EPIDEMIOLOGICAL TYPING

Correlation of MAb subgroups with genotype in closely related *Legionella pneumophila* serogroup 1 strains from a cooling tower

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Summary. *Legionella pneumophila* serogroup 1 strains isolated from a cooling tower during the investigation of an outbreak of Legionnaires' disease were shown previously to be related closely or indistinguishable by hybridisation-based restriction fragment length polymorphism analysis. However, these strains could be differentiated into five different MAb subgroups by comparison of their reactivity patterns with a recognised panel of monoclonal antibodies (MAbs). Pulsed-field gel electrophoresis (PFGE) of genomic fragments obtained after cleavage with rare-cutting restriction endonucleases also differentiated these strains. Four different restriction patterns were obtained with *Sfi*I, *Eag*I and *Sma*I, three restriction patterns with *Not*I, *Apa*I and *Sac*II, and two patterns with *Nae*I. Generally, the restriction patterns were related closely, differing in only one or two bands. The combined results of the restriction endonuclease digestions allowed the strains to be differentiated into groups that correlated to the MAb subgroups. Both PFGE patterns and MAb subgroups were found to be stable markers. The findings demonstrated that the MAb variability seen amongst the *L. pneumophila* serogroup 1 strains from this cooling tower was not solely phenotypic.

Introduction

Legionella pneumophila serogroup 1 is the most frequent cause of Legionnaires' disease, and also the member of the Legionellaceae family isolated most frequently from environmental sources.¹ The ubiquity of legionellae in natural and artificial aquatic environments complicates the establishment of an epidemiological link between isolates from the environment and those from patients. Several techniques, including monoclonal antibody (MAb) subgrouping,²⁻⁵ electrophoretic alloenzyme typing,⁶ plasmid analysis^{1,7} and analysis by pulsed-field gel electrophoresis (PFGE)^{1,8,9} have been described for the subtyping of *L. pneumophila* serogroup 1.

Harrison *et al.*⁷ reported the isolation of a number of *L. pneumophila* serogroup 1 strains from the same environmental source. Although these strains were indistinguishable by a genotypic typing method, based on comparison of restriction fragment length polymorphism (RFLP) patterns obtained after hybridisation with cloned DNA fragment probes, they could be differentiated phenotypically into different MAb

subgroups. Harrison *et al.*⁷ thus concluded that phenotypic variation of a single parent strain had occurred within the environmental site. Similar results were reported later by Struelens *et al.*⁴ during an investigation of a nosocomial outbreak of Legionnaires' disease.

This report presents evidence that PFGE analysis can be used successfully for subtyping the *L. pneumophila* serogroup 1 strains isolated by Harrison *et al.*⁷ which were indistinguishable by hybridisation-based RFLP analysis. The PFGE groups obtained are compared with the MAb subgroups for the same set of strains.

Materials and methods

Bacterial strains

The nine *L. pneumophila* serogroup 1 strains used in this investigation are listed in the table. All these strains were isolated from a cooling tower in London.⁷

RFLP typing

The strains listed in the table were typed by probing *Nci*I-restricted chromosomal DNA with cloned *Eco*RI

Table. Correlation of MAb phenotype and genotype in closely related *L. pneumophila* serogroup 1 strains isolated from a cooling tower in London

Strain no.	LRU* no.	MAb type	PFGE type	Restriction pattern type obtained with enzymes							RFLP pattern
				<i>Sfi</i> I	<i>Not</i> I	<i>Sma</i> I	<i>Apa</i> I	<i>Eag</i> I	<i>Nae</i> I	<i>Sac</i> II	
1	LC751a	Bellingham	A	1	1	1	1	1	1	1	47
2	LC751b	Philadelphia	B	2	2	2	2	2	2	2	1
3	LC751c	Benidorm	C	3	3	3	3	3	2	3	1
4	LC751e	OLDA	D	2	2	4	2	2	2	2	1
5	LC751f	Oxford	E	4	3	1	2	4	2	2	1
6	LC751j	Benidorm	C	3	3	3	3	3	2	3	1
7	LC752f	OLDA	D	2	2	4	2	2	2	2	1
8	LC752y	Oxford	E	4	3	1	2	4	2	2	1
9	LC752o	Oxford	E	4	3	1	2	4	2	2	1

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fragments (probes λ NS20 and λ NS21) derived from *L. pneumophila* strain Knoxville-1.¹⁰

MAb subgrouping

MAb subgrouping was performed with the MAbs of the standardised subgrouping scheme.² These MAbs were either prepared in our laboratory, obtained from m-tech, Atlanta, GA, USA (MAb 3), or kindly provided by Jean Joly, University of Quebec, Canada (MAb 5 and MAb 7).⁵ The procedure used for MAb subgrouping was the indirect immunofluorescence test, as described previously.⁵

PFGE analysis of genomic DNA

Intact genomic DNA was prepared¹¹ from *L. pneumophila* serogroup 1 strains and restricted with *Sfi*I, *Not*I, *Sma*I, *Apa*I, *Eag*I, *Nae*I or *Sac*II (New England Biolabs). Cleavage was for 15 h at 50°C for *Sfi*I, at 25°C for *Sma*I, and at 37°C for *Not*I, *Apa*I, *Eag*I, *Sac*II and *Nae*I, according to the manufacturer's instructions. The plugs containing cleaved DNA were loaded on agarose (GTG-agarose; Biometra, Göttingen, Germany) 1% w/v in Tris-borate-EDTA buffer (44.5 mM Tris, 44.5 mM boric acid, 1 mM EDTA, pH 8.3) gels for *Sfi*I and *Not*I, or on agarose 1.3% w/v gels for the remaining enzymes. PFGE was performed with Rotaphor equipment (Biometra) at 13°C. Runs were carried out with the following parameters: for *Sfi*I, electrophoresis for 34 h with pulse times of 60–5 s, angles of 100–125° and a voltage of 200–180 v, all with logarithmic ramping; for *Not*I, electrophoresis for 34 h with pulse times of 100–5 s, angles of 100–125° and a voltage of 200–180 v, all with logarithmic ramping; and for the other enzymes, electrophoresis for 30 h with pulse times of 3–2 s, angles of 120–130° and a voltage of 180–140 v, all with linear ramping. Yeast chromosomes (*Saccharomyces cerevisiae* WAY 5-4A) and Lambda concatemers (Biometra) were used as DNA size markers. PFGE gels were stained with ethidium bromide, photographed under UV light, and subjected to visual analysis. Different patterns were assigned the numbers 1–4. Eclectic PFGE types,

determined by the combination of data obtained from individual PFGE patterns, were assigned the letters A–E.

Results

The results of the hybridisation-based RFLP analysis of the strains used in this study have been published previously.⁷ Strains belonging to MAb subgroups Philadelphia, Benidorm, OLDA and Oxford were

Fig. 1. PFGE of *Sfi*I-cleaved DNA of *L. pneumophila* serogroup 1 strains. Lane numbers correspond to the strain numbers listed in the table.

Fig. 2. PFGE of *NotI*-cleaved DNA of *L. pneumophila* serogroup 1 strains. Lane numbers correspond to the strain numbers listed in the table.

indistinguishable (RFLP pattern 1). The strain belonging to the Bellingham MAb subgroup produced a similar, but distinguishable RFLP pattern (RFLP pattern 47), differing in only one band from that of the other strains.

The results of the MAb subgrouping and the PFGE analysis, based on the *SfiI*, *NotI*, *ApaI*, *SacII*, *SmaI*, *EagI* and *NaeI* profiles of the genomic DNA of the nine strains tested, are summarised in the table. Examples of the restriction patterns of the strains are given in figs. 1 and 2. Enzymes *SfiI*, *SmaI* and *EagI* produced four restriction patterns; *NotI*, *ApaI* and *SacII* produced three patterns; and *NaeI* produced two patterns. Digestion with *SfiI* and *EagI* produced indistinguishable restriction patterns for strains belonging to the Philadelphia and the OLDA subgroups. Strains belonging to the Bellingham and Oxford subgroups were indistinguishable when their *SmaI* cleavage patterns were compared. PFGE analysis of the strain belonging to the Bellingham subgroup produced restriction patterns that differed most clearly from those of the strains belonging to the other four MAb subgroups. PFGE analysis with *NaeI* differentiated the strains into two groups corresponding to their RFLP patterns. The eclectic PFGE groups A, B,

C, D and E corresponded to the MAb subgroups Bellingham, Philadelphia, Benidorm, OLDA and Oxford, respectively. All strains of the same MAb subgroup displayed identical PFGE restriction patterns whichever restriction enzyme was employed. The reproducibility of the *NotI* and *SfiI* restriction patterns obtained for each strain was demonstrated by achieving identical results on two separate occasions.

Discussion

The thorough investigation of outbreaks or single cases of nosocomial or community-acquired Legionnaires' disease requires a combination of epidemiological and microbiological data. In order to identify a given environmental reservoir (e.g., hot water supply system, cooling tower, evaporative condenser, whirlpool spa or decorative fountain) as the source of infection, a comparison of the phenotypic and genotypic characteristics of clinical isolates with isolates obtained from that environmental source is required. The ability of a specific technique to differentiate unrelated strains into different groups is integral to the usefulness of a technique in outbreak investigation. Thus, the differentiating power of MAb subgrouping can be considered limited as only 10 subgroups have been defined.^{2,10} In contrast, RFLP analysis^{7,10} and electrophoretic alloenzyme typing⁶ have been reported to differentiate *L. pneumophila* serogroup 1 strains into 47 and 62 subtypes, respectively. However, MAb subgrouping has the advantage that it is easy and quick to perform, and can be used directly in antigen detection assays of patient specimens.^{12,13} Such assays are of great benefit when the causative agent cannot be isolated by culture.

The usefulness of MAb subtyping has been questioned in several studies in which genotypically homologous *L. pneumophila* serogroup 1 strains, isolated from the same environmental source, expressed different MAb phenotypes (MAb reactivity patterns).^{4,7} These studies assumed that MAb phenotypic variation occurred amongst genetically homogeneous strains.^{4,7} To investigate this assumption further, PFGE analysis was applied to the subtyping of *L. pneumophila* serogroup 1 strains isolated originally from the same cooling tower in London, since PFGE analysis has been used previously for the successful subtyping of *L. pneumophila* serogroups 1 and 6.^{3,8,9}

All strains used in this study displayed very similar PFGE types, with differences in only one or two bands (figs. 1 and 2), supporting the suggestion of Harrison *et al.*⁷ that these strains were derived originally from a single parent strain. Although none of the seven restriction endonucleases used could differentiate the strains—in which there were representatives of five MAb subgroups—into more than four different PFGE subtypes, a strong correlation between MAb subgroup and PFGE subtype was found. This link suggests that the observed variation in MAb phenotype was ac-

accompanied by minor changes in the genome structure, and, therefore, that MAb reactivity may be a useful indicator of diverging genotypic lines amongst genotypically closely related strains. As all strains of a given MAb subgroup showed indistinguishable restriction patterns with each of the restriction endonucleases applied, spontaneous mutations leading to the alteration of restriction sites within the genome of the strains during subculturing could be excluded. The observation that similar PFGE types were found with different restriction endonucleases also supports this conclusion. Thus *Sfi*I and *Eag*I, despite recognising different restriction sites, both differentiated the strains into four PFGE subtypes, each subtype comprising the same strains with both enzymes. PFGE analysis also indicted that the genomic structure of these *L. pneumophila* strains was stable over multiple passages on artificial media, concurring with previous findings from RFLP analysis¹⁰ and PFGE analysis.¹⁴ Furthermore, technical variations during preparation of DNA and during electrophoresis did not influence the restriction patterns significantly as restriction with *Not*I and *Sfi*I yielded identical profiles on two separate occasions.

The MAbs used in the standardised subgrouping scheme² recognise major lipopolysaccharide epitopes on the surface of *L. pneumophila* serogroup 1 strains. The stability of these epitopes, as determined by their reactivity with the MAbs, has been debated by various groups. Several workers have reported stability of the MAb reactivity of strains, even after multiple passages on either BCYE agar,^{2, 15} at various temperatures,¹⁶ or

in cultured *Acanthamoeba* cells (personal unpublished results). In contrast, Rodgers *et al.*¹⁷ and Mintz and Zou¹⁸ reported changes in the MAb reactivity patterns of strains during in-vitro cultivation. Neither of these two latter groups reported whether the observed MAb phenotypic variations were accompanied by genotypic change. Thus, the question of whether legionellae change the epitopes of their surface antigen composition *in vivo* remains unclear. The results of the present study argue for the co-existence of genotypically distinguishable—albeit closely related—strains of *L. pneumophila* serogroup 1 in a specific environmental reservoir, rather than genotypically homologous organisms exhibiting phenotypic variation.

In keeping with the findings of other groups,^{4, 9} PFGE analysis was found to detect genotypic differences in *L. pneumophila* serogroup 1 strains indistinguishable by hybridisation-based RFLP analysis. No evidence of MAb phenotypic variation amongst genotypically homologous strains was found, and it was concluded that MAb subgrouping can still be considered a useful and stable tool for the differentiation of *L. pneumophila* serogroup 1 strains. However, it is clear that a combined use of genotypic and MAb typing methods in epidemiological studies relating to cases of Legionnaires' disease remains preferable.

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