MOLECULAR TYPING AND EPIDEMIOLOGY

Molecular typing of *Legionella pneumophila* serogroup 1 by pulsed-field gel electrophoresis with *Sfi*I and comparison of this method with restriction fragment-length polymorphism analysis

A. S. DE ZOYSA and T. G. HARRISON

Respiratory and Systemic Infection Laboratory, PHLS Central Public Health Laboratory, 61 Colindale Avenue, London NW9 5HT

A panel of 131 well-characterised *Legionella pneumophila* serogroup 1 strains, previously used to evaluate a restriction fragment-length polymorphisms (RFLP) typing scheme, was examined by pulsed-field gel electrophoresis (PFGE) with the restriction endonuclease *Sfi*I. The data obtained show that PFGE with *Sfi*I is a highly discriminatory method yielding an index of discrimination (IOD) of 0.992 and 0.975, with 100% and 90% similarity thresholds respectively, compared with an IOD of 0.909 for the RFLP typing method. Reproducibility of PFGE profiles within gels was excellent and it was possible to compare the profiles visually. However, the reproducibility of the technique between gels was poor and visual comparison of the patterns was extremely difficult. Computer-aided analysis assisted the assessment of inter-gel reproducibility. Of 11 duplicates examined only four pairs showed 100% similarity, although 9 of 11 showed $\geq 90\%$ similarity. In an attempt to determine if the PFGE banding patterns were sufficiently unambiguous to allow the method to be used as a definitive typing method, 20 coded strains were examined. At a 90% similarity level, 16 of these were placed in the correct PFGE type and four were not allocated to a type. Partial digestion of DNA by *Sfi*I was noted despite careful control of DNA and enzyme concentrations, suggesting that an alternative enzyme might give more reproducible results.

Introduction

Legionnaires' disease (LD) is an uncommon but important form of pneumonia caused by bacteria of the genus *Legionella*, and is manifest as both community-acquired and nosocomial infection [1, 2]. Legionellae are ubiquitous in the environment and a wide variety of natural and man-made water distribution systems have been implicated as sources of infection [3, 4]. Although >40 species of *Legionella* have been identified, *Legionella pneumophila*, particularly serogroup 1, is the species and serogroup most often associated with human disease [5]. As this serogroup is also isolated frequently from environmental specimens, various typing methods have been developed to investigate epidemiological links between cases and potential environmental sources [6–14].

Since 1988, this laboratory has used restriction fragment-length polymorphisms (RFLP) analysis [15, 16] to type strains of *L. pneumophila* and has examined >1000 isolates, most of which were serogroup 1. The method, which uses two randomly cloned, chromosomal DNA probes to reveal a simple pattern of bands on Southern blots of *Nci*I-digested DNA, has been evaluated thoroughly and shown to be highly discriminatory (~100 types), reproducible and stable [15, 16]. The patterns obtained are simple enough to be identified easily and allocated to a definitive type by visual examination of blots. The method has been used widely in the investigation of outbreaks and clusters of legionellosis in the UK [15, 17, 18].

Although the discriminatory power of the RFLP technique appears to be good, giving an index of discrimination (IOD) [19] of 0.909, in practice it has been found that some RFLP patterns are seen more frequently than others (e.g., RFLP type 1 [15]). In 1995, Lück and colleagues [20] demonstrated that a

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Corresponding author: Mrs A. S. De Zoysa.

panel of isolates obtained from a single source, and considered to be indistinguishable by RFLP typing [21], could be discriminated further by PFGE with *Sfi*I.

These data raised the following questions: (i) are isolates of these common RFLP types really representatives of frequently encountered strains or rather, as the PFGE study suggested, is the observation merely indicative of the lower discrimination of the RFLP method compared with PFGE? (ii) if the PFGE technique is more discriminatory, can PFGE be used like RFLP as a definitive typing method, allowing long-term comparison of strains, or is its application confined to comparative typing or 'fingerprinting' where strains can be compared only when examined in parallel?

Although used widely, PFGE has not been evaluated systematically as a typing method for *L. pneumophila*. In this study, a large panel of *L. pneumophila* serogroup 1 strains used previously to evaluate the RFLP typing method was examined by PFGE with *Sfi*I. The results obtained by PFGE were compared with the available RFLP typing data [15]. In addition, the PFGE data were analysed to determine if the PFGE banding patterns were sufficiently unambiguous to allow the method to be used as a definitive typing method rather than a fingerprinting technique. The computer software programme GelCompar was used to aid analysis of the PFGE patterns.

Materials and methods

Bacterial isolates

Isolates of *L. pneumophila* serogroup 1 submitted to the Respiratory and Systemic Infection Laboratory between 1979 and 1988 from sources in Europe, North America, Asia and Australia became part of the study. Additional strains were obtained from the National Collection of Type Cultures, Colindale. The identity of all isolates was confirmed by nutritional, biochemical and serological characteristics [5]. The isolates, which were stored on glass beads at -70°C , were recovered by inoculation on to BCYE Agar (Oxoid) and incubated for 72 h at 37°C before the growth was harvested.

All but seven of the strains examined previously for the RFLP typing study [15] were available for examination by PFGE; seven isolates could not be recovered from frozen beads. Thus 131 isolates were included, of which 99 were considered to be unrelated as they had not been isolated from patients or environmental sources with any geographical or temporal connection to any other isolate included in the study (Table 1). The remaining 32 isolates comprised sets of related isolates and were used to determine the data analysis parameters for GelCompar,

and to evaluate the reproducibility of the PFGE method (Table 2).

Pulsed-field gel electrophoresis

The method used was essentially that described by Lück and colleagues [25], but with the pulse times reported in their later study [20] and with some modifications to the gel running conditions.

Briefly, bacteria were grown on BCYE agar and harvested in SE buffer (75 mM NaCl, 25 mM EDTA, pH 7.4). Bacterial cells were washed twice in the same buffer and the turbidity was adjusted to an OD_{600} of 1.5. An equal volume of cell suspension and molten low melting point (LMP) agarose (Life Technologies) 2% w/v in SE buffer were mixed in a 50°C water bath, making a final concentration of LMP agarose in the blocks of 1%. The suspension was pipetted into a multiple-well plug former ($10 \times 6 \times 1$ mm) and allowed to set at 4°C for 10 min. The agarose blocks were then transferred in 3 ml of NDS buffer (Sarkosyl 1% w/v, 0.5 M EDTA, pH 9.5, containing proteinase K 2 mg/ml) and incubated overnight at 50°C . The plugs were washed three times (30 min each at 4°C) with TE buffer (10 mM Tris, 0.5 mM EDTA, pH 7.4) and stored at 4°C .

Sections (2 mm) were excised from each agarose plug and equilibrated for 1 h on ice with 100 μl of restriction buffer, after which the buffer was replaced with 100 μl of fresh buffer and 60 U of *Sfi*I (Boehringer). The mixture was incubated overnight at 50°C . DNA fragments were separated in an agarose (Ultra pure; Life Technologies) 1% gel in $0.5 \times$ Tris-borate-EDTA buffer with a CHEF DRII or a CHEF DRIII system (BioRad, Herts).

The gel was run at 14°C for 40 h with an initial pulse time of 60 s and a final pulse time of 5 s, stained in ethidium bromide 1 $\mu\text{g}/\text{ml}$ for 45 min and photographed on Polaroid type 667 instant sheet film. Photographs of the PFGE profiles were analysed visually and with the assistance of computer software packages.

Data capture for computer analysis

The PFGE profiles were prepared for analysis as follows. The Polaroid photographs were scanned (Scanmaker E6, Microtek Lab) into Adobe Photoshop at 150 dpi with 256 levels of grey. The contrast of the scanned photographs was optimised (by the automatic facility of the software) and the resulting images were saved as TIFF files. Each TIFF file was then converted, normalised and analysed by GelCompar (Version 4.0, Applied BioMaths, Kortrijk, Belgium). Normalisation within each gel was achieved with λ concatemer markers (BioRad) which were placed in every third gel track. One gel marker track (selected at random)

Table 1. Summary of the 99 unrelated isolates used in the estimation of discrimination

Designation	Source*	RFLP type	Arbitrary PFGE type 100% (PF100) [†]	Arbitrary PFGE type 90% (PF90) [‡]	MAb subgroup [§]
ML 56	C (UK)	1	1	1	Philadelphia
ML 93	C (Eu)	1	2	2	Philadelphia
ML 124	C (UK)	1	3	3	Philadelphia
ML 133	C (Ch)	1	3	3	Philadelphia
LC 26	C (Fr)	1	4	3	Philadelphia
LC 32	C (Fr)	1	5	4	Philadelphia
LC 81	C (UK)	1	6	3	Philadelphia
LC 433	C (UK)	1	3	3	Philadelphia
LC 631	E (UK)	1	7	5	Philadelphia
LC 41	C (Fr)	1	8	6	Allentown
LRU 16	E (UK)	1	3	3	Allentown
LC 28	C (Fr)	1	3	3	Benidorm
A310/80	C (UK)	1	9	7	Benidorm
LC 20	E (Fr)	1	10	8	France
LC 620	C (UK)	1	3	3	France
LC 629	E (UK)	1	11	9	Olda
LC 54	C (Fr)	1	12	10	Oxford
LC 400	E (UK)	1	13	11	Oxford
LC 470	E (UK)	1	14	3	Oxford
LC 6	C (UK)	1	15	12	Heysham
LC 260	C (UK)	1	15	12	Heysham
LC 636	E (UK)	1	16	11	Heysham
A29/81	E (UK)	1	17	13	Heysham
LRU 7	E (UK)	1	19	15	Camperdown
LC 359	? (USA)	1	18	14	Other
LC 521	E (UK)	1	16	11	Other
LC 376	C (UK)	2	20	16	Philadelphia
NCTC 11191 (Pontiac-1)	E (USA)	2	21	16	Philadelphia
ML 51	C (Czech)	3	22	17	Knoxville
NCTC 11286 (Knoxville-1)	C (USA)	3	23	17	Knoxville
LC 647	E (UK)	3	24	18	Bellingham
LC 358	E (Sing)	4	25	19	Bellingham
NCTC 11404 (Bellingham-1)	C (USA)	4	26	17	Bellingham
LC 423	C (UK)	5	27	20	Philadelphia
LC 480	E (UK)	5	28	21	Philadelphia
LC 536	C (UK)	5	29	20	Philadelphia
LC 639	C (UK)	5	30	22	Philadelphia
LC 660	C (UK)	5	29	20	Philadelphia
LRU 78	C (UK)	5	29	20	Philadelphia
NCTC 11192 (Philadelphia-1)	C (USA)	5	31	21	Philadelphia
LC 512	C (UK)	5	29	20	Philadelphia
LC 99	C (Fr)	5	32	8	Allentown
LC 31	C (Fr)	5	33	8	France
LC 57	C (Fr)	5	33	8	France
LC 173	C (Fr)	5	33	8	France
LC 360	? (USA)	6	34	15	Other
LC 409	E (UK)	7	35	23	Allentown
LC 474	E (UK)	7	36	24	Allentown
LRU 5	E (UK)	7	37	4	Oxford
LC 235	C (Po)	8	38	25	Allentown
ML 70	C (UK)	10	39	26	Benidorm
LRU 89	C (UK)	10	40	27	Benidorm
LC 380	E (UK)	10	41	28	Bellingham
A32/81	E (UK)	11	42	29	Knoxville
A100/81	E (Aus)	12	43	17	Bellingham
LC 19	C (Fr)	13	44	30	Bellingham
LC 51	C (Fr)	13	45	31	Bellingham
LC 80	C (Sp)	14	46	32	Benidorm
LC 230	C (Eu)	14	47	33	Benidorm
A167/80	C (Eu)	14	48	34	Benidorm
LC 412	C (Gr)	14	49	32	Benidorm

continued overleaf

Table 1. (continued)

Designation	Source*	RFLP type	Arbitrary PFGE type 100% (PF100) [†]	Arbitrary PFGE type 90% (PF90) [‡]	MAb subgroup [§]
A218/80	E (UK)	15	50	35	Bellingham
176/79	C (USA)	16	51	36	Philadelphia
LC22	C (Fr)	17	52	34	Bellingham
LC 435	C (Gr)	18	53	37	Philadelphia
LC 372	C (UK)	19	54	34	Benidorm
LC 642	E (UK)	19	55	34	Benidorm
ML 47	C (UK)	20	56	38	Benidorm
LC 36	C (Fr)	21	57	39	France
LC 27	C (Fr)	22	58	19	Bellingham
LC 457	E (Sing)	23	59	40	Philadelphia
ML 72	C (I)	23	60	41	Benidorm
LC 436	C (Yu)	24	61	42	Allentown
ML 66	C (UK)	25	62	43	Knoxville
LC 669	C (UK)	25	62	43	Knoxville
LC 421	C (UK)	25	63	22	Knoxville
ML 54	C (UK)	26	64	44	Philadelphia
LC 185	C (Fr)	26	33	8	Philadelphia
LC 460	C (Fr)	26	65	45	Philadelphia
LC 190	C (Fr)	26	66	46	France
LC 199	C (Fr)	26	67	47	France
LC 62	C (N)	26	68	47	Knoxville
LC 661	E (UK)	26	69	47	Bellingham
A358/80	C (Fr)	26	70	47	Bellingham
A175/79	C (UK)	27	71	2	Benidorm
LC 547	E (UK)	27	72	48	Olda
LC 567	E (UK)	27	73	49	Olda
LC 514	E (Sing)	27	74	14	Oxford
ML 68	C (UK)	28	75	29	Philadelphia
ML 150	C (N)	28	76	50	Benidorm
LC 564	E (Ost)	29	77	51	Allentown
ML 87	E (HK)	31	3	3	Olda
LC 607	E (UK)	32	78	52	Other
LC 657	E (Tk)	35	79	42	Olda
LC 635	C (Yu)	36	79	42	Benidorm
LC 638	E (UK)	37	80	53	Bellingham
LC 667	C (Dom)	38	81	54	Benidorm
LC 518	E (UK)	39	82	54	Bellingham
LC 615	C (Gr)	Untypable	83	55	Allentown

*C, clinical isolate; E, environmental isolate; ?, source not known. The country of origin (environmental isolates) or country in which a patient was thought to have contracted their illness (clinical isolate) is indicated as follows: UK, United Kingdom; Eu, continental Europe (more than one country visited during the incubation period); Ch, Switzerland; Fr, France; USA, United States of America; Czech, Czech Republic; Sing, Singapore; Po, Portugal; Aus, Australia; I, Italy; Sp, Spain; Gr, Greece; Yu, Yugoslavia; N, the Netherlands; Ost, Austria; HK, Hong Kong; Tk, Turkey; Dom, Dominican Republic.

[†]PF100, strains were considered to be indistinguishable if they were 100% similar.

[‡]PF90, strains were considered to be indistinguishable if they were $\geq 90\%$ similar.

[§]MAb subgroup as designated by Joly *et al.* [12] except 'Other' which indicates that the pattern of reaction did not fit into the described scheme.

was used as the normalisation standard for 'between-gel' normalisation of all gels in the study. The bands for each PFGE profile were first identified by the auto-search facility in the Analysis module (set for minimum of 15% profile, 5% total area) followed by review and modification after a careful visual comparison of the gel photograph and GelCompar image.

RFLP typing and monoclonal antibody subgrouping

RFLP typing and monoclonal antibody (MAb) subgrouping data obtained for these strains as part of an earlier study [15] were used in this study for comparison with the PFGE profiles.

Table 2. Summary of the 'related' strains examined to assess reproducibility and to determine analysis criteria

Designation	Source	Category	RFLP type	Arbitrary PFGE type		MAB subgroup
				PF 100*	PF 90*	
LC 230	C (Sp)	Direct plating of sputum	14	47	47	Benidorm
LC 231		Amoebal enrichment	14	47	47	Benidorm
LC 462	C (Sp)	Direct plating of sputum	14	84 [†]	54	Benidorm
LC 463		Amoebal enrichment	14	84 [†]	54	Benidorm
LC 260	C (UK)	Lung washings	1	15	15	Heysham
LC 308		Sputum	1	15	15	Heysham
LC 372	C (UK)	Sputum	19	54	54	Benidorm
LC 373		Sputum	19	54	54	Benidorm
LC 669	C (UK)	Sputum	25	62	62	Knoxville
LC 676		Post-mortem lung	25	62	62	Knoxville
ML 64	C (UK)	Clinical isolates from	25	62	62	Knoxville
ML 65		Stafford District Hospital	25	62	62	Knoxville
ML 66		outbreak [22]	25	62	62	Knoxville
LC 436	C (Yu)	Clinical isolates from Yugoslavian	24	61	61	Allentown
LRU 88		hotel outbreaks [‡]	24	61	61	Allentown
LC 65	E (N)	Clinical and environmental isolates from Dutch Hospital outbreak [6]	26	68	68	Knoxville
LC 66	E (N)		26	68	68	Knoxville
LC 67	C (N)		26	68	68	Knoxville
LC 68	E (N)		26	68	68	Knoxville
LC 69	C (N)		26	68	68	Knoxville
LC 70	E (N)		26	68	68	Knoxville
LC 71	C (N)		26	68	68	Knoxville
LC 536	C (UK)	Clinical isolates from Portland Place outbreak [23]	5	29	29	Philadelphia
LC 537			5	29	29	Philadelphia
LC 539			5	29	29	Philadelphia
LC 540			5	29	29	Philadelphia
LC 541			5	29	29	Philadelphia
LC 568			5	29	29	Philadelphia
LC 583			5	29	29	Philadelphia
LC 584			5	29	29	Philadelphia
LC 660	C(UK)	Clinical isolate from Lostock outbreak [24]	5	29	29	Philadelphia
LC 671			5	29	29	Philadelphia

*See footnote to Table 1.

[†]No representative of this set of isolates was included in the estimation of discrimination, therefore a new 'type' was designated.[‡]Communicable Disease Surveillance Centre (1987), confidential report.

Results

Visual discrimination of PFGE profiles

The PFGE profiles obtained with *Sfi*I each comprised 10–12 bands (Fig. 1). In most cases the bands were clear and unambiguous. However, in a significant proportion of strains, faint bands were visible and in a few cases there was only partial digestion of the bacterial DNA.

From a simple visual inspection of the profiles of the unrelated strains, it was apparent that PFGE with *Sfi*I was a highly discriminatory method. However, even with this quite modest dataset of 99 isolates, it was not easy to compare profiles obtained on different gels and it was certainly not possible to quantify the degree of discrimination or to allocate a 'type' designation to a particular pattern. Therefore, visual analysis of the gels was not considered further.

Strategy for analysis of PFGE profiles by GelCompar

As PFGE profiles comprise a series of discrete bands of a specific sequence length it was considered inappropriate to compare isolates by measuring the correlation between the entire densitometric image of each gel track. Rather the individual bands of each gel track were identified (automatically first, then modified by visual comparison) and GelCompar was then used to order the band patterns by their degree of similarity. To achieve this, various parameters such as the algorithm used for determining similarities, cluster analysis and dendrogram construction, and the level of acceptable band tolerance, had first to be determined.

Table 2 shows 10 sets of isolates which, from epidemiological data and earlier studies [15], were considered to be representatives of the 'same strain' and should therefore be indistinguishable. A selection

Fig. 1. PFGE *Sfi*I profiles of 16 of the 26 *L. pneumophila* serogroup 1 RFLP type 1 strains shown in Table 1. The figure illustrates the discriminatory power of PFGE compared with RFLP. Lanes 1, 4, 7, 10, 13, 16, 19, 22 and 25 contain λ concatemer as a size standard (sizes indicated on the left). The remaining tracks show PFGE profiles of the following strains: 2, LC 400; 3, LC 470; 5, LC 6; 6, LC 260; 8, LC 636; 9, A29/81; 11, LRU 7; 12, LC 359; 14, LC 521; 15, LC 376; 17, NCTC 11191; 18, ML 51; 20, NCTC 11286; 21, LC 647; 23, LC 358; 24, LC NCTC 11404.

of matching coefficients and band tolerances was investigated to determine the optimum parameters that would give maximum discrimination between the 99 unrelated isolates, but allocate related and visually indistinguishable isolates into distinct groups. For this study the optimum was determined to be the Dice coefficient with a 3.0% error tolerance for the migration distance of each band. These settings were used throughout the rest of the study. Cluster analysis was undertaken with the unweighted pair-group method with averages (UPGMA).

Discrimination of PFGE compared with RFLP

According to the analysis criteria outlined above, and with isolates considered to be indistinguishable only if they were 100% similar, the 99 unrelated isolates were discriminated into 83 arbitrary PFGE types (designated 'PF100 types') giving an IOD of 0.992. This compared with 35 RFLP types, with an IOD of 0.909. When isolates were considered to be indistinguishable by PFGE if they were $\geq 90\%$ related, the 99 isolates were separated into 55 arbitrary PFGE types (designated 'PF90 types') with an IOD of 0.975 (Table 1).

It was demonstrated clearly that PFGE can discriminate within the more common RFLP types. For example, the 26 unrelated RFLP type 1 isolates (Table 1) [15] were divided into multiple PFGE types (Table 1, Figs. 1 and 2). Thus 19 PF100 types and 15 PF90

types were identified. However, overall, there was some concordance between the PFGE and RFLP types such that where multiple strains of the same PFGE type were identified (e.g., PF100 type 3, and PF90 types 3, 8, 16 and 17) they also fell within the same RFLP type. This suggests, at least for some isolates, that both the RFLP and PFGE types reflect a high degree of homology in their genetic architecture.

Reproducibility of PFGE

When examined on the same gel 'related strains' appeared indistinguishable (Fig. 3 and Table 2). As expected, this was confirmed in the computer-assisted analysis as the analysis criteria used had been selected to achieve this.

To determine the level of reproducibility that might be achieved when indistinguishable isolates were encountered unexpectedly (i.e., they were not run in adjacent gel tracks and not analysed with the knowledge that they were likely to be indistinguishable), 11 unrelated strains, chosen at random and examined 'blind', were analysed in duplicate with the full panel of unrelated strains. Four of the 11 duplicates were 100% similar; 8 of 11 were $\geq 95\%$ similar and 9 of 11 were $\geq 90\%$ similar. The remaining two duplicate pairs showed $< 70\%$ similarity; however, on examination of the gels, the DNA for the one strain in each pair appeared to be only partially digested.

Fig. 2. Dendrogram generated from the PFGE profiles of the 26 RFLP type 1 strains detailed in Table 1 showing percentage similarity of the strains. Shaded area illustrates 19 PFGE types identified at 100% similarity (PF100) and 15 PFGE types identified at 90% similarity (PF90).

Definitive typing

If PFGE is to be used as a definitive typing method, it will be necessary to allocate an unknown isolate to a particular 'PFGE type' by comparison of its PFGE profile with those of the 'PFGE type strains'. To determine the feasibility of this approach, 20 of the unrelated isolates were chosen at random, and an attempt was made to type them. Previously extracted, undigested, DNA was used as starting material and the identity of the strains was blinded by coding.

Photographs of the PFGE profiles for the 20 strains were scanned, normalised and analysed with the Identification module of GelCompar. The 20 isolates were first matched with the PF100 type strain library (this comprised a single representative of each of 83 PF100 types). Only six of 20 were assigned to the correct PF100 type. One isolate was misidentified (i.e., it showed 100% similarity with PF100 type 70 although it was originally designated as PF100 type 69) and the remaining 13 isolates were not identified. The analysis was repeated with the PF90 type strain library (comprising a single representative of each of

55 PF90 types) with $\geq 90\%$ similarity required for identification. In this case 16 of the 20 strains were correctly identified and the remaining four strains were not allocated to any PF90 type. On review, it appeared that partially digested DNA confounded the identification of two of the isolates.

Relationship of MAb subgroup and PFGE type

As anticipated, MAb subgroups showed little correlation with PFGE or RFLP types. Hence the combination of MAb subgrouping and molecular analysis effectively increased the IODs from 0.909 to 0.979 (56 combined types) for combined RFLP/MAb typing, 0.992 to 0.997 (90 combined types) for PF100/MAb typing and 0.975 to 0.992 (75 combined types) for PF90/MAb typing.

Discussion

The primary reason for the epidemiological typing of *Legionella* isolates is to identify environmental source in order to prevent further infections. Most cases of LD are caused by *L. pneumophila* and the vast majority

Fig. 3. PFGE *Sfi*I profiles of some of the related *L. pneumophila* serogroup 1 strains (Table 2) showing within gel reproducibility of the technique. Lanes 3, 6, 9, 12, 15, 18, 19, and 21 contain λ concatemer as a size standard (sizes indicated on the right). The remaining tracks show PFGE profiles of the following: 1, LC 65; 2, LC 66; 4, LC 67; 5, LC 68; 7, LC 70; 8, LC 71; 10, LC 536; 11, LC 537; 13, LC 539; 14, LC 540; 16, LC 541; 17, LC 568; 19, LC 583; 20, LC 660; 22, LC 671.

of these strains are serogroup 1 [5]. Furthermore, *L. pneumophila* serogroup 1 is very frequently isolated from environmental sources, hence it is necessary to differentiate this serogroup further to establish epidemiological links which are convincing.

Numerous studies have reported the application of molecular typing methods to distinguish between strains of *L. pneumophila* [6-14]. Typically in such studies, relatively few isolates, collected over a short period of time, have been examined in parallel and the data obtained used to answer a specific epidemiological question. Was X or Y the most probable source of infection? While such 'comparative typing' or 'fingerprinting' studies have yielded valuable information in the investigations of specific outbreaks of LD, they have little value beyond that individual outbreak. In contrast, the ability to designate isolates to definitive types allows data to be compared between outbreaks and incidents, allowing strain-specific traits to be identified. The recognition that isolates of a particular *L. pneumophila* serogroup 1 MAb subgroup [12, 26] (the so-called MAb2⁺ strains) are more likely to cause infection than are MAb2⁻ strains demonstrates the value of definitive typing as applied to *L. pneumophila* [27]. Another advantage of definitive typing is the ability to compare isolates examined in different laboratories rather than having to study all isolates in the same laboratory. This is particularly important in the investigation of outbreaks of travel-associated LD where, if rapid results are required, simultaneous

analysis of samples frequently needs to be undertaken in laboratories in several different countries [18, 28].

We have used RFLP analysis [15] to examine isolates of *L. pneumophila* for many years [15-18] and the ability to 'type' rather than 'fingerprint' has facilitated the identification and monitoring of environmental sources which only occasionally, but persistently, have given rise to cases of LD. Furthermore, provisional analysis of the RFLP results of almost 600 unrelated isolates indicates that some RFLP types are more likely to cause infection than are others (authors' unpublished observations). However, although the RFLP method has demonstrated its usefulness, some RFLP types (e.g., RFLP type 1) do occur very frequently and methods with higher discriminatory potential are desirable.

Of the many molecular typing methods applied to *L. pneumophila*, PFGE is probably the most widely used and is generally considered to be highly discriminatory [20, 25, 29-32]. A study by Lück and colleagues demonstrated that a panel of isolates considered to be indistinguishable by RFLP typing could be discriminated further by PFGE with *Sfi*I, suggesting that this method was more precise than RFLP typing [20]. However, despite its widespread use there does not appear to have been a detailed evaluation of PFGE typing as applied to *L. pneumophila*. Consequently, a comprehensive evaluation of PFGE typing of *L. pneumophila* serogroup 1 isolates with *Sfi*I was undertaken to determine if it offered better discrimi-

nation than RFLP. Furthermore, the present study aimed to determine if computer-assisted PFGE analysis could be used as a definitive typing method rather than just a comparative tool.

It is clear from the data presented in this study that PFGE with *Sfi*I is a highly discriminatory method, yielding IODs of 0.992 and 0.975 (for 100% and 90% similarity thresholds, respectively) compared with 0.909 for the RFLP method. Within-gel reproducibility of PFGE was also excellent and it was possible to interpret and compare PFGE profiles on the same gel by visual examination. However, inter-gel reproducibility was poor and, consequently, although visual examination was theoretically possible, with the large number of isolates in the study, meaningful visual analysis was clearly impractical.

In an attempt to improve 'inter-gel' comparisons GelCompar was used to analyse the PFGE profiles. Given the wide variety of strategies for analysis that can be used with such software packages, a pragmatic approach was taken and parameters were chosen that ensured that isolates known to be related were found to be 100% similar when examined on the same gel. Under these conditions, the inter-gel reproducibility determined with coded duplicates was found to be quite reasonable. Nine of 11 isolates showed $\geq 90\%$ similarity, the remaining two isolates were partially digested.

If PFGE is to be used as a definitive typing method, the unknown isolates will have to be compared against a reference library of PFGE type profiles. Only six of 20 duplicates were allocated to the correct PF100 type, but 16 of 20 had the correct PF90 type. These data illustrate the usual trade-off between the discriminatory power of a technique and its reproducibility. It may be that alternative strategies of data analysis might have given even better results and this warrants further study.

Some of the limitations noted with the PFGE method used here appear to be related specifically to the use of *Sfi*I. Although this enzyme generated PFGE profiles comprising 10–12 bands of reasonable intensity, close examination of the gel photographs revealed that, despite careful control of DNA and enzyme concentrations, *Sfi*I sometimes yielded partial, rather than complete, digests.

The choice of enzyme for PFGE analysis of legionellae has been addressed in several studies. Most workers have chosen to use *Sfi*I or *Not*I, or both [30, 32–37], although Johnson and colleagues [31], who screened over 30 enzymes including *Sfi*I and *Not*I, concluded that *Bss*HI, *Sal*I and *Spe*I gave the best results. However, it should be noted that Johnson and colleagues only examined eight isolates, some of which were related. Most of the other studies have

also looked at only a small number of isolates; this contrasts with the present study, in which a total of 131 strains of diverse origin was analysed. Interestingly, it has recently been reported that *Sfi*I uses an unusual mechanism to cleave target DNA and this often results in partial rather than complete digestion [38]. Although the problems encountered in this study caused by partial digestion should not be overstated, consideration should be given to selecting an alternative restriction endonuclease for future studies.

The combined use of MAb subgrouping and either molecular typing method led to increased discrimination. However, whereas the discrimination obtained with the MAb/RFLP combination was significantly better than for RFLP alone, the MAb/PFGE combination added little (<0.02 IOD) to the already high discrimination of PFGE. However, as MAb subgrouping is a rapid and simple technique, it seems sensible to use this method to screen isolates in outbreak investigations before PFGE analysis, as this would avoid complex PFGE analyses being undertaken on strains that were clearly unrelated.

With further efforts to standardise both the methodology and particularly the data analysis, it seems likely that PFGE could be used as the basis for a highly discriminatory definitive typing method. However, at present, for analysis of isolates in the context of an outbreak investigation, it would be advisable to examine strains thought to be related on the same gel.

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