/T, primer 23S-r-115). Two alternative primers described by Riffard *et al.* [26] complementary to regions 1522–1542 (*E. coli* numbering) on the 16S rRNA gene (5'-TGC GGC TGG ATC CCC TCC TT; primer FGPS 1490-72) and 115–132 on the 23S rRNA gene (5'-CCG GGT TTC CCC ATT CGG; primer FGPL 132'-38) were also used to amplify genomic DNA in some reactions. The PCR mixture contained 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 3.5 mM MgCl₂, 200 µM of each deoxynucleoside triphosphate (dATP, dCTP, dGTP and dTTP; Boehringer Mannheim), 110 pmols of each primer (PE Applied BioSystems, Warrington) and 1.25 U *Taq* polymerase (Life Technologies). Template DNA (*c.* 100 ng) was added and the mixture was overlaid with mineral oil (Sigma). Reaction mixtures with no added DNA served as negative controls. Alternatively, reaction mixtures without template and *Taq* polymerase were prepared and overlaid with wax (Biowax; Biozym BV, Landgraaf; The Netherlands) and stored at –30°C until use. Amplification was performed in a Trio-Thermoblock (Biometra) in 50-µl volumes under the following conditions: pre-denaturation for 5 min at 95°C, then 27 cycles consisting of denaturation for 40 s at 95°C, annealing for 42 s at 55°C, extension for 1 min at 72°C, followed by a final extension for 10 min at 72°C. The effect of varying the amount of DNA template (1, 10, 50 and 100 ng) in the PCR on the banding pattern was also examined.

Analysis of PCR products

PCR products (*c.* 10% of the reaction mixture) were separated by electrophoresis in poly(NAT)TM gels (Elchrom Scientific, Cham, Switzerland) 6% w/v in 30 mM Tris-acetate, 0.75 mM EDTA buffer, pH 8.2, for 17 h at 25 V. Electrophoresis was performed horizontally in a SEA 2000TM tank (Elchrom Scientific) connected to an external circulating waterbath at 20°C. Molecular size markers (100-bp ladder, Life Technologies) were included in the outer two lanes of each eight-lane mini-gel (Elchrom Scientific). Following electrophoresis, gels were stained with ethidium bromide (0.5 µg/ml), destained in distilled water and photographed with Polaroid film Type 667 (Sigma).

S1 nuclease treatment of PCR products

The published method [14] treated the amplification products with S1 nuclease before separation by electrophoresis. To evaluate the use of this treatment, PCR-amplification products from strains of *L. pneumophila* were incubated with S1 nuclease (Boehringer Mannheim) with the recommended conditions [14, 27] for increasing times (1–15 min) and then compared to untreated samples.

Computer-assisted analysis of patterns

Black and white photographs of the PCR gels were scanned with a ScanMaker (Microtek Lab) into Adobe Photoshop with 256 levels of grey and 150 dpi. The resultant tagged image file format (TIFF) files were then interpreted with four of the programs within the Taxotron[®] package (Taxolab[®]; Institut Pasteur, Paris, France). These were RestrictoScan[®] (with no smoothing) for band detection and migration measurements; RestrictoTyper[®] for interpolation of fragment sizes from migration values (with the Schaffer and Sederoff algorithm [28]); Adanson[®] for clustering by single-linkage and Dendrograph[®] for constructing dendrograms. To ensure the highest quality of the results, the following steps were taken: (1) each molecular size 'standard lane' fitting curve was examined closely to verify that all data points were on or very close to the curve; (2) the schematic representation of bands drawn by RestrictoTyper[®] was visually compared to the actual bands on the photograph to detect any missing or artifactual bands. The problem of weak or unresolved bands is universal to the electrophoretic analysis of nucleic acid fragments or their resulting blots. The Taxotron[®] program allows the designation of bands as weak or doubtful, represented as thin lines by RestrictoTyper[®]. Where confirmed visually, weak bands were included in the numerical analyses.

Identification of patterns

A database file was constructed containing the molecular sizes of bands resulting from the PCR with DNA from type strains of the 43 species (Table 1). Separate files containing the molecular sizes of bands resulting from 52 well-characterised strains (dataset I, see above) and 46 recent isolates (dataset II, see above) were prepared and then matched against this 'type strain database'. Preliminary analyses were done with various tolerance values (ranging from 1.0% to 10.0%) to determine an optimal value that would give maximum discrimination between type strains and the highest number of correct identifications for strains in dataset I. The optimum error tolerance was determined to be 3.0% (see below) and this value was used for the rest of the study.

Results

Reproducibility of methodology

The gel electrophoresis conditions used were shown to be highly reproducible. An example of a molecular size standard lane fitting curve with the 100-bp ladder is shown in Fig. 1. All the reference points were on, or very close to, the standard curve. Variation within the poly(NAT)TM gels was determined by calculating the molecular sizes of bands from replicate samples (i.e., same PCR mixture) on the same gel. Mean molecular size estimates (from five replicates) of the three bands from ISR amplification of template DNA, e.g., *L. pneumophila* (NCTC strain 11191), were calculated to be 1453 (SD 4) bp, 878 (SD 5) bp and 845 (SD 2) bp. This corresponded to an SD of < 0.6% within gels. Variation between the poly(NAT)TM gels was determined by calculating the molecular sizes of bands from replicate samples (i.e., same PCR mixture) on five different gels. Mean molecular size estimates of the three bands described above were calculated to be 1433 (SD 13) bp, 878 (SD 7) bp and 848 (SD 6) bp, which corresponded to an SD of < 1.0% between gels. Mean molecular size estimates of three bands resulting from ISR amplification of the recent isolate LC4048 on three separate occasions, with template DNA from the same extraction, were calculated to be 1258 (SD 6) bp, 1063 (SD 5) bp and 456 (SD 5) bp. This corresponded to an SD of < 1.2% for different amplifications. Varying the

amount of DNA template from 1 to 100 ng (i.e., 1, 10, 50, 100 ng) in the PCR had no significant effect on three of the four bands from the type strain of *L. pneumophila*; which yielded bands of 1412 (SD 2) bp, 863 (SD 1) bp and 828 (SD 3) bp, i.e., an SD of < 0.6%. However, with 1 ng of DNA template, the lowest molecular size band, calculated as 395 (SD 2) bp from the three results above, was very faint, such that it was not recognised as a band by RestrictoScan[®].

Intergenic spacer region patterns from type strains of *Legionella*

The ISR method yielded relatively simple patterns, and analysis of the type strains of the 43 *Legionella* spp. confirmed that most could be differentiated visually (Fig. 2). To determine the optimum parameters for computer-assisted analysis, a file (type strain database) comprising molecular sizes of all the bands from the 43 type strains was compiled, against which well characterised non-type strains (dataset I) could be compared with a variety of fixed error tolerances. With a 1% tolerance, 39 of the 43 type strains could be identified, but only three of the 52 dataset I strains were identified to the correct species. Conversely, increasing the tolerance to 5% allowed the identification of 17 of the 52 dataset I strains to the correct species or species group, but discriminated between only 15 type strains (plus two groups of ≥ 10 type strains). Therefore, the

Fig. 1. Example of a standard lane fitting curve (100-bp ladder) drawn by Taxotron[®] by the Schaffer and Sederoff method [28] showing the relationship between the inverse of migration distance (represented by pixel number) and fragment size. SD = 27 bp.

Fig. 2. Examples of results of electrophoretic analysis following PCR-amplification of the 16S–23S rDNA ISR from strains of *Legionella*. Lanes: **M**, marker (100-bp ladder); **1**, *L. fairfieldensis*^T NCTC 12488; **2**, *L. dumoffii*^T NCTC 11370; **3**, *L. pneumophila* NCTC 11976; **5**, *L. pneumophila* NCTC 11191; **6**, *L. donaldsonii*^T 86/35784; **7**, *L. pneumophila* NCTC 11986; **8**, *L. pneumophila* NCTC 11985; **9**, LLAP-12; **10**, *L. israelensis*^T NCTC 12010; **11**, *L. hackeliae*^T NCTC 11979; **12**, *L. jordanis*^T NCTC 11533; **13**, *L. saintiocrucis*^T NCTC 11989; **14**, *L. quinlivanii*^T NCTC 12433; **15**, *L. erythra*^T NCTC 11977; **16**, *L. steigerwaltii*^T NCTC 11991; **17**, *L. spiritensis*^T NCTC 11990; **18**, *L. shakopeae*^T NCTC 12829.

value of 3% was selected as the optimum to give the greatest discrimination and likelihood of correct identification of the maximum number of species. With this value, 26 of the 43 type strains of *Legionella* could be differentiated (Fig. 3). The remaining 17

Legionella spp. clustered into four groups: group I (10 species), containing all the blue-white autofluorescent species, i.e., *L. anisa*, *L. bozemanii*, *L. cherrii*, *L. dumoffii*, *L. genomospecies 1*, *L. gormanii*, *L. parisiensis*, *L. steigerwaltii*, *L. tucsonensis* and one non-

Fig. 3. Dendrogram and schematic representations (drawn by RestrictoTyper[®]) of PCR-amplification of 16S–23S rDNA ISR from the type strains of 43 *Legionella* species. Patterns were clustered by the single linkage method with a fixed error tolerance of 3%. Analyses were performed with the Taxotron[®] software package (Institut Pasteur). BW, blue-white autofluorescent species; R, red autofluorescent species.

autofluorescent species *L. cincinnatiensis*; group II (three species), *L. gratiana*, *L. longbeachae* and *L. oakridgensis*; group III (two species), *L. lansingensis* and *L. maceachernii*; and group IV (two species), *L. feeleeii* and '*L. donaldsonii*'. A dendrogram together with the schematic representation of bands drawn by RestrictoTyper[®] from the 43 type strains of *Legionella* with a fixed error tolerance of 3% is shown in Fig. 3.

Use of the primers described by Riffard *et al.* [26] also resulted in intra-species heterogeneity, i.e., ISR patterns distinct from the type strain, in *Legionella* spp. (data not shown).

S1 nuclease treatment of PCR products

PCR-amplification products from strains of *L. pneumophila* incubated with S1 nuclease for increasing times (1–15 min) were electrophoresed with the conditions described above. The resulting bands were smeared compared to the untreated samples, leading to less distinguishable bands and patterns (data not shown). Consequently, all the banding patterns presented here are from PCR products untreated with S1 nuclease.

Data analysis of well-characterised strains of Legionella

Molecular size files of ISR amplification products from well-characterised 'non-type' strains of *L. pneumophila*, *L. bozemanii*, *L. dumoffii*, *L. erythra*, *L. feeleeii*, *L. hackeliae*, *L. longbeachae*, *L. lytica*, *L. micdadei*, *L. quinlivanii*, *L. rubrilucens*, *L. sainthelensi* and *L. spiritensis* were matched against the type strain database with an error tolerance of 1.0–10%. Results from the comparison of these 'non-type' strains (dataset I) against the database of type strains with an error tolerance of 3% are shown in Table 2. Strains were identified to the correct species (or species group) in all cases for only seven of these 13 species. Strains from the other species were mis-identified in some or all cases.

Data analysis of recent isolates of Legionella

Recent isolates (identified by conventional methods) were identified to the presumed correct species (or species group) in only a minority of cases by the PCR-based method. Patterns from recent isolates of *Legionella* (dataset II) were matched against the 'type strain database' (see above). Results from the comparison of these strains against the database of type strains with a fixed error tolerance of 3% are shown in Table 3. Molecular size files of amplification products of the ISR from recent isolates were identified to the presumed correct species (or species group) for some strains belonging to the following 12 species: *L. anisa*, *L. bozemanii*, *L. dumoffii*, *L. erythra*, *L. gormanii*, *L. jordanis*, *L. lytica*, *L. micdadei*, *L. nautarum*, *L.*

oakridgensis, *L. spiritensis* and *L. steigerwaltii*. Identification to the presumed correct species (or species group) was not achieved with any strains representing the following nine species: *L. birminghamsensis*, *L. cherrii*, *L. feeleeii*, *L. maceachernii*, *L. rubrilucens*, *L. quinlivanii*, *L. pneumophila*, *L. sainthelensi* and *L. wadsworthii*.

ISR identification of L. pneumophila

Of the 27 strains examined (including both well characterised and recent isolates) of *L. pneumophila*, 14 appeared to show similar patterns to the type strain (sharing the three major bands). However, with a fixed error tolerance of 3%, only eight were recognised by ISR identification as *L. pneumophila* (Fig. 4).

ISR identification of L. lytica and the Legionella-like amoebal pathogens

Of the 11 strains of *Legionella*-like amoebal pathogens (LLAPs) tested, only two (LLAP-7 and LLAP-11) were identified as *L. lytica*. Patterns from all the remaining LLAPs were distinct from the type strain (data not shown).

Discussion

The principal aim of the study was to evaluate critically a published method for its suitability in the identification to species level of recent isolates of *Legionella*. Deviations from the previous method are fully acknowledged, i.e., method of DNA preparation, make of thermocycler, S1 nuclease digestion of PCR products, electrophoresis conditions and method of analysis. Such deviations were made only after preliminary investigation had shown that they led to improvements in reproducibility and data analysis.

Many of the ISR banding patterns from the type strains could be distinguished visually. However, a practical identification scheme for *Legionella* spp. should allow comparison of test strains against a previously characterised panel without the necessity of testing all previously characterised species at the same time. Thus some type of computer-assisted analysis is required. With the conditions determined to be optimal for this study (a fixed error tolerance of 3%), the type strains could be differentiated for only 60% (26 of 43) of *Legionella* spp. This was not a result of variation resulting from the gel running conditions, as these were highly reproducible (< 1.5%), but rather because several species produced similar patterns with only small differences in the molecular size of some bands. These remaining species fell into four groups (comprising 10, three, two and two species, respectively). All the species in group I (except *L. cincinnatiensis*) demonstrated the phenotypic property of blue-white autofluorescence

Table 2. Results of identification (with the Taxotron[®] package) of well-characterised strains of *Legionella* (dataset 1) with a fixed error tolerance of 3%

NCTC no.	Original designation	<i>Legionella</i> species	Serogroup	Identification to <i>Legionella</i> species or species group			Closest match within group	Reference
				<i>Legionella</i> spp.	Variation (%)			
11191	Pontiac-1	<i>L. pneumophila</i>	1	<i>L. pneumophila</i>	2.5	...		
11230	Togus-1	<i>L. pneumophila</i>	2	No match	...	...		
11232	Bloomington-2	<i>L. pneumophila</i>	3	No match	...	...		
11233	Los Angeles-1	<i>L. pneumophila</i>	4	<i>L. pneumophila</i>	3.0	...		
11417	Cambridge-2	<i>L. pneumophila</i>	5	No match	...	...		
11287	Oxford-1	<i>L. pneumophila</i>	6	No match	...	...		
11406	Chicago-2	<i>L. pneumophila</i>	6	No match	...	...		
11984	Chicago-8	<i>L. pneumophila</i>	7	No match	...	...		
11985	Concorde-3	<i>L. pneumophila</i>	8	<i>L. pneumophila</i>	3.0	...		
11986	IN-23-GI-C2	<i>L. pneumophila</i>	9	No match	...	...		
12000	Leiden-1	<i>L. pneumophila</i>	10	<i>L. pneumophila</i>	2.3	...		
12179	797-PA-H	<i>L. pneumophila</i>	11	No match	...	...		
12180	570-CO-H	<i>L. pneumophila</i>	12	No match	...	...		
12181	82A3105	<i>L. pneumophila</i>	13	No match	...	...		
12174	1169-MN-H	<i>L. pneumophila</i>	14	No match	...	...		
	Lansing-3	<i>L. pneumophila</i>	15	<i>L. pneumophila</i>	3.0	...		[2]
	Jena-1	<i>L. pneumophila</i>	16	<i>L. pneumophila</i>	2.3	...		[15]
11405	Dallas-1E	<i>L. pneumophila</i> subsp. <i>fraseri</i>	5/8/10	<i>L. pneumophila</i>	2.5	...		
12273	U8W	<i>L. pneumophila</i> subsp. <i>pascullei</i>		<i>L. pneumophila</i>	1.5	...		
11369	MI-15	<i>L. bozemanii</i>	1	No match	...	...		
11975	Toronto-3	<i>L. bozemanii</i>	2	Group I	0.2	<i>L. dumoffii</i>		
11402	TEX-KL	<i>L. dumoffii</i>		Group I	1.0	<i>L. dumoffii</i>		
	LC1317	<i>L. erythra</i>		No match	...	...		[18]
	LC709	<i>L. erythra</i>		<i>L. erythra</i>	0.2	...		[18]
11978	691-WI-H	<i>L. feeleeii</i>	2	Group III	1.5	<i>L. feeleeii</i>		
11980	798-PA-H	<i>L. hackeliae</i>	2	<i>L. hackeliae</i>	1.3	...		
11530	Tucker-1	<i>L. longbeachae</i>	2	<i>L. lytica</i>	2.0	...		
	LLAP-1	<i>L. lytica</i>		No match	...	...		[17]
	LLAP-2	<i>L. lytica</i>		No match	...	...		[17]
	LLAP-3	<i>L. lytica</i>		No match	...	...		[17]
	LLAP-4	<i>L. lytica</i>		<i>L. erythra</i>	2.0	...		[17]
	LLAP-6	<i>L. lytica</i>		No match	...	...		[16, 17]
	LLAP-7	<i>L. lytica</i>		<i>L. lytica</i>	0.6	...		[17]
	LLAP-8	<i>L. lytica</i>		No match	...	...		[17]
	LLAP-9	<i>L. lytica</i>		No match	...	...		[17]
	LLAP-10	<i>L. lytica</i>		No match	...	...		[17]
	LLAP-11	<i>L. lytica</i>		<i>L. lytica</i>	1.6	...		[17]
	LLAP-12	<i>L. lytica</i>		No match	...	...		[17]
11372	PPA	<i>L. micdadei</i>		<i>L. micdadei</i>	2.3	...		
11403	HEBA	<i>L. micdadei</i>		<i>L. micdadei</i>	0.4	...		
12434	LC870	<i>L. quinlivanii</i>	2	No match	...	...		
	LC704	<i>L. rubrilucens</i>		No match	...	...		[18]
	LC705	<i>L. rubrilucens</i>		No match	...	...		[18]
	LC706	<i>L. rubrilucens</i>		No match	...	...		[18]
	LC707	<i>L. rubrilucens</i>		No match	...	...		[18]
	LC708	<i>L. rubrilucens</i>		No match	...	...		[18]
	LC805H	<i>L. rubrilucens</i>		No match	...	...		[18]
	LC805N	<i>L. rubrilucens</i>		No match	...	...		[18]
	LC985	<i>L. rubrilucens</i>		No match	...	...		[18]
	LC1092	<i>L. rubrilucens</i>		No match	...	...		[18]
12450	1489-CA-H	<i>L. sainthelensi</i>	2	No match	...	...		
12082	ML76	<i>L. spiritensis</i>	2	<i>L. spiritensis</i>	2.2	...		

Identification was attempted by searching in the database for matching patterns, noting the percentage variation in size between the fragments in the tested pattern and that in the matching database pattern. The maximum percentage variation in fragment size is indicated. References for well characterised strains that are not available from the NCTC or ATCC are given.

(Fig. 3), and these species have been shown to be closely related phylogenetically by 16S rDNA sequence analysis [21, 29].

Differences in the ISR banding patterns of the type strains compared to those obtained by Hookey *et al.* [14] were apparent for most species. Although this could be caused by a number of factors, the most

likely are the use of S1 nuclease and the electrophoresis conditions. The use of S1 nuclease was reported to digest unwanted single-stranded PCR products to give clearer banding patterns [14]. However, S1 nuclease can digest double-stranded DNA and cleave double-stranded nucleic acids at nicks or small gaps [27]. When applied in the present study, this treatment led to smearing of the banding patterns and could,

Table 3. Results of identification test with the Taxotron[®] package of a set of recent isolates identified by standard methods (dataset II)

APU no.	Identification by standard methods	Serogroup	Identification to <i>Legionella</i> species or species group		Closest match within group
			<i>Legionella</i> sp.	Variation (%)	
EUL22	<i>L. pneumophila</i>	1	No match	...	...
EUL26	<i>L. pneumophila</i>	1	No match	...	...
EUL56	<i>L. pneumophila</i>	1	No match	...	...
EUL61	<i>L. pneumophila</i>	1	No match	...	...
EUL66	<i>L. pneumophila</i>	1	No match	...	...
EUL75	<i>L. pneumophila</i>	1	No match	...	...
EUL96	<i>L. pneumophila</i>	1	No match	...	...
EUL31	<i>L. pneumophila</i>	1	No match	...	...
LC3962	<i>L. pneumophila</i>	3	No match	...	...
LC3934	<i>L. anisa</i>		Group I	0.9	<i>L. anisa</i>
LC1276	<i>L. anisa</i> / <i>L. parisiensis</i>		Group I	0.5	<i>L. tucsonensis</i>
LC2720	<i>L. birminghamensis</i>		No match	...	...
LC3266	<i>L. bozemanii</i>	1	Group I	1.1	<i>L. steigerwaltii</i>
LC3932	<i>L. bozemanii</i>	1	No match	...	...
LC777C	<i>L. cherrii</i>		No match	...	...
LC3664	<i>L. cherrii</i>		No match	...	...
LC455	<i>L. dumoffii</i>		Group I	1.3	<i>L. parisiensis</i>
LC3719	<i>L. erythra</i>		<i>L. erythra</i>	2.2	...
LC797	<i>L. feeleeii</i>	2	No match	...	...
LC458	<i>L. gormanii</i>		<i>L. gormanii</i>	2.3	...
LC1436	<i>L. jamestowniensis</i>		No match	...	...
LC3940	<i>L. jordanis</i>		<i>L. jordanis</i>	1.6	...
LC818A	<i>L. maceachernii</i>		No match	...	...
LC3743	<i>L. maceachernii</i>		No match	...	...
LC858	<i>L. micdadei</i>		<i>L. micdadei</i>	0.3	...
LC3745	<i>L. micdadei</i>		No match	...	...
LC1128	<i>L. nautarum</i>		No match	...	...
LC3779	<i>L. nautarum</i>		<i>L. nautarum</i>	1.0	...
LC3845	<i>L. nautarum</i>		No match	...	...
LC1251	<i>L. oakridgensis</i>		No match	...	...
LC3741	<i>L. oakridgensis</i>		No match	...	...
LC3780	<i>L. oakridgensis</i>		<i>L. oakridgensis</i>	0.7	...
LC2763	<i>L. parisiensis</i>		No match	...	...
LC3505	<i>L. quinlivanii</i>	2	No match	...	...
LC3764	<i>L. rubrilucens</i>		No match	...	...
LC3851	<i>L. rubrilucens</i>		No match	...	...
LC3936	<i>L. sainthelensi</i>	2	No match	...	...
LC4042	<i>L. spiritensis</i>	1	No match	...	...
LC738	<i>L. steigerwaltii</i>		No match	...	...
LC777B	<i>L. steigerwaltii</i>		Group I	2.0	<i>L. anisa</i> / <i>L. steigerwaltii</i>
LC1329	<i>L. tucsonensis</i>		No match	...	...
LC121	<i>L. wadsworthii</i>		No match	...	...
LC4045*	<i>Legionella</i> sp.		<i>L. erythra</i>	2.8	...
LC4046†	<i>Legionella</i> sp.		No match	...	...
LC4048‡	<i>Legionella</i> sp.		No match	...	...
LC4049§	<i>Legionella</i> sp.		No match	...	...

APU, Atypical Pneumonia Unit, Respiratory and Systemic Infection Laboratory, CPHL, London.
*Turin I no. 1, †Montbeliard A1, ‡Greoux II D13, and §Mulhouse B26; represent putative new species, subspecies or serogroups (J. Etienne, personal communication).

potentially, lead to the loss of lower molecular size bands and the digestion of higher molecular size bands to artificially lower sizes, thus it was not used for the main study. In the authors' laboratory, the use of poly(NAT)TM pre-cast gels and controlled electrophoresis conditions resulted in greatly increased resolution, sensitivity and reproducibility compared to electrophoresis by the conditions in the method published previously [14] (data not shown). These factors, combined with use of the Taxotron[®] software package, enabled precise size estimation of the band sizes to be achieved. The error tolerance of 3% used in the study was chosen empirically. Extensive analyses were done with various values (1.0–10.0%) to maximise the potential for an unknown pattern to

be matched correctly. However, given the great similarity of many of these patterns, any additional 'true matches' achieved at higher tolerance levels were always accompanied by a greater number of mismatches.

Well-characterised non-type strains from 14 species were identified to the correct species (or species group) in some cases. However, strains from the eight other species for which strains were available were not identified, or identified incorrectly. In contrast to the previously published method [14], discrimination of the type strains of the two red autofluorescent species, *L. erythra* and *L. rubrilucens*, was achieved by the method described here. This difference is likely to be

Fig. 4. Dendrogram illustrating results of PCR-amplification of 16S-23S rDNA obtained with 28 strains of *L. pneumophila*.

related to the increased resolution of the gels and running conditions used in this study. However, although two recent isolates of *L. erythra* (LC709 and LC1317 [18]) were examined and correctly identified, none of the nine strains of *L. rubrilucens* was identified correctly.

It has been proposed that LLAPs should not all be placed in the single species *L. lytica*, but should be split into at least five species [17, 20]. Whilst some of the ISR patterns obtained in this study showed correlation with the proposed new species, the agreement was not absolute (personal unpublished observations). However, the analysis of the ISR here is not a phylogenetic one and the relationships revealed by such analyses must be treated with caution.

The small number of strains of *L. pneumophila* recognised correctly by ISR identification may demonstrate the heterogeneity of this species (Fig. 4). An alternative and more likely explanation is that as *L. pneumophila* was the only species with a large number of isolates available for testing, this result may indicate what would be found for many other species of *Legionella* if multiple recent isolates were available for analysis.

The choice of primers obviously affects the resulting band patterns in ISR analysis. There are several well-characterised regions that can be used as targets to amplify all copies of the 16S–23S rDNA spacer positions. In the 16S rRNA gene, there are three highly conserved positions, i.e., 1390–1407, 1525–1541 and 1491–1506 (*E. coli* numbering [30, 31]). From the available 23S rRNA gene sequence data, six conserved regions within the first 520 bp of the gene have been described [30]. Gürtler *et al.* [31] recommended optimum primer target regions for amplification of the 16S–23S rDNA spacer region. Neither of the primers chosen by Hookey *et al.* [14] targeted these regions and, furthermore, the forward primer has one mis-match with *Legionella* spp. 16S rDNA in this region. However, it appears unlikely that this factor alone would explain the failure of the ISR method. The purpose of this study was to evaluate the published method [14] for its use in the identification of new isolates, hence the primers of Hookey *et al.* [14] were used. However, to take account of the recommendation above [31], an alternative primer pair [26] was examined, but also demonstrated intra-species variation in the ISR patterns obtained.

The identification and typing of bacteria by PCR-amplification of the ISR of the 16S and 23S rRNA genes has been described for several genera [31, 32]. Such banding patterns obtained with 16S and 23S rDNA primers described previously are assumed to be the result of specific amplification of these target regions. However, in the absence of supporting sequence data or adequate description of the rRNA

operons, the presence of multiple, often weak bands, must be regarded with caution. It may be that some of the resulting bands are not genuine amplicons from the 16S–23S rDNA intergenic region. That such methods have been demonstrated to be successful for the identification of other genera to species level may reflect true homogeneity within those species or in some cases may reflect the paucity of strains examined.

The patterns resulting from the amplification of the 16S–23S rDNA ISR do appear to be distinct and stable for some species of *Legionella*. However, intra-species heterogeneity and a high degree of inter-species similarity of patterns for many of the species makes identification by this method unsuitable for the genus as a whole. This study illustrates the importance of testing recent isolates (preferably in large numbers) when investigating any potential identification system. It is possible that the apparent intra-species variation observed in many of the species could be exploited for epidemiological typing for these particular species. However, for the family Legionellaceae, identification to the species level remains a challenge which may be better addressed by investigating alternative target sites in the genome.

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