MOLECULAR TYPING AND EPIDEMIOLOGY

Molecular typing of *Legionella pneumophila* serogroup 1 isolates from patients and the nosocomial environment by arbitrarily primed PCR and pulsed-field gel electrophoresis

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The ubiquity of *Legionella pneumophila* in aquatic habitats means that epidemiological evaluation is important for the investigation and control of nosocomial outbreaks of legionellosis. Pulsed-field gel electrophoresis (PFGE) of chromosomal DNA following digestion with *Sfi*I is considered to be one of the most discriminative methods for detecting DNA polymorphisms amongst *L. pneumophila* serogroup 1 (*Lp*1) isolates. This paper describes an arbitrarily primed PCR (AP-PCR) method with three different primers (20-mers) for detecting DNA polymorphisms of *Lp*1 isolates. The AP-PCR assay was compared with PFGE analysis. Both experimental methods were found to have good discriminatory power (discrimination index of 98% and 94.3%, respectively) with 27 unrelated isolates from different geographical areas collected between 1987 and 1997. Furthermore, when the AP-PCR was used in the epidemiological investigation of nosocomial cases of infection, convergent results with the three primers allowed an epidemiological link to be established between isolates from patients and their environment. The AP-PCR method, which is rapid and easy to perform, gave results at least as discriminatory as those obtained with the PFGE method and is proposed for use in the molecular typing of *Lp*1 outbreaks.

Introduction

Legionella pneumophila was first isolated following an outbreak of severe pneumonia in Philadelphia in 1976 [1]. Although many *Legionella* species are found in the natural environment, *L. pneumophila* is responsible for >85% of cases of Legionnaires' disease [2]. Outbreaks of community-acquired and nosocomial *L. pneumophila* infection have been described [1, 3]. During such outbreaks, epidemiological studies are required to identify the environmental source of the infection and a possible epidemiological link between the cases. Serotyping of *L. pneumophila* isolates with specific antibodies is used widely, but is of limited value as most *L. pneumophila* infections involve serogroup 1 [2]. Other epidemiological markers used in the investigation of legionellosis include monoclonal antibody subtyping of serogroup 1 [4], analysis of allo-enzymes [5] and

plasmids [6], and restriction endonuclease digestion of chromosomal DNA [3]. Restriction fragments of *L. pneumophila* DNA have been used as probes for molecular typing of nosocomial isolates belonging to the same serogroup (serogroup 3) [7]. Ribotyping has also been used to study the epidemiology of Legionnaires' disease [7, 8], but with conflicting results.

PCR fingerprinting with arbitrary primers can also be performed easily when epidemiological investigation is required [9]. A combination of ribotyping and arbitrary primed PCR (AP-PCR) with one arbitrary primer [10, 11] has been used previously for molecular typing of *L. pneumophila* epidemic isolates. In the present study, an attempt was made to improve the AP-PCR method by the use of three arbitrary primers. To validate this method, AP-PCR patterns of related and unrelated isolates were compared with those obtained after restriction endonuclease digestion of chromosomal DNA and analysis by pulsed-field gel electrophoresis (PFGE). PFGE is considered to be one of the most discriminative methods for detecting DNA

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polymorphisms among *L. pneumophila* serogroup 1 (*Lp1*) isolates [8, 12, 13]. The AP-PCR method was then used to study the epidemiological links and the environmental origin of *L. pneumophila* isolates involved in nosocomial cases of infection in two different hospitals.

Materials and methods

Clinical isolates

In total, 49 *Lp1* isolates were studied (Table 1). Five unrelated strains from different geographical areas and

the reference strain Philadelphia 1 (ATCC strain 33152) were obtained from the French National Reference Center in Lyon. Twenty-one clinical isolates were obtained in this laboratory from bronchoalveolar lavage fluids or bronchial aspirates from patients hospitalised in the Paris area. These isolates, obtained in 1987–1997 from patients in 19 different hospitals, were considered to be unrelated. In addition, isolates from two nosocomial investigations were included. The clinical isolate of *L. pneumophila* from hospital A was compared with two environmental isolates by PFGE and AP-PCR. The investigation was then continued with the AP-PCR assay alone on 10 other *Lp1*

Table 1. Epidemiological data and molecular characterisation of the *L. pneumophila* serogroup 1 isolates studied

Isolate designation	Area*	Hospital designation	Source	Date of isolation	AP-PCR type			Pulsotype
					IR6110	LEG-2	B1245	
Unrelated isolates								
C1	P	C	Patient	06/1996	A	1	a	I
D1	P	D	Patient	06/1991	A	1	a	I
E1	P	E	Patient	06/1988	A	1	a	I
A14	P	A	Patient	04/1997	A	1	a	I
F1	P	F	Patient	09/1995	B	1	a	I
G1	P	G	Patient	06/1996	C	1	a	I
H1	P	H	Patient	07/1994	E	2	b	IIa
J1	P	J	Patient	06/1992	E	2	c	IIb
A15	P	A	Patient	09/1996	E	11	d	IIc
A16	P	A	Patient	07/1994	E	2	d	IIa
K1	P	K	Patient	10/1995	E	2	e	IIb
L1	P	L	Patient	04/1996	E	2	f	IIb
B10	P	B	Patient	07/1994	F	2	d	IIe
M1	P	M	Patient	10/1993	F	2	d	IIe
N1	P	N	Patient	01/1997	F	2	g	IIf
O1	P	O	Patient	07/1987	G	2	h	III
P1	P	P	Patient	10/1991	E	3	i	IV
Q1	P	Q	Patient	12/1995	H	4	j	V
R1	P	R	Patient	03/1996	I	5	k	VI
S1	P	S	Patient	05/1996	J	6	l	VII
T1	P	T	Patient	10/1987	K	7	m	VIII
U1	T	U	Patient	05/1997	O	8	q	X
V1	O	V	Patient	05/1997	P	9	r	XI
W1	O	W	Patient	03/1997	Q	9	a	XII
X1	O	X	Patient	06/1997	R	10	s	XIII
Y1	O	Y	Patient	05/1997	S	9	t	XIV
ATCC 33152			Ref. strain		E	2	u	IId
Nosocomial investigation A								
A1	P	A	Patient	10/1995	A	1	a	I
A2	P	A	Environment	11/1995	A	1	a	I
A3	P	A	Environment	11/1995	A	1	a	I
A4	P	A	Environment	11/1995	A	1	a	...
A5	P	A	Environment	11/1995	A	1	a	...
A6	P	A	Environment	05/1996	A	1	a	...
A7	P	A	Environment	05/1996	A	1	a	...
A8	P	A	Environment	05/1996	A	1	a	...
A9	P	A	Environment	11/1995	L	1	n	...
A10	P	A	Environment	05/1996	M	1	o	...
A11	P	A	Environment	05/1996	M	1	o	...
A12	P	A	Environment	05/1996	M	1	o	...
A13	P	A	Environment	05/1996	M	1	o	...
Nosocomial investigation B								
B1	P	B	Patient	12/1996	E	2	b	IIa
B2	P	B	Patient	03/1996	E	2	b	IIa
B3	P	B	Patient	03/1996	E	2	b	IIa
B4	P	B	Patient	03/1996	E	2	c	IIb
B5	P	B	Environment	03/1996	E	2	b	IIa
B6	P	B	Environment	03/1996	E	2	b	IIa
B7	P	B	Environment	03/1996	E	2	b	...
B8	P	B	Environment	03/1996	D	8	p	...
B9	P	B	Environment	03/1996	D	8	p	...

*P, Paris area; T, Tunisia; O, other French areas.

environmental isolates. Similarly, four clinical isolates and two environmental isolates obtained from hospital B over a 3-month period were compared by both methods. Three additional environmental isolates were studied by AP-PCR alone (Table 1).

All clinical specimens and the reference strain were cultured on buffered charcoal-yeast extract agar supplemented with α -ketoglutaric acid (BCYE- α) [14]. Environmental isolates were plated as recommended in the French standard AFNOR T90-431 for isolation of *Legionella* from potable water [15]. Serogrouping was based on reactivity with serogroup-specific fluorescent antisera (Centers for Disease Control, Atlanta, GA, USA).

AP-PCR assay

DNA was extracted from individual colonies with a DNA extraction reagent containing Chelex anion-exchange resin (Perkin Elmer, Branchburg, NJ, USA) [16]. Bacteria were harvested from BCYE- α agar and suspended in 100 μ l of distilled water. The Chelex reagent (200 μ l) was added to the suspension and, after boiling for 25 min and centrifugation at 2000 *g* for 2 min, the supernates were used for AP-PCR analysis (see below).

Three primers were used for the AP-PCR method. Primer B1245 (5'-AGGTGGCGTCGAGGAAGAC-3') has been used previously for AP-PCR analysis of *Mycobacterium avium* [16]. Primer IR6110 (5'-CCCGCATGTCCGGAGACTC-3') corresponds to the inverse repeat sequence of IS6110 found in the *M. tuberculosis* complex [17] and has already been used for AP-PCR analysis of *L. pneumophila* [11]. Primer LEG-2 (5'-CTGGCTTCTTCCAGCTTCA-3') has been used previously, combined with primer LEG-1, for the specific amplification of DNA from *L. pneumophila* strains [18].

PCR amplifications were performed in a total volume of 50 μ l. Each PCR mixture consisted of 50 mM Tris-HCl (pH 8.5), 17 mM (NH₄)₂SO₄, 2 mM MgCl₂, 6.7 μ M EDTA, 10 mM β -mercaptoethanol, bovine serum albumin (BSA) 0.1 mg/ml, gelatin 0.1% w/v, 200 μ mol of each deoxynucleoside triphosphate, 100 pmol of arbitrary primers, and 0.5 U of *Taq* DNA polymerase (Perkin Elmer). A 10- μ l portion of the DNA eluted from Chelex resin was added. Distilled water was used as a negative PCR control in place of *Legionella* DNA. The reaction mixtures were overlaid with 100 μ l of paraffin oil and incubated for 5 min at 94°C, followed by 36 cycles of 92°C for 1 min, 35°C for 1 min and 72°C for 2 min, with an additional final step at 72°C for 7 min (Hybaid Thermal Cycler; Hybaid Ltd, Teddington, Middlesex). DNA fragments were separated in agarose 2% w/v gels by electrophoresis and visualised by ethidium bromide staining. Gels were photographed

and band patterns were compared visually. Very weak DNA bands were not taken into account. Two isolates were considered to have the same AP-PCR type when patterns with the three primers were identical. Some experiments were repeated after a new DNA extraction and the patterns were compared after co-migration on the same agarose gel to confirm the interpretation of the results.

Chromosomal PFGE analysis

Genomic DNA was prepared in agarose plugs [19]. Plugs were incubated in 3 ml of lysis solution (6 mM Tris-HCl, pH 7.6, 1 M NaCl, 100 mM EDTA, pH 7.5, Brij 58 0.5% w/v, sodium deoxycholate 0.2% w/v, sodium lauroyl sarcosine 0.5% w/v, RNAase 20 μ g/ml, lysosyme 1 mg/ml) for 24 h at 37°C. This solution was replaced by 3 ml of EPS solution (0.5 M EDTA, pH 9, proteinase K 50 μ g/ml, sodium lauroyl sarcosine 1% w/v), incubated for 24 h at 50°C, and then washed three times with 5 ml of TE buffer (10 mM Tris-HCl, pH 7.6, 1 mM EDTA). DNA was digested with 10 U of *Sfi*I (Pharmacia) by incubation overnight at 50°C. DNA fragments were separated in agarose 1.2% w/v gels prepared and run in 0.5 $\times$ Tris-borate-EDTA buffer in a contour-clamped homogeneous field apparatus (CHEF-DR2; BioRad). Electrophoresis parameters were 200 V for 23 h at 4°C with pulse times ramped from 7 to 74 s. Gels were then stained with ethidium bromide and photographed under UV illumination. Isolates with patterns that differed by three or fewer restriction fragments were classified as subtypes of the same pulsotype (Ia, Ib, Ic, etc.), while isolates differing by more than three restriction fragments were considered sufficiently divergent to warrant a separate pulsotype designation (I, II, III, etc.) [20, 21].

Discrimination index

The discriminatory power of each typing method was evaluated by calculating the index of discrimination (ID) proposed by Hunter and Gaston [22]. Subtypes of the same pulsotype were considered as different types when calculating this index for the PFGE method.

Results and discussion

Of the 10 primers tested with the reference strain in the AP-PCR assay, only three gave patterns with more than four bands (data not shown) and these were used subsequently in this study.

The performance and convenience of the AP-PCR method was evaluated by comparing it with the PFGE method for 27 unrelated isolates. The PFGE method yielded 13 pulsotypes, including one (pulsotype II) with six subtypes, resulting in an ID of 94.3% (Fig. 1 and Table 1). The AP-PCR assay yielded 15 patterns

Fig. 1. Representative pulsotypes of (a) unrelated and (b) related *L. pneumophila* isolates after digestion by *Sfi*I. Letters refer to strains described in Table 1. Lane M, size markers (kb).

with primer IR6110, 11 patterns with primer LEG-2 and 18 patterns with primer B1245. The combination of the AP-PCR patterns with the three primers resulted in 23 AP-PCR types (Fig. 2 and Table 1), resulting in an ID of 98%. In total, 21 patterns obtained with the AP-PCR method corresponded to single isolates; the two remaining patterns were obtained from two clusters of four and two isolates, respectively (Table 1). Cluster A1a included four clinical isolates obtained in different hospitals in 1988–1997 in the Paris area and corresponded to a

single pulsotype (pulsotype I). The second cluster included two isolates from two different hospitals from the Paris area with the same AP-PCR type F2d and the same pulsotype IIe. Although the isolates in these clusters were distant in time and were not isolated from the same hospital, an epidemiological link could not be excluded.

PFGE is time-consuming and requires expensive equipment that is not available in all microbiology laboratories. The AP-PCR method, which was easy to

Fig. 2. Representative AP-PCR types of *L. pneumophila* unrelated (left) and related (right) strains with (a) primer IR6110, (b) primer LEG-2, (c) primer B1245. Letters refer to strains described in Table 1. Lane M, size markers (kb). N, negative controls.

perform and gave fast results, appeared to be more convenient for descriptive epidemiological studies. Therefore, this method was chosen to identify the environmental origin of isolates associated with

nosocomially acquired Legionnaires' disease. The PFGE method was then used to confirm the results of the AP-PCR method on chosen isolates (mentioned when used).

The first case studied occurred in hospital A, where a *Lp1* isolate was obtained from bronchoalveolar lavage fluid. The patient concerned was hospitalised for several weeks in the rehabilitation department. The AP-PCR patterns of the clinical isolate were compared to the patterns obtained with 12 environmental isolates obtained from the water supply in the same and different buildings of the hospital, before and after thermal shock. The same AP-PCR profile, A1a, was obtained for the clinical isolate (A1) and for isolates from the patient's close environment, i.e., from his bedroom shower and sink hot water taps (isolates A2 and A3). These three isolates were also identical by PFGE. However, while some *Lp1* isolates from the hospital environment had different patterns (isolates A9–A13), environmental isolates from other buildings before and after the thermal shock were also found to have the A1a AP-PCR profile (isolates A3–A8) (Table 1). Three *L. pneumophila* serogroup 2 isolates and seven *L. pneumophila* serogroup 3 isolates obtained from the hospital environment showed different patterns to the clinical isolate and the *Lp1* environmental isolates (data not shown).

The second study was performed in hospital B, where three cases of Legionnaires' disease occurred in the haematology department in 3 months. These three patients were transferred to an intensive care unit, where a fourth case occurred. Five strains were isolated later from the environment. The AP-PCR patterns of the three clinical isolates (B1, B2, B3) and the isolate (B5) obtained from the hot water supply of the patients' bedroom in the haematology department were identical with the three primers used for AP-PCR (AP-PCR type E2b) and with the PFGE method (pulsotype IIa). Identical patterns were also obtained by AP-PCR and PFGE for an isolate (B6) from the fibroscopy room water supply. The isolate from the fourth patient differed in its AP-PCR pattern with primer B1245 (one strong extra band; AP-PCR type E2c) and by PFGE subtype (IIb) (Fig. 2 and Table 1). This case is difficult to interpret as only minor differences were observed compared to the outbreak strain. However, differences were observed by both molecular typing methods. Furthermore, it is noteworthy that this fourth patient had never been hospitalised in the haematology department. In contrast, the other three patients developed Legionnaires' disease in the haematology department, and the environmental origin of the infection was localised to this department.

Van Belkum and Pruckler have shown that PFGE is more discriminative than AP-PCR, but only one or two arbitrary primers were used [9, 11]. In the present study, the AP-PCR method was found to have a higher ID than the PFGE method (98% and 94.3%, respectively), even when subtypes were considered as different. Although AP-PCR with primer B1245 was more discriminatory than AP-PCR with the two other

primers, convergent results with the three primers confirmed the results and some isolates could be discriminated only by the use of primer IR6110 (AP-PCR types B1a and C1a or types E2d and F2d) or primer LEG-2 (AP-PCR types E11d and E2d). When unrelated clinical isolates were studied by both molecular typing methods, some were found to form clusters. Whether this resulted from the fact that the number of virulent *Lp1* strains isolated from patients was smaller than the number of environmental strains remains to be determined. Another possibility is the existence of common unknown environmental origins of legionellosis in the Paris area.

In conclusion, the major advantages of the AP-PCR method include technical simplicity, wide availability of equipment and reagents, and speed. This study assessed the method in comparison with PFGE and demonstrated that AP-PCR could be used with efficacy to demonstrate an epidemiological link between clinical isolates during an outbreak and to identify the environmental origin. These molecular typing methods should help to elucidate how Legionnaire's disease occurs and why these organisms are the cause of many community and nosocomial outbreaks of infection.

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