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Assessment of fluorescent amplified fragment length polymorphism analysis for epidemiological genotyping of *Legionella pneumophila* serogroup 1

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ABSTRACT

This study assessed the reproducibility and epidemiological concordance of double-enzyme fluorescent amplified fragment length polymorphism (fAFLP) analysis for genotyping of *Legionella pneumophila* serogroup (sg) 1. fAFLP fragment analysis was performed on three different sequencing platforms (one gel- and two capillary-based) in different laboratories with a well-characterised set of 50 strains of *L. pneumophila* sg 1. fAFLP data were analysed with the Pearson correlation similarity coefficient, using a range of parameters, and dendrogram outputs were converted to arbitrary types after selection of a specified percentage similarity threshold. The results obtained were compared with those obtained by the standard non-fluorescent AFLP method and were found to be broadly concordant. Using optimised settings for each fAFLP method to analyse the panel of 50 strains, epidemiological concordance (*E*) and reproducibility (*R*) values of 1.00 were obtained, and the number of types ranged from nine to 15, compared with *E* = 1.00 and *R* = 1.00, with 16 types, for the non-fluorescent AFLP protocol. The study demonstrated the potential of fAFLP for typing strains of *L. pneumophila* sg 1 on all three platforms; however, inter-platform comparison of fAFLP data was not achieved. fAFLP analysis may have a role in the fingerprinting of multiple isolates during *Legionella* outbreak investigations, but further work is required before type designations and identification libraries can be developed.

Keywords Amplified fragment length polymorphism analysis, fAFLP, genotyping, *Legionella pneumophila*, typing

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INTRODUCTION

The requirement for a genotypic method for the differentiation of *Legionella pneumophila* isolates is well-documented [1,2]. *L. pneumophila* causes the vast majority of cases of legionellosis worldwide, and most of these isolates belong to serogroup (sg) 1 [3]. Thus, in order to confirm or refute an epidemiological relationship between clinical cases and suspected environmental sources, differentiation below the level of serogroup and monoclonal antibody type is usually required. Many factors influence the choice of technique employed by laboratories for this purpose, e.g., labour, time, cost, access to equipment and level of expertise. These techniques can be divided into two main groups, termed 'fingerprinting' and 'epidemiological typing'. While many definitions of these terms may be found in the literature, the term 'fingerprinting' can be defined in this context as a genotypic method suitable for use on all the isolates being evaluated, at one time, by a single laboratory, and would include highly discriminatory, but poorly reproducible, techniques, e.g., PCR using an arbitrary primer (AP-PCR) [4]. In contrast, true epidemiological typing allows the allocation of an isolate to a previously

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defined type, making possible the construction of on-line identification libraries [5]. Such libraries are suitable for long-term epidemiological studies, and allow remote comparisons without the need for the transfer of isolates between laboratories.

One genotypic method, amplified fragment length polymorphism (AFLP) analysis, has been standardised for epidemiological typing of *L. pneumophila* and is used widely by members of the European Working Group for *Legionella* Infections (EWGLI; http://www.ewgli.org). This method uses a non-fluorescent single-enzyme (*Pst*I) approach, with AFLP types being allocated based on the mean band sizes of the profiles obtained following agarose gel electrophoresis with a defined protocol.

Fluorescent AFLP (fAFLP) has been reported to be a reproducible and highly discriminatory method suitable for typing [6,7]. However, this approach requires considerable technical manipulation in both the optimisation and completion of reactions, as well as in the data analysis. Therefore, it may not be sufficiently robust or transportable to allow true epidemiological typing. Although various platforms capable of fluorescent detection of labelled DNA fragments are now available commercially, there are few data regarding the comparative performance of these platforms for epidemiological typing, including for *L. pneumophila* [8]. The present study compared the results obtained with a double-enzyme fAFLP protocol performed on three different platforms (one gel- and two capillary-based) in different laboratories, using a well-characterised set of 50 strains of *L. pneumophila* sg 1. The aims of the study were: (1) to assess the reproducibility (*R*) and epidemiological concordance (*E*) of the fAFLP protocol on each platform; and (2) to assess the suitability of fAFLP for fingerprinting, or typing, of *L. pneumophila* sg 1. To facilitate these assessments, results were compared with those obtained from a previous study using the standardised non-fluorescent method [9].

MATERIALS AND METHODS

Participants

Participants from three institutions (Institute of Environmental Medicine and Hospital Epidemiology, Freiburg, Germany; Health Protection Agency, Centre for Infections, London, UK; National Institute for Infectious Diseases, Rome, Italy) took part in the study. Each of these centres acts as a local or national reference laboratory for *Legionella* infections. The study was coordinated at the Health Protection Agency, Respiratory and Systemic Infection Laboratory, Centre for Infections, London, UK.

Bacterial strains

A collection of 50 *L. pneumophila* sg 1 isolates from nine European countries was used. The characteristics of these isolates have been described previously [4,9]. The collection comprised one reproducibility panel of 20 isolates and one epidemiologically related panel of 30 isolates (Table 1). The reproducibility panel comprised ten duplicate isolates, and the epidemiologically related panel comprised nine epidemiologically related sets and one additional set comprising variants of the same strain. All isolates were designated by a unique European Union *Legionella* culture collection number (EUL no.) [9]. Replicate cultures were prepared on Buffered Charcoal Yeast Extract (BCYE) agar (Oxoid, Basingstoke, UK) slopes, were randomly allocated a study code number, and were distributed to the participating laboratories by courier. Following receipt, the isolates were cultured by inoculation on to BCYE agar (Oxoid) plates and incubated for 2–4 days at 37°C in either CO_2 5% v/v or a moist environment.

Genomic DNA preparation

DNA was prepared after harvesting fresh bacterial growth, either by the method of Ausubel *et al.* [10], as described by Valsangiacomo *et al.* [11], or with the Nucleon BACC2 Kit (Amersham Pharmacia Biotech, Little Chalfont, UK), according to the manufacturer's recommendations, including treatment with RNase A. Genomic DNA preparations from both methods were resuspended in 1 × TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and the DNA concentration was determined spectrophotometrically by measuring the absorbance at 260 nm.

Each centre examined the coded isolates by fAFLP analysis, using the protocols described below, on one of three platforms: (1) an ALF Express DNA Sequencer (Amersham Pharmacia Biotech); (2) a CEQ 8000 DNA Analysis System (Beckman Coulter, Fullerton, CA, USA); or (3) an ABI 3100 Analysis System (Applied Biosystems, Foster City, CA, USA), referred to hereafter as ALF, CEQ and ABI, respectively. A standardised fAFLP method was devised (see below), based on the method described by Arnold *et al.* [6], and was distributed by the coordinating centre. Any deviation from this method was recorded and reported to the coordinating centre. Evaluation of the data using the fAFLP protocol on each of the platforms was based on the Consensus Guidelines of the European Study Group on Epidemiological Markers [12] as described previously [4,9]. For each platform, dendrogram outputs were converted to arbitrary 'types' by selecting a percentage similarity threshold at (or above) which strains were considered to be indistinguishable (i.e., belonging to the same type). The reproducibility (*R*) was defined as the ability of the method to correctly identify epidemiologically related isolates as the same type. The epidemiological concordance (*E*) was defined as the number of epidemiologically related strains found to be indistinguishable, divided by the total number of such sets in the study. Intra-run variation and inter-run variation were determined with *L. pneumophila* strain EUL no. 137 on the ALF and CEQ, or with *Escherichia coli* W3110

Table 1. Reproducibility and epidemiologically related panels, showing details of replicates, variants and epidemiologically related sets of clinical and environmental isolates of *Legionella pneumophila* sg 1

EUL no.	Sender designation	Study code no.	Country of origin	Characteristics and source of isolate
Reproducibility panel				
001	IBS-2	22	Switzerland	Clinical isolate
001	IBS-2	33		Duplicate of above
002	IBS-25	41	Switzerland	Clinical isolate
002	IBS-25	47		Duplicate of above
019	94/51727	3	Scotland	Clinical isolate
019	94/51727	34		Duplicate of above
031	L51	38	France	Clinical isolate
031	L51	7		Duplicate of above
036	1	40	Italy	Clinical isolate
036	1	46		Duplicate of above
051	16140/95	35	Spain	Clinical isolate
051	16140/95	36		Duplicate of above
086	R270	39	Denmark	Clinical isolate
086	R270	16		Duplicate of above
096	L810	12	Denmark	Clinical isolate
096	L810	23		Duplicate of above
101	LD10/94	17	Sweden	Clinical isolate
101	LD10/94	8		Duplicate of above
121	R4-Augsburg1	37	Germany	Clinical isolate
121	R4-Augsburg1	45		Duplicate of above
Epidemiologically related panel				
073	LC3832a	9	England & Wales	Clinical isolates from same patient;
078	LC3832b	18		each isolate is a single colony picked from
079	LC3832c	4		the primary isolation plate
071	LC3868	24	England & Wales	Clinical isolates from same patient;
076	LC3869	13		isolated from sputum by direct culture
077	LC3870	10		Isolated from sputum via amoebal co-culture
				Isolated from faeces by direct culture
048	006/96	25	Spain	Clinical isolates from same patient
056	17/96	6		
040	5	19	Italy	Clinical isolate
047	12	26		Related environmental isolate
124	LC462	14	England & Wales	Clinical isolates from same patient
125	LC463	27		
126	ML64	28	England & Wales	Clinical isolates from the Stafford District
127	ML65	15		General Hospital outbreak
128	ML66	1		
129	LC436	29	England & Wales	Clinical isolates from hotel outbreak in Croatia
130	LRU88	52		
131	LC536	30	England & Wales	Clinical isolates from the BBC outbreak,
132	LC537	31		London
133	LC539	42		
134	LC540	48		
135	Corby RA/LC4404	43	England & Wales	Corby strain variant after seven passes in amoebae
136	Corby CA/LC4405	49		Corby strain variant after 100 passes on MH
137	Corby CAC/LC4406	50		Corby strain variant after 100 passes on BCYE
138	Corby Rif/LC4407	5		Corby strain variant from a rifampicin mutant
139	Corby 3/1-/LC4408	2		Corby strain variant from a mAb3/1^{-} mutant
140	1956X/96	11	Spain	Clinical isolate from Madrid outbreak, Spain
141	2099X/96	32		Clinical isolate
142	208/96	44		Related environmental isolate
143	209/96	51		Related environmental isolate

(Applied Biosystems) on the ABI. Variation was calculated by analysing results from the same strains from the same run and different runs, and then taking the percentage similarity threshold at (or above) which all the profiles clustered together into the same type. Variation was expressed as 100% minus this percentage threshold. The *R* and *E* values, as well as the number of types obtained, were compared with data from previous studies using a non-fluorescent AFLP method [9,13].

fAFLP protocols

At the start of this study, the complete genomic sequence of *L. pneumophila* was not available. Therefore, the choice of restriction endonucleases and primers for fAFLP analysis was based on those recommended for AFLP analysis of organisms with a mol% G + C content of 38–47 [14,15], since *L. pneumophila* had a reported mol% G + C of 39 [16]. The original AFLP method used radioisotopic end-labelling of the primers [14,15], but the present study used fluorescent labelling and fragment analysis protocols recommended by the manufacturer of each of the three platforms described above. Where required, all reaction mixes were made up to volume with PCR-grade water (Promega, Southampton, UK; Carlo Erba Reagenti, Milano, Italy; or DeltaSelect GmbH, Pfullingen, Germany).

fAFLP using the ALF Express

The initial restriction endonuclease digestion was performed at 37°C for 2 h in a total volume of 20 µL. Each reaction mix contained *c.* 500 ng of genomic DNA, 5 U of *Mse*I (New England Biolabs, Beverly, MA, USA), 0.2 µL of bovine serum albumin (BSA) (New England Biolabs) 10 mg/mL, 1.0 µL of

DNase-free RNase A (Sigma, Poole, UK) 10 mg/mL and 1 × *Mse*I buffer (New England Biolabs). Following incubation, the second digestion was achieved by adding 2 μL of 0.5 M Tris-HCl, pH 7.2, 2.5 μL of 0.5 M NaCl and 5 U of *Eco*RI (New England Biolabs) to the initial mix (final volume, 25 μL), followed by incubation for a further 2 h at 37°C. Mixes were then incubated at 65°C for 10 min to inactivate the endonucleases, and then kept on ice prior to ligation. Ligation mixes comprised 25 μL of the double-digest mix, 5 pmol of *Eco*RI adaptor (*Eco*RI-1, 5′-CTCGTAGACTGCGTACC-3′; *Eco*RI-2, 5′-AATTGGTACGCAGTC-3′), 50 pmol of *Mse*I adaptor (*Mse*I-1, 5′-GACGATGAGTCCTGAG-3′; *Mse*I-2, 5′-CTACTCAGGACT-CAT-3′), 40 U of T4 DNA ligase and 1 × T4 ligase buffer (New England BioLabs). Reaction mixes were incubated at 12°C for 18 h, heated at 65°C for 10 min and then stored below −20°C until required. This restriction–ligation mix was used as template DNA (2.5 μL) in the PCRs.

Amplification was performed in a reaction volume of 25 μL. Each mix contained 2.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 200 μM each deoxynucleotide, 16.6 pmol of labelled *Eco*RI-C primer (5′-GACTGCGTACCAATTCC-3′), 100 pmol of *Mse*I-A primer (5′-GATGAGTCCTGAGTAAA-3′) and 0.65 U of *Taq* DNA polymerase (Invitrogen, Paisley, UK). The *Eco*RI-C primer was labelled at the 5′-terminus with Cy-5 (MWG Biotech, Ebersburg, Germany). Amplification was performed in a DNA engine PTC-200 (MJ Research; Bio-Rad, Waltham, MA, USA) with denaturation at 94°C for 2 min, followed by 30 cycles of 20 s at 94°C, 30 s at the annealing temperature (66°C to 56°C), 2 min at 72°C, and a final extension at 60°C for 30 min. Touchdown PCR was used; i.e., the initial annealing temperature of 66°C (for 30 s) was decreased by 1°C for each of the next nine cycles; an annealing temperature of 56°C was used for the final 20 cycles.

Detection of fluorescently-labelled products was by automated laser fluorescence analysis. Amplification products (1 μL) were denatured and then separated on a denaturing polyacrylamide gel in the ALF Express. Gels were prepared using polyacrylamide 5.5% (Long-Ranger; FMC Bioproducts, Rockland, ME, USA) containing 6 M urea in 0.6 × TBE (0.53 mM Tris-borate, pH 8.3, 1.2 mM EDTA). Samples were loaded together with 50-bp and 300-bp internal standards (Amersham Pharmacia Biotech). Electrophoresis conditions comprised 800 V at 55°C for 480 min using 0.6 × TBE buffer. A 50-bp ladder (Amersham Pharmacia Biotech) was used as an external standard. Gel images were converted to tagged information file format (TIFF) files with the Alf2tiff software tool (Amersham Pharmacia Biotech) and processed with GelCompar II or BioNumerics software v. 3.5 (Applied Maths, St-Martens-Latem, Belgium). Fragment sizes were calculated following normalisation using the internal and external standards. Data sets were analysed with a background subtraction disk size of 3% without least square filtering. Group analysis was performed with the Pearson correlation coefficient and the unweighted pair-group method with averages (UPGMA) clustering method. An optimisation setting of 0.02% was used, and only fragments between 50 and 300 nucleotides (nt) in size were included in the analyses.

fAFLP using the Beckman CEQ 8000 DNA Analysis System

The fAFLP protocol was followed as described above, except that the *Eco*RI-C primer was labelled at the 5′-terminus with D4-PA (Invitrogen) for detection on the Beckman CEQ 8000. Amplification products (0.5–1.0 μL) were mixed with 40 μL of sample loading solution (SLS, Beckman Coulter) containing a 600-nt marker (Beckman Coulter) 0.5% (v/v), and were then separated using the FRAG-4 program. Densitometric curves were imported into BioNumerics using the ABI conversion filter. Settings for background subtraction were a disk size of 20%, a least square filtering cut-off of $<0.04\%$, and a power of 2. Analysis was performed with the Pearson correlation coefficient and the UPGMA clustering method, with an optimisation setting of 0.11. Only fragments within the size range 60–640 nt were included.

fAFLP using the Applied Biosystems 3100

fAFLP was performed with the AFLP Microbial Fingerprinting kit (Applied Biosystems), according to the recommendations of the manufacturer. Reference DNA from *E. coli* W3110 (Applied Biosystems) was used as an internal control throughout the analysis. Briefly, 10 ng of purified *Legionella* DNA was restricted simultaneously with *Eco*RI and *Mse*I (New England Biolabs) and ligated to the adaptor–linker primers with T4 DNA ligase (New England Biolabs) for 2 h at 37°C in a final volume of 11 μL. After addition of 150 μL of nuclease-free water, 4 μL of the diluted ligation mix was amplified with the *Eco*RI and *Mse*I core primers (Applied Biosystems) in a final volume of 20 μL. Non-selective pre-amplification was performed at 72°C for 2 min, followed by 20 cycles of 94°C for 20 s, 56°C for 30 s and 72°C for 2 min. The presence of multiple-sized amplification products was checked by electrophoresis on agarose 1.5% w/v gels in 1 × TBE buffer. After addition of 150 μL of nuclease-free water, 1.5 μL of the diluted pre-selective amplification product was used as template for selective amplification. PCR was performed in 10-μL volumes containing 1.5 μL of template DNA, 0.5 μL of NED-labelled *Eco*RI-C selective primer (1 mM), 0.5 μL of *Mse*I-A selective primer (5 mM), and 7.5 μL of AFLP Core Amplification Mix (all reagents from Applied Biosystems). Touchdown PCR cycling conditions were used for amplification, as described above, using a GeneAmp 9600 thermocycler (Perkin-Elmer, Emeryville, CA, USA). Five microlitres of the labelled PCR mix were mixed with 12 μL of Ultrapure Formamide and 0.5 μL of Genescan Rox500 internal lane standard (Applied Biosystems) in a Microamp optical 96-well reaction plate. Separation and detection of AFLP fragments were performed with a model 3100 capillary electrophoresis system equipped with a 36-cm capillary loaded with the POP-4 polymer (Applied Biosystems). Samples were injected for 22 s at 1 kV and separated for 108 min at 100 mA. Densitometric curves were imported into BioNumerics as described above. Settings for background subtraction were a disk size of 10%, a least square filtering cut-off of $<2.95\%$, and a power of 2. Analysis was performed with the Pearson correlation coefficient and the UPGMA clustering method, and an optimisation setting value of zero. Only data within the size range >35 nt to <500 nt were included.

Data analysis

Gel images or densitometric traces were exported from each platform and analysed with either GelCompar II (Applied Maths) or BioNumerics (Applied Maths) software. Only peaks within the range of the molecular size marker used were included in the initial analyses. The curve-based Pearson correlation similarity coefficient was used with the UPGMA

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clustering method, using the range of parameters described above. Dendrogram outputs were converted to arbitrary types after selection of a specified percentage similarity threshold at (or above) which strains were considered to belong to the same type. The optimal percentage threshold for each method is given above. All analyses were undertaken before the code was broken. All data were subjected to visual analysis by the coordinating centre, to determine the presence of any reaction 'failures', which were scored as 'fail'. Repeat testing of any such isolates was performed, together with a number of related and unrelated isolates, to allow final scoring of the same maximum number of sets. Results were compared with those obtained by the EWGLI non-fluorescent AFLP method for the same panel of isolates [9].

RESULTS

Fig. 1 shows examples of the results obtained with each of the three platforms. Although each of the isolates in the study was typeable by all three of the fAFLP protocols, there were two 'failures' in individual sample runs, resulting in 'fail' scores. Thus, the results for the initial run of study code no. 16 (EUL no. 086) and study code no. 31 (EUL no. 132) could not be evaluated following fAFLP analysis on the CEQ. Duplicate or related sets containing such failures were excluded from the analyses. Upon retesting of these two isolates, study code no. 16 (EUL no. 086) clustered at >85% with its replicate, i.e., study code no. 39 (EUL no. 086), while study code no. 31 (EUL no. 132) clustered at >85% with the isolates in its epidemiologically linked set (study code nos. 30, 42 and 48). These values were above the threshold used to assign types on this platform; therefore, these isolates were scored subsequently as a match for the reproducibility value and epidemiological concordance values.

The reproducibility (R) value is related to the percentage similarity threshold (defined above). Optimal percentage similarity threshold values were different for each platform. Values of $R = 1.00$ were achieved at thresholds of 90%, 85% and 79% for the ALF express, CEQ and ABI, respectively. Using these thresholds, an epidemiological concordance (E) value of 1.0 was achieved on all three platforms, with the number of distinct types obtained being 12 (ALF), nine (CEQ) and 15 (ABI). Increasing the thresholds reduced the reproducibility value and/or the epidemiological concordance values for each platform (data not shown). Intra- and inter-gel variation values (calculated from within and between gel data from a control strain) were 7% (100% to 93%), 3% (100% to 97%) and 2% (100% to 98%) for the ABI, CEQ and vertical gel-based ALF express system, respectively.

Evidence for epidemiological typing

Use of comparable 'type' designations by all three centres provided evidence for true

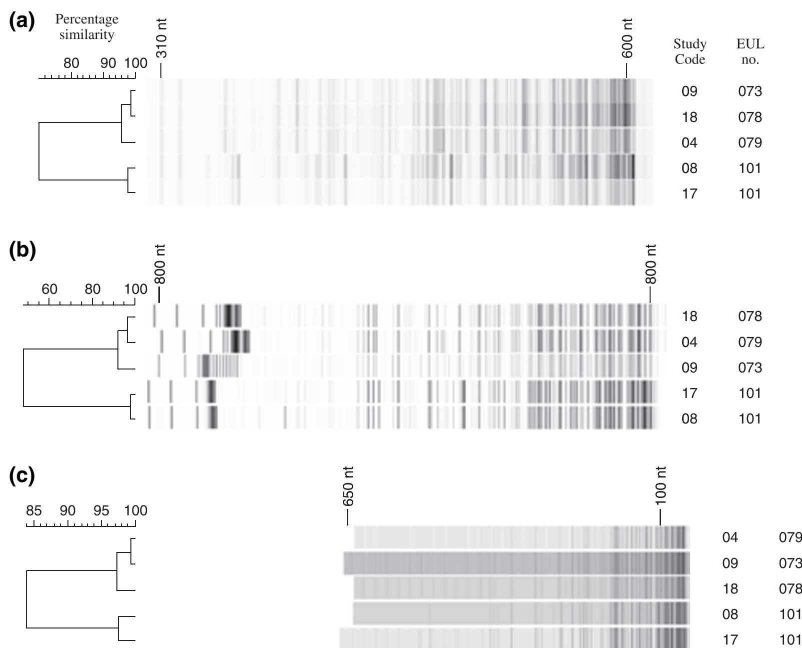

Fig. 1. Examples of normalised patterns obtained following fAFLP analysis of *Legionella pneumophila* sg 1 isolates from the reproducibility and epidemiologically related panels. Study code nos. 8 and 17 are replicates of the same strain. Study codes nos. 4, 9 and 18 are a set of epidemiologically related strains. (a) ALF Express (gel). (b) ABI Prism 3100 (capillary). (c) CEQ 8000 (capillary). nt, nucleotide.

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epidemiological typing, as distinct from fingerprinting. Comparable type designations were assigned by all three centres for two sets of isolates included in the epidemiologically related panel (Table 2). The first set (designated type C, A or G on the ALF, CEQ or ABI platforms, respectively) comprised three isolates from the same patient obtained by different culture methods (study code nos. 10, 13 and 24), and two clinical isolates from a hotel outbreak in Croatia (study code nos. 29 and 52). The second set (designated type H, D or L) comprised three isolates derived from single colonies on a primary isolation plate from the same patient (study code nos. 4, 9 and 18), and three isolates from a well-characterised hospital outbreak in Stafford, UK (study code nos. 1, 15 and 28). Complete concordance between the three platforms was not observed.

Comparison with non-fluorescent AFLP method

Of the three fAFLP platforms, the ALF express platform correlated most closely with the EWGLI AFLP method in terms of comparable type designations; for example, EWGLI AFLP 013 London was scored as type H on three of four occasions, and 009 London was scored as type A on both of two occasions. Interestingly, one of the two sets where comparable type designations were used by all three fAFLP platforms (C, A or G) was subdivided into two EWGLI AFLP types, 010 London and 014 London. The CEQ was the only platform to subdivide the EWGLI type 013 London into two types (D and I).

The discriminatory index of fAFLP was not addressed specifically in this study because of the relatively small sample size. However, a comparison of the number of types obtained by each of the fAFLP platforms with the maximum number of types obtained by the EWGLI AFLP method ($n = 16$) was made in order to provide an estimate of their relative discriminatory power. The ABI platform appeared to be the most discriminatory approach, dividing the 20 sets into 15 types, followed by the ALF express (12 types) and the CEQ platform (nine types).

Table 2. Results of fAFLP analysis of *Legionella pneumophila* sg 1 using three platforms, compared with a previously described non-fluorescent AFLP method [10], showing correlation coefficient, percentage similarity threshold, type designation and number of types; sets of strains designated as having the same type by all three fAFLP platforms are in bold type

EUL no.	Study code no.	EWGLI[a] AFLP Dice (90%)	ALF Express Pearson (90%)	CEQ 8000 Pearson (85%)	ABI Prism 3100 Pearson (79%)
001	22	001 Lugano	E	E	B
001	33	001 Lugano	E	E	B
002	41	002 Lugano	I	C	B
002	47	002 Lugano	I	C	B
031	38	004 Lyon	F	B	D
031	7	004 Lyon	F	B	D
019	3	003 Glasgow	H	D	M
019	34	003 Glasgow	H	D	M
036	40	005 Rome	D	A	F
036	46	005 Rome	D	A	F
051	35	002 Lugano	I	C	H
051	36	002 Lugano	I	C	H
040	19	012 Rome	L	H	N
047	26	012 Rome	L	H	N
048	25	011 Madrid	G	B	K
056	6	011 Madrid	G	B	K
071	24	010 London	**C**	**A**	**G**
076	13	010 London	**C**	**A**	**G**
077	10	010 London	**C**	**A**	**G**
073	9	013 London	**H**	**D**	**L**
078	18	013 London	**H**	**D**	**L**
079	4	013 London	**H**	**D**	**L**
086	39	006 Copenhagen	F	B	E
086	16	006 Copenhagen	F	B[b]	E
096	12	007 Copenhagen	I	C	A
096	23	007 Copenhagen	I	C	A
101	17	008 Stockholm	K	A	C
101	8	008 Stockholm	K	A	C
121	37	009 London	A	G	A
121	45	009 London	A	G	A
124	14	009 London	A	G	O
125	27	009 London	A	G	O
126	28	013 London	**H**	**D**	**L**
127	15	013 London	**H**	**D**	**L**
128	1	013 London	**H**	**D**	**L**
129	29	014 London	**C**	**A**	**G**
130	52	014 London	**C**	**A**	**G**
131	30	013 London	H	I	L
132	31	013 London	H	I[b]	L
133	42	013 London	H	I	L
134	48	013 London	H	I	L
135	43	015 Dresden	J	C	I
136	49	015 Dresden	J	C	I
137	50	015 Dresden	J	C	I
138	5	015 Dresden	J	C	I
139	2	015 Dresden	J	C	I
140	11	016 Madrid	B	F	J
141	32	016 Madrid	B	F	J
142	44	016 Madrid	B	F	J
143	51	016 Madrid	B	F	J
No. of types		16	12	9	15

[a]European Working Group for *Legionella* Infections.
[b]Initial result not evaluable; result of repeated sample is shown.

DISCUSSION

Since its initial description, AFLP analysis has been used as a fingerprinting method for human forensic and paternity testing [17], for monitoring inheritance of genetic markers in plants [18], and in studies of microorganisms [19–21]. Microbial applications of AFLP include investigations of genomic relatedness and diversity, genotyping, characterisation of outbreak strains, and polyphasic taxonomy. EWGLI members have previously

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investigated single-enzyme non-fluorescent AFLP as a typing tool for *L. pneumophila* sg 1 [4,9,13]. This method was adopted as the first internationally agreed typing method for *L. pneumophila* sg 1, and is currently used by many centres in Europe and Australia [22]. However, proficiency panel evaluation of this method revealed problems in achieving optimal results, associated with user-defined band scoring, which is, by its nature, subjective.

fAFLP has been reported to be reproducible, more discriminatory and capable of higher throughput than other molecular typing methods [7,23]. After initial optimisation, this method should also be more amenable to automated analysis using software, and should require less user intervention in, for example, the scoring and assignment of types. However, there are few studies on the use of fAFLP as an epidemiological typing tool, and to our knowledge, no reports on its use for typing isolates of *L. pneumophila* sg 1. The present study investigated the performance of three fAFLP platforms, two capillary and one vertical gel-based, for genotyping isolates of *L. pneumophila* sg 1. Performance was assessed by comparison of reproducibility and epidemiological concordance scores using a coded panel of isolates.

The results demonstrated that all three platforms were capable of achieving high reproducibility ($R = 1.0$) and epidemiological concordance ($E = 1.0$) scores. The assignment of isolates to the same epidemiological type by all three methods also demonstrated the potential of this approach as an epidemiological typing system. One of the aims of the study was to compare the performance of the three platforms, so variation in the procedures was kept to a minimum wherever possible. Two centres used a commercial kit for DNA extraction and one used a published organic extraction method. Both methods have been shown previously to yield high molecular size DNA from *Legionella*. Two centres used the same supplier for the unlabelled oligonucleotides. Two centres used thermocyclers manufactured by MJ Research and the other used a thermocycler manufactured by Perkin-Elmer. The most significant variation in the fAFLP protocols was the inclusion of a non-selective pre-amplification step in the ABI protocol with *Eco*RI and *Mse*I core primers. Other variations included the method of DNA extraction, the fluorescent labels, the inclusion of a non-selective amplification step (one method), the molecular size markers, the method of separation (capillary or gel), and the settings used for analysis. Some of these variations (such as the method of DNA extraction) have been shown previously to have no apparent effect on AFLP profiles visualised on agarose gels [9]. Other sources of variation (e.g., fluorescent labels and the method of separation) are associated with the intrinsic differences between platforms. It is noteworthy that, although intra- and inter-run variations obtained with a control strain were between 2% and 7%, values obtained with the reproducibility panel suggest that the variation can be much higher (10–21%). These data illustrate clearly the requirement to evaluate a range of isolates to determine the level of variation in such systems. One factor that was found to have a significant effect on the performance of fAFLP was the amount of sample loaded on to the gel; for example, one centre tried loading volumes of 3 μL and 7 μL, and the larger volume was found to be sub-optimal on the ABI platform (data not shown). The critical differences between the three platforms were in the different analysis settings required. Although the different platforms might be expected to produce similar results, cross-platform analyses could not be performed, as all the profiles from the same platform clustered together (data not shown).

The study illustrated the potential of the fAFLP methodology to function as a true epidemiological genotyping tool for *L. pneumophila* sg 1, as judged by good reproducibility and epidemiological concordance scores, as well as the ability to assign strains to the same type in a coded trial. However, meaningful comparison of profiles between the platforms studied was restricted. Apart from the differences noted above, the most likely explanation for differences between platforms is the mobility of the labelled fragments through the gel matrix and their subsequent normalisation. To assess the performance of the fAFLP method more accurately, the fragment sizes could be determined *in silico*, using the *L. pneumophila* genome sequence [24], with subsequent comparison of fragment sizes in terms of base-pairs. Further studies to determine the discrimatory index of such systems would also need to be performed.

Comparison of the fAFLP data with data obtained by the non-fluorescent method revealed broad concordance between these two

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techniques. This was despite major differences, in particular, the inclusion of single- vs. double-enzyme AFLP, band sizes, and the correlation coefficients used for analyses. Complete concordance between the non-fluorescent method and any of the fluorescent methods was not observed. However, both AFLP and fAFLP show potential for epidemiological typing of *L. pneumophila* sg 1.

In conclusion, the double-enzyme fAFLP protocol used in the present study appeared to be applicable to different detection platforms. Careful optimisation of reaction conditions and analysis are required, but once achieved can provide excellent results. Comparing data between platforms remains difficult, but designation of profiles to defined types could allow the development of a typing system using densitometric curves or profiles based on fragment sizes in base-pairs. The fAFLP method also allows for the potential characterisation of many isolates or colonies from an outbreak investigation at a relatively low cost, particularly when using the multiple capillary platforms. These methods could therefore be used to screen large numbers of *L. pneumophila* isolates in an outbreak situation, prior to further investigation of indistinguishable isolates by other genotyping methods, including sequence-based typing [25]. fAFLP may also be applicable to the investigation of legionellosis caused by non-sg 1 isolates of *L. pneumophila* or other non-*pneumophila* species of *Legionella*.

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