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Phenotypic variation amongst genotypically homogeneous *Legionella pneumophila* serogroup 1 isolates: implications for the investigation of outbreaks of Legionnaires' disease

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SUMMARY

One hundred and seventy-nine isolates of *Legionella pneumophila* serogroup 1, obtained from a site associated with an outbreak of Legionnaires' disease, were examined by monoclonal antibody subgrouping, restriction fragment length polymorphism typing, restriction endonuclease analysis and plasmid content. Nine distinct phenotypes were detected but at the genotypic level all strains were closely related. The data presented indicate that phenotypic variation of a single parent strain can occur within an environmental site. The implications of these findings are discussed in relation to the investigation of outbreaks of Legionnaires' disease.

INTRODUCTION

In the investigation of outbreaks of Legionnaires' disease (LD) it is considered important to seek microbiological data to support the conclusions drawn from the epidemiological studies [1]. Thus 'typing' methods have been developed to distinguish between strains of *L. pneumophila*, particularly those of *L. pneumophila* serogroup 1 (Sgp1) [2–4]. In the investigation of a recent outbreak of Legionnaires' disease in central London isolates were obtained from several patients and found to be indistinguishable using a variety of typing methods. The initial epidemiological findings suggested that the source of the outbreak was likely to be a cooling tower within a particular area. Water samples were therefore collected from cooling towers within the area and examined to determine whether any contained legionellae indistinguishable from the clinical isolates.

This paper reports the results obtained from the detailed examination of legionellae isolated from one suspected cooling tower, and discusses the implications of these findings for the microbiological investigation of outbreaks of LD.

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METHODS

Isolation of legionellae

Duplicate two litre samples of water (designated A and B) were collected from the pond of a suspect cooling tower and were submitted to this laboratory for examination. Legionellae were concentrated 100-fold from the samples by filtration onto 0·2 μm pore-size nylon membranes (Sartorius Ltd) as previously described [5]. The sample concentrates were then inoculated, undiluted or diluted 1/100, onto BCYE agar or BCYE agar containing antibiotics (Oxoid SR 118) without pretreatment or after (*a*) acid-pretreatment or (*b*) heat-pretreatment [5]. The inoculated culture plates were incubated in a humidified environment at 37 °C and examined using a plate-microscope at 24, 36 and 48 h, and then every 24 h for 6 days. Colonies with a morphology typical of legionellae were picked onto BCYE agar, BCYE agar without L-cysteine and, into PBS containing 2% formalin for serological identification [6].

Serological identification of legionellae

Formalin-killed preparations of each putative legionella colony from the primary isolation plate were identified serologically using FITC-labelled monoclonal antibodies specific for either *L. pneumophila* (Genetic Systems Inc) or *L. pneumophila* Sgp1 (DMRQC, Colindale). The identity of these isolates was subsequently confirmed by their nutritional requirements and serologically using rabbit antisera as previously described [6].

Restriction endonuclease digest analysis (REA)

DNA was extracted from bacterial pellets using a recently described rapid method [7]. The isolated DNA was digested to completion with the restriction endonucleases *Nci* I or *Hin*d III (Anglian Biotech Ltd) according to the manufacturers instructions. The restriction fragments were subjected to agarose gel electrophoresis (0·8% gel, 1 V/cm for 16 h) and stained with 0·5 μg/ml ethidium bromide for 30 min.

Restriction fragment length polymorphism (RFLP) typing

Restriction fragment length polymorphism typing was carried out as previously described [8] using the probes λNS20 and λNS21 derived from the *L. pneumophila* strain Knoxville-1 (NCTC 11286). The probe λLEG1, selected from a library of λ fragments derived from the *L. pneumophila* strain Philadelphia-1 (NCTC 11191) was also employed.

Monoclonal antibody (MAb) subgrouping

MAb subgrouping was carried out as described by Joly and colleagues [2] using reagents kindly supplied by Dr R. M. McKinney, Dr J. R. Joly and Dr J. O'H. Tobin.

Plasmid analysis

Plasmid DNA was isolated by the method of Kado and Liu [9], subjected to electrophoresis in 0·7% agarose gels cast in Tris/borate buffer (50 mM Tris,

50 mM borate, 1 mM-EDTA) at 10 V cm^{-1} for 2 h and stained with ethidium bromide (0·5 μg ml^{-1}) for 30 min. *Escherichia coli* strain 39R861 (NCTC 50192), which bears plasmids of 98, 42, 23·9 and 4·6 MDa and *E. coli* strain R27 (NCTC 50010) which carries a plasmid of 112 MDa were analysed concurrently in order to provide molecular-weight standards.

RESULTS

Selective media inoculated with sample concentrate or diluted sample concentrate which had received no pretreatment, became overgrown with contaminants within 48 h. All non-selective media became overgrown within 72 h. Colonies with the typical morphological appearance of legionellae were first seen on the remaining culture plates after 72 h incubation. There was no obvious difference between the acid or heat pretreatments in the time taken for colonies to become visible, however more colonies grew from the heat treated than from the acid treated samples. The total number of colonies seen and the number of colonies examined further are shown in Table 1.

All 179 colonies examined showed positive fluorescence with both the *L. pneumophila* specific and Sgp1 specific FITC-MAb conjugates. However MAb subgrouping revealed that the isolates could be divided into five of the ten described subgroups (Table 2). Although pretreatment had no effect on the numbers of 'Benidorm' and 'Oxford' isolates obtained the data show that colonies of 'OLDA' were isolated more frequently from the heat treated sample than from the acid treated sample (χ^2, Yates' correction = 5·4, $P < 0·02$). Conversely more 'Philadelphia' isolates were obtained from the acid treated samples but this difference was not statistically significant (χ^2, Yates' correction = 3·63, $0·05 < P < 0·1$). An additional noteworthy point is that the first batch of seven isolates (all those colonies large enough to be examined at 72 h) were of either the 'Oxford' or 'OLDA' subgroups. It was only when further isolates were examined on the next working day that 'Benidorm', 'Philadelphia' and 'Bellingham' subgroup isolates were also detected.

Examination of the plasmid content of the 179 isolates showed that they could be subdivided further (Fig. 1). Although the molecular weights of such large plasmids could only be estimated crudely using an agarose gel, REA confirmed the existence of three distinct plasmids (unpublished observations). Plasmid-containing and plasmidless representatives were found for subgroups 'Philadelphia', 'Oxford' and 'OLDA'. The 'Benidorm' isolates all contained either one or two plasmids and the single 'Bellingham' isolate contained one plasmid. Thus nine phenotypes could be distinguished by a combination of MAb subgroup and plasmid content (Table 3).

Fifty-one of the 179 isolates, including representatives of each subgroup/plasmid combination, were selected for further analysis. RFLP typing using the probe λNS20/21 and *Nci* I endonuclease showed that all but one were RFLP type 1 and thus indistinguishable. The single isolate of subgroup 'Bellingham' was distinguishable from the other 50 isolates however the RFLP pattern obtained (RFLP type 47) differed only in the size of single band (Fig. 2) and has not been seen in any previous studies. Further RFLP analyses using λNS20/21 and

Table 1. *Summary of the numbers of colonies isolated (and examined) using each pretreatment method*

Sample	Number of colonies examined/number of colonies seen			
	No treatment	Acid treatment	Heat treatment	Total
A	0*	36/43	67/109	103/152
B	0	48/48	28/28†	76/76
Total	0	84/91	95/137	179/228

* No colonies isolated as culture plate overgrown by contaminants.
† More colonies (>100) were seen in this specimen but they could not be accurately counted or examined further due to fungal overgrowth.

Table 2. *Monoclonal antibody subgroups of the 179 colonies examined*

Sample treatment	MAB subgroup*					Total
	Philadelphia	Benidorm	OLDA	Oxford	Bellingham	
Acid	15	34	7	27	1	84
Heat	7	38	21	29	0	95
Total	22	72	28	56	1	179

* MAB subgroups as defined by Joly and colleagues [2].

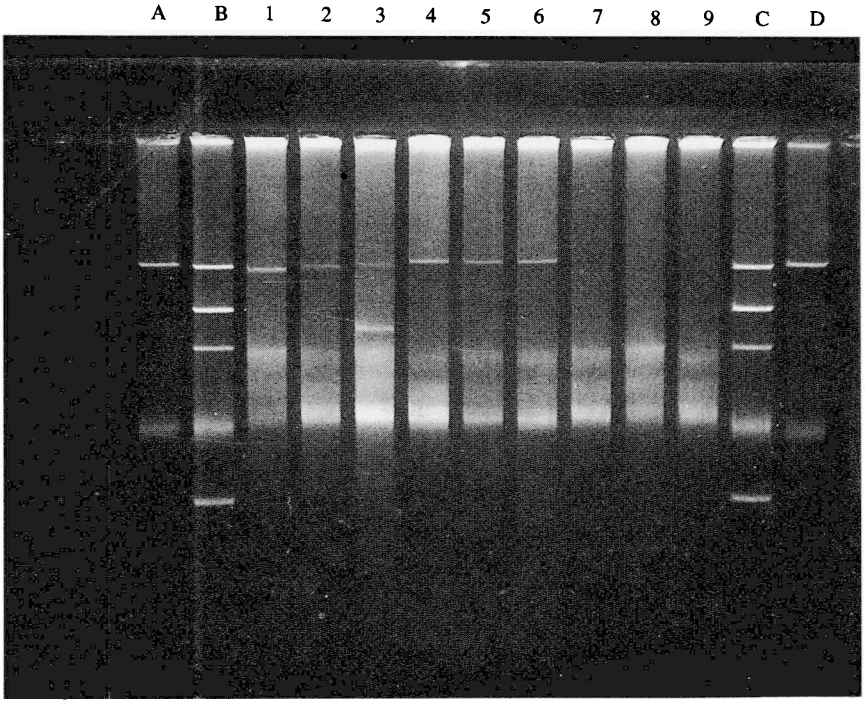

Fig. 1. Agarose gel electrophoresis of plasmid DNA from nine representatives of the 179 isolates examined. Lanes A and D contain a 112 MDa plasmid and lanes B and C contain plasmids of 98, 42, 23·5 and 4·6 MDa. Estimated from these standards; lane 1 contains a ~90 MDa plasmid, lane 2 a ~110 MDa plasmid, lane 3 a ~110 MDa and a ~35 MDa plasmid, lanes 4–6 a ~130 MDa plasmid and lanes 7–10 no plasmids. The order in which the isolates are shown corresponds to that given in Table 3.

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Table 3. *Summary of the characteristics and frequency of the nine phenotypes recognized*

	Isolate(s)
1. *L. pneumophila* Sgp1, MAb 'Bellingham', plasmid A (~90 Mdal)	1
2. *L. pneumophila* Sgp1, MAb 'Benidorm', plasmid B (~110 Mdal)	69
3. *L. pneumophila* Sgp1, MAb 'Benidorm', 2 plasmids (~110 Mdal+35 Mdal)	3
4. *L. pneumophila* Sgp1, MAb 'Philadelphia', plasmid C (~130 Mdal)	21
5. *L. pneumophila* Sgp1, MAb 'Oxford', plasmid C	15
6. *L. pneumophila* Sgp1, MAb 'OLDA', plasmid C	25
7. *L. pneumophila* Sgp1, MAb 'Oxford', no plasmid	41
8. *L. pneumophila* Sgp1, MAb 'Philadelphia', no plasmid	1
9. *L. pneumophila* Sgp1, MAb 'OLDA', no plasmid	3

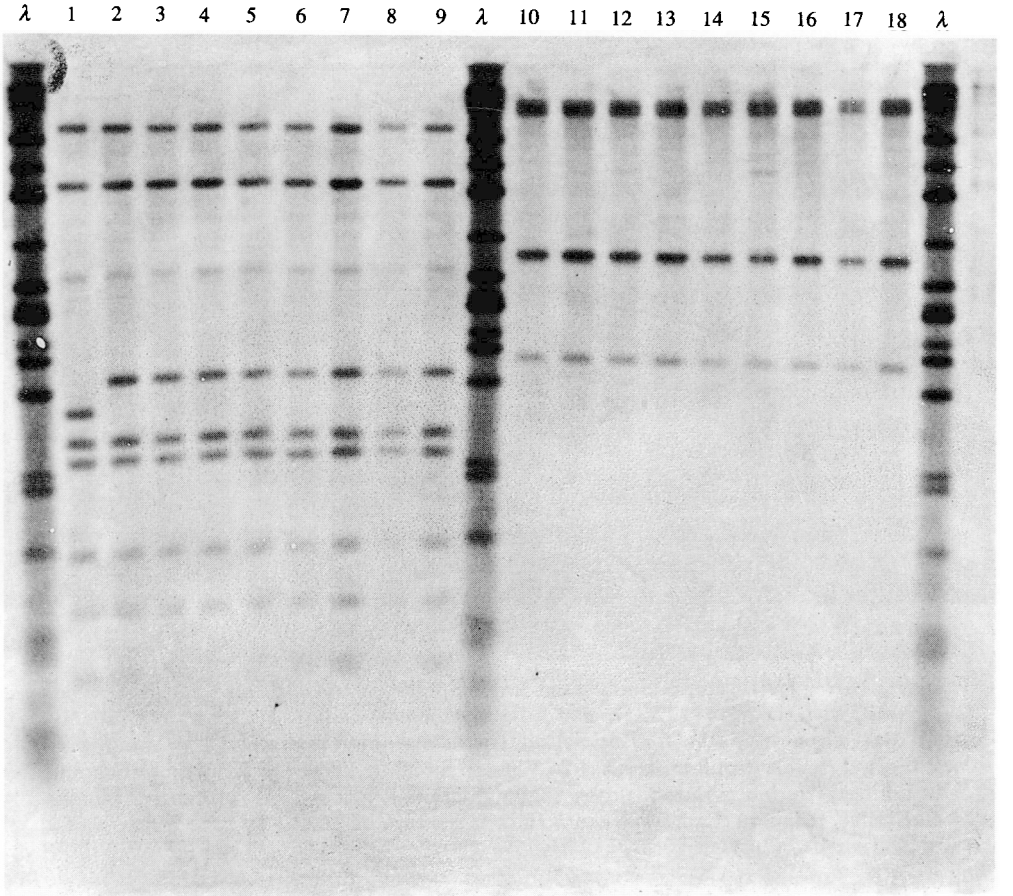

Fig. 2. *Nci* I (lanes 1–9) and *Hin*d III (lanes 10–18) restriction endonuclease fragments, from each of the nine phenotypes, detected with the probes λNS20 and λNS21. The order in which the isolates are shown corresponds to that given in Table 3. Lanes 1 and 10 contain the single 'Bellingham' subgroup isolate. Restriction fragments (approximately 1 μg/lane) were subjected to electrophoresis at 1·25 V cm^{-1} for 16 h. The DNA was blotted and hybridized to biotinylated probe (300 ng). Bound probe was visualized by the BluGENE reagents. Lanes marked λ show a mixture of *Eco*R I and *Pst* I digests of λ phage DNA.

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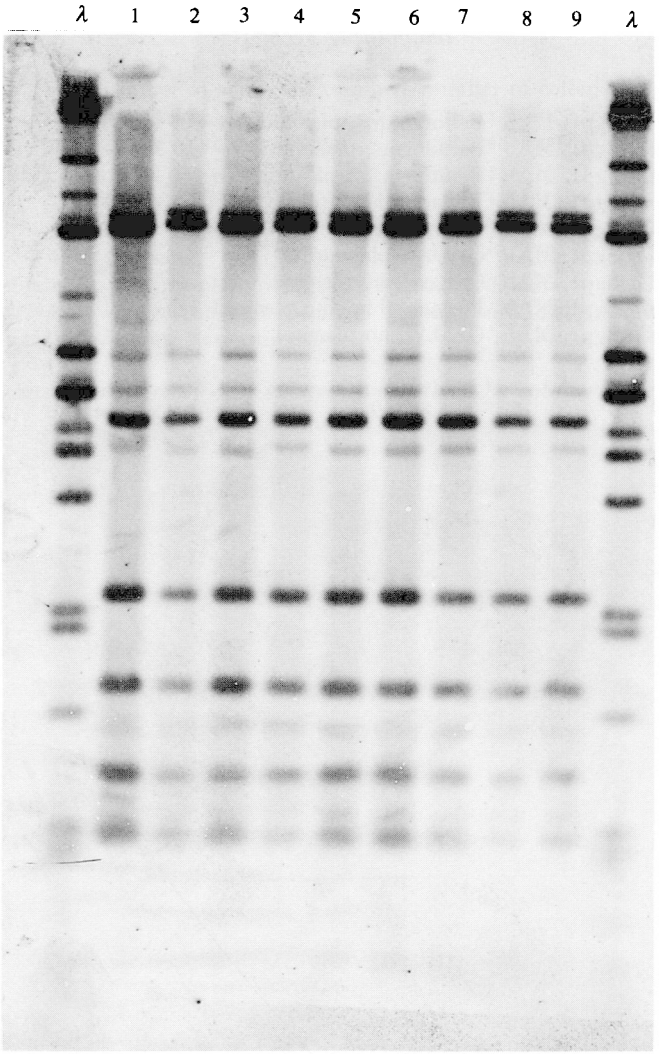

Fig. 3. *Nci* I restriction endonuclease fragments from each of the nine phenotypes detected with the probe λLEG1. The order in which the isolates are shown corresponds to that given in Table 3. Restriction fragments (approximately 1 μg/lane) were subjected to electrophoresis at 1·25 V cm^{-1} for 16 h. The DNA was blotted and hybridized to biotinylated probe (300 ng). Bound probe was visualized by the BluGENE reagents. Lanes marked λ show a mixture of *Eco*R I and *Pst* I digests of λ phage DNA.

Hind III (Fig. 2) or λLEG1 with either *Nci* I (Fig. 3) and *Hind* III (not shown) failed to distinguish between the nine phenotypes. When the DNA content of these isolates was compared by REA using either *Nci* I or *Hind* III, no significant differences could be discerned (Fig. 4). Minor differences between the high-molecular-weight bands of plasmid-containing and plasmidless strains could be seen (not discernible in Fig. 4), but REA of *Nci* I digests of these plasmids suggested that their presence/absence would result in the minor differences observed (unpublished observations).

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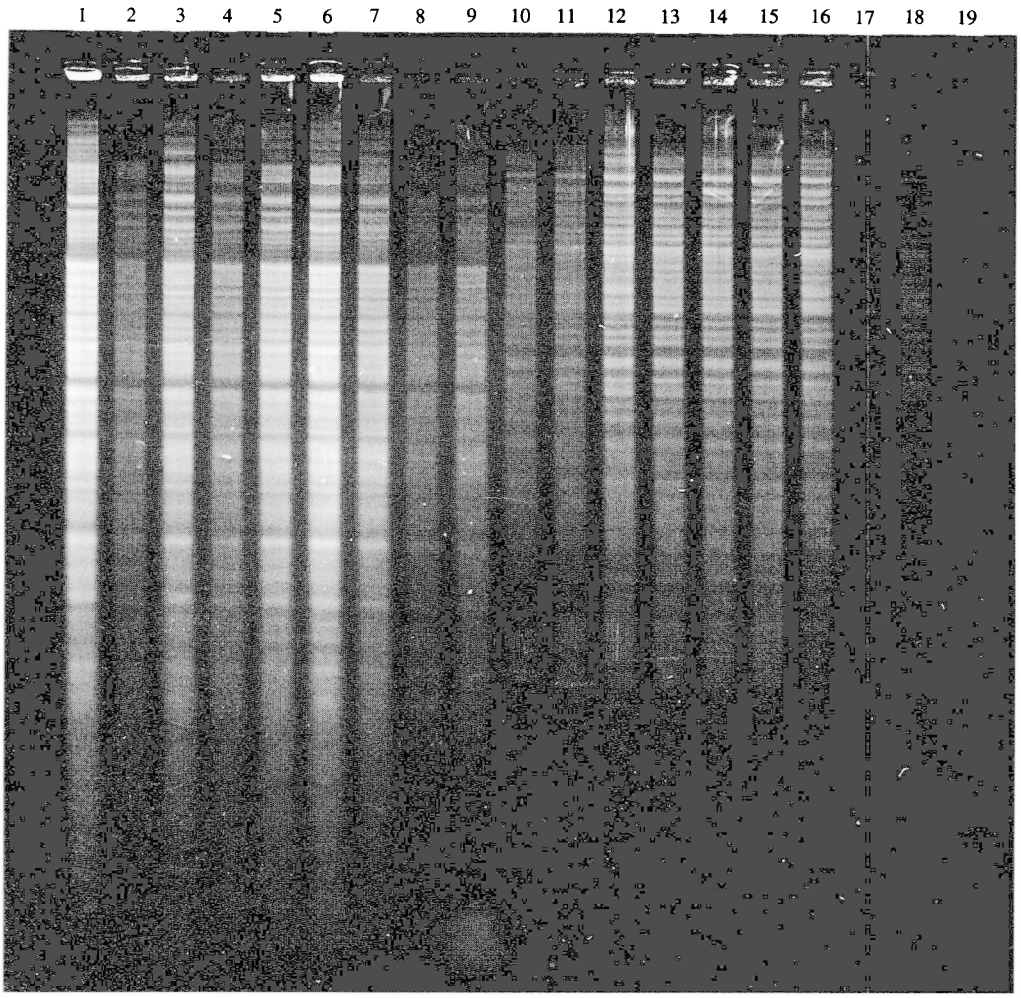

Fig. 4. *Nci* I (lanes 1–9) and *Hind* III (lanes 10–18) restriction endonuclease fragments from each of the nine phenotypes. The order in which the isolates are shown corresponds to that given in Table 3. Restriction fragments (approximately 2 μg/lane) were subjected to electrophoresis at 1 V cm^{-1} for 16 h and stained with 0·5 μg/ml ethidium bromide for 30 min.

DISCUSSION

The microbiological investigation of an outbreak of LD usually involves a search for a particular phenotype and/or genotype of *L. pneumophila* in a number of large, complex water systems (e.g. cooling towers or domestic hot water systems). It is well recognized that legionellae of different species or serogroups may be isolated from the same source [10], however this study demonstrates that, on detailed examination, water samples may be found to contain a phenotypically diverse population of *L. pneumophila* Sgp1 bacteria. Since *L. pneumophila* Sgp1 is the most commonly isolated serogroup, three problems concerning the microbiological examination of environmental specimens arise from this observation. First, it is necessary to obtain a sample(s) that is representative of the system;

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second, legionellae that are representative of the sample must be recovered; and third, enough colonies must be examined to identify the various phenotypes of legionella that are present.

Considerable progress has been made in the first two areas and suitable procedures have been published [5]. However work has mainly been directed towards the recovery of maximal numbers of legionellae and not to the determination of whether the various media and pretreatments used preferentially select particular phenotypes/genotypes. The data presented here show that significantly more 'OLDA' subgroup isolates were obtained from the heat treated specimens, indicating that the pretreatment method may influence the recovery of different subgroups.

In this study the seven colonies first examined were distinguishable from the patient isolates by MAb subgrouping. It was only on examination of further isolates that indistinguishable isolates (by MAb subgrouping) were identified. Review of the specimens sent to the PHLS Legionella Reference Unit showed that in most instances only one or two isolates per water sample are submitted for typing. The above data demonstrate that examination of such small numbers of colonies might give rise to misleading results.

Although in theory all colonies from every sample should be examined it clearly is not practical to do this. In considering the statistical aspects of the microbiological examination of food, which is analogous to water sampling, Jarvis [11] noted

> ...when mixed cultures are present and some of the organisms of different types have apparently similar colonial morphologies, then several colonies may need to be picked. In some laboratory manuals (Hall, 1975) no reference is made to the number of colonies to pick; others (ISO, 1979) recommend selection of the square root of the number of 'typical' colonies, whilst ICMF (1978) recommend picking of at least two typical colonies and ISO (1978) recommends picking of at least five typical colonies.

Thus there is no concensus of opinion. Jarvis [11] has calculated that if a sample size of $\sqrt{N}$ colonies is examined, where a culture plate contains between 30 and 150 (N) 'typical' colonies, between 43 and 21% of them, respectively, must be of the type sought if there is to be a 95% chance of including at least one in the sample. A sample size of $\sqrt{N}$ colonies would therefore appear to be acceptable, albeit that the suspect strain might only be detected when present as a substantial proportion ($>20\%$) of the legionellae in the water system. Whatever number of colonies is chosen this should be clearly stated when reporting results.

Successful isolation of *L. pneumophila* from a site epidemiologically associated with the outbreak is often cited as corroborative evidence that the site was the source of infection [12, 13]. In early studies patient and environmental isolates were shown to be similar by serogrouping [14], but it rapidly became apparent that *L. pneumophila* Sgp1 was the most common serogroup isolated from both clinical and environmental sources. More discriminating typing methods have therefore been utilized to distinguish between strains of this serogroup [1, 15, 16]. However, the validity of the conclusions drawn from these studies depends upon the stability of the epidemiological markers used.

Examination of restriction enzyme digests of *L. pneumophila* Sgp1 DNA has shown that many distinct genotypes can be demonstrated [17, 18]. Furthermore,

the RFLP typing method used here (with λNS20/21 and *Nci* I) has previously been shown to reveal more than 40 distinct RFLP types [8]. The DNA preparations from 50 of 51 isolates examined in this study, were found to be indistinguishable by both total and probed restriction enzyme digest analysis. The single isolate which was distinguishable from the others by RFLP analysis using λNS20/21 with *Nci* I digestion was, however, indistinguishable by REA and by RFLP analysis using both λNS20/21 with *Hin*d III digestion, and λLEG1 with either *Nci* I or *Hin*d III digestion.

The likelihood that the cooling tower examined here was contaminated with nine phenotypically distinct strains, eight of which were of the same RFLP type and the ninth being of a very similar RFLP type, is remote. It is therefore probable that some or all of the isolates examined here were derived by phenotypic variation of a single parent organism. If this is the case then neither MAb subgroup or plasmid content can be constant characteristics of a particular strain. The presence within the cooling tower of strains having two slightly different RFLP patterns indicates that genotypic variation may also have occurred. Only a single base-change would be required to generate the observed difference in RFLP type. The alternative hypothesis that strains with different RFLP types contaminated the tower cannot, however, be ruled out.

Restriction endonuclease analysis of the three plasmid types observed showed striking similarities between them. Thus it is possible that these are variants of a single plasmid derived by recombination and rearrangements of its sequence. The plasmidless isolates may have arisen by spontaneous curing of the plasmid bearing stock.

Evidence that legionellae can undergo changes that result in a different MAb subgroup has also been reported by others. Edelstein and colleagues [19] demonstrated that the reactivity of a strain with two monoclonal antibodies varied depending on the incubation temperature used. Similarly, Colbourne and colleagues [20] reported that legionellae examined directly in a water sample had a different pattern of reactivity with a panel of MAbs, than did legionellae isolated from that sample after heat treatment. In both the above studies the MAb subgroup of the entire population appeared to alter. However we have found that several different MAb subgroups may be recognized among isolates of a single genotype occurring in the same sample. This suggests that environmental conditions can modify the expression of surface antigens in a number of different ways concurrently.

Previous studies have shown that the MAb subgroup of both outbreak-associated patients isolates, and repeatedly subcultured laboratory isolates are stable [8]. However, if MAb subgrouping is to be used to implicate an environmental source, the stability of these and other epidemiological markers in the environment must be investigated further.

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