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Molecular epidemiology of *Legionella pneumophila* environmental isolates representing nine different serogroups determined by automated ribotyping and pulsed-field gel electrophoresis

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SUMMARY

The purposes of the study were (i) to describe the abundance and epidemiology of Legionellaceae in the man-made environment in a northern Italian area, (ii) to assess the concordance between pulsed-field gel electrophoresis (PFGE) and automated ribotyping (AR) techniques for genotyping *L. pneumophila* and (iii) to investigate the correlation between serogrouping and genotyping data. Water was sampled from reservoirs in 12 buildings across an area of 80-km radius. Despite the water temperature always being maintained above 55 °C, all of the buildings sampled were contaminated with Legionellaceae on at least one occasion and 63 *L. pneumophila* isolates representing nine different serogroups were collected. The two DNA methods revealed a high degree of genetic heterogeneity, even though identical *L. pneumophila* clones were recovered at different sites. The AR technique provided a fairly reliable approximation of PFGE results (73% concordance), however there was poor correlation between serogrouping and genotyping data as identical DNA fingerprints were shared by isolates of different serogroups.

INTRODUCTION

Legionella pneumophila is widespread in aqueous environments and is a common cause of nosocomial and community-acquired pneumonia. It is responsible for over 85% of the cases of Legionnaires' disease and 15 serogroups have been described thus far, with serogroup (Sg) 1 accounting for almost 90% of cases [1]. For epidemiological investigations accurate discrimination between *L. pneumophila* isolates is important for identifying cases with common sources, routes of diffusion of the organism [2, 3], and for evaluating the environmental spread in water distribution networks [4]. The most commonly used techniques are based on the detection of genomic DNA polymorphisms [5] and currently amplified fragment length polymorphisms (AFLP) is the established reference method for genotyping *L. pneumophila* Sg1 [6], although pulsed-field gel electrophoresis (PFGE) of DNA macrorestriction digests remains the most used owing to its high discriminatory power [7, 8]. Conventional ribotyping was previously used for *L. pneumophila* genotyping [9], but in recent years automated ribotyping (AR) has been successfully applied to genotype several bacterial species [10–14], although its use has not been reported for *L. pneumophila*. A number of methods have been used to subgroup *L. pneumophila* Sg1 isolates but relatively few studies have addressed subtyping of non-Sg1 isolates [2, 3, 7, 9, 15–18].

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Table 1. *Frequency, viable counts and serogroups of* Legionella pneumophila *in water samples from different buildings*

Building (source of ground water)	Water samples positive for *L. pneumophila*/samples tested (%)	Range (c.f.u./l)	Serogroups
Hospital A (1)	36/98 (37)	20–16 000	1, 3, 5, 6, 8
Hospital B (4)	1/6 (16)	400	1
Hospital C (2)	4/20 (20)	20–700	6, 8, 12
Hospital D (5)	3/12 (25)	20–800	1, 8, 9
Hospital J (3)	2/10 (20)	80–800	1, 6
Nursing home E (6)	1/5 (20)	20	10
Nursing home F (2)	1/5 (20)	500	6
Nursing home G (3)	3/15 (20)	50–900	1, 10
Nursing home H (7)	1/5 (20)	300	12
Nursing home K (1)	2/10 (20)	40–200	8
Hotel I (9)	1/5 (20)	400	13
Hotel L (10)	8/20 (40)	20–900	1, 13
Total	63/211 (29·8)	20–16 000	1, 3, 5, 6, 8, 9, 10, 12, 13

There is little recent information about the frequency and distribution of *L. pneumophila* in the man-made environment from areas of northern Italy and such data may be of interest for international comparison. Indeed, different genotyping techniques have never been applied to a large set of environmental *L. pneumophila* isolates of different serogroups collected over a large geographical area. We collected *L. pneumophila* isolates from water sources of different buildings in an area of northern Italy and characterized them by serogroup and genotype using PFGE and AR methods. We determined the frequency of *L. pneumophila* in water samples and evaluated the concordance between the genotyping methods and serogroup distribution.

METHODS

Microbiological surveillance

Between August 1999 and April 2002, water samples for bacteriological analysis were collected from hot-water taps and showers in six hospitals (designated by letters A–D, H and J, Table 1), four nursing homes for the elderly (E–G and K) and two hotels (I and L) located within a 80-km radius in northern Italy. All the buildings are supplied by independent wells which are filled by ground water (designated by numbers 1–10, Table 1). All water sources are distinct, with the exception of those that provide water to the establishments A and K, C and F, G and J, which are supplied by ground-water sources 1, 2 and 3 respectively (Table 1). In keeping with national guidelines [19] to suppress colonization with Legionellaceae, the circulating water temperature in all buildings is kept above 55 °C. The temperature level is checked once a month and no major discrepancies were observed in the surveillance period. In total, 211 water samples from 12 buildings were collected in accordance with the guidelines for the isolation of *Legionella* spp. from water [19] as part of an environmental monitoring programme that provided for a six-monthly assessment of water systems in all public buildings within the province. According to the dimension of the building, one or more (from different areas of the building) water samples were collected at a time. Briefly, 5 l of water was collected aseptically from selected taps and showers in a sterile receptacle; 4990 ml of each sample was filtered through a 0·2 μm membrane and this was resuspended in 10 ml of the original water sample. After vortexing briefly 0·1 ml was spread on selective agar medium. Isolates were cultured and identified in accordance with the ISO 11731 [20]. Serogrouping was performed by agglutination with commercial specific monoclonal antibodies (Pro-lab Diagnostics, Neston, UK). Quality controls for *L. pneumophila* identification and quantification were periodically performed with commercially available controls (Oxoid, Basingstoke, UK). A decontamination protocol was carried out in the

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buildings where the concentrations of *L. pneumophila* were $\geqslant 10^4$ c.f.u./l [19] and surveillance cultures were performed subsequently to monitor the efficiency of eradication.

Automated ribotyping

Automated ribotyping was performed with the RiboPrinter Microbial Characterization System (DuPont Qualicon, Wilmington, DE, USA), as previously described [21], in accordance with the manufacturer's instructions [22]. Extracted bacterial DNA was subjected to *Eco*RI restriction enzyme digestion. The fragments were separated on agarose gel, transferred to a nylon membrane, hybridized with a rDNA probe, visualized with a chemiluminescent detection system and subjected to densitometric scanning. Preliminary ribotype groups (ribogroups) were furnished by the Riboprinter, which compares isolate patterns with those of others in the system database and generates groups based on similarities in the number, position and intensity of the bands [23]. For this study, however, definitive ribogroups were identified by computerized pattern analysis using Bionumerics software (version 3.0, Applied Maths, Sint-Martens-Latem, Belgium), which generates a dendrogram of similarity of the isolates based on the unweighted pair group method using arithmetic averages (UPGMA) using the Dice similarity coefficient for band matching [24]. Isolates were assigned to the same ribogroup if they had similarity coefficients of $\geqslant 0{\cdot}95$.

Chromosomal PFGE analysis

Patterns were obtained by *Sfi*I (Promega, Madison, WI, USA) digestion using an established protocol [9] with minor modifications. Briefly, bacteria embedded in a 1% PFGE-certified agarose gel (Bio-Rad, Hercules, CA, USA) were treated for 16 h with proteinase K (final concentration 100 μg/ml) in TE buffer [10 mM Tris–HCl (pH 8·0), 0·1 mM EDTA] and 1% *N*-laurosylsarkosine. After extensive washing and equilibration for 1 h at room temperature with *Sfi*I buffer [10 mM Tris–HCl (pH 7·5), 10 mM $MgCl_2$, 50 mM NaCl], the DNA in each plug was digested with 30 U of *Sfi*I for 16 h at 50 °C. The plug was then washed for 30 min in TE buffer and loaded into slots of a 1% agarose gel in sterile 0·5× Tris–borate EDTA buffer. Lambda concatemers (New England Biolabs, Beverly, MA, USA) were used as size markers. DNA fragments were separated in the contour-clamped homogeneous electric field system (CHEF Mapper, Bio-Rad) with running conditions of 200 V for 27 h at 14 °C with switch times of 1 s (initial) and 35 s (final). After ethidium bromide staining, the gels were photographed with a UV light source. Isolates with patterns differing by no more than three bands were considered to have the same pulsotype [25]. Macrorestriction patterns were analysed using Bionumerics software (version 3, Applied Maths) in a similar manner as that used for ribotyping.

RESULTS

Microbiological surveillance

During 3 years of environmental monitoring each of the 12 buildings were found to be contaminated on at least one occasion by *L. pneumophila* (Table 1). Of 211 samples collected, 63 were positive for *L. pneumophila* (29·8%), with Hospital A being the most frequently contaminated (37% of positive water samples) and on a single occasion (July 2001) a count of $1{\cdot}6 \times 10^4$ c.f.u./l of *L. pneumophila* was recorded in this building. A decontamination protocol was applied for three consecutive days, the water temperature was maintained at 70 °C and all taps were allowed to run for at least 30 min/day [19]. This procedure was insufficient for complete eradication of the *L. pneumophila* populations as subsequent surveillance cultures grew the species but with reduced counts of $< 10^3$ c.f.u./l (Table 2).

Molecular epidemiology of isolates

All isolates were successfully typed with both molecular methods adopted. Preliminary studies of reproducibility were performed on 10 random isolates which were typed twice by AR and PFGE. Similarity coefficients ranging from 0·95 to 1·00 (data not shown) were obtained with the pairs of tests indicating that each method had excellent intra- and inter-gel reproducibility. Two to four fragments ranging in size from 6·0 to 24·5 kb were visualized for the 63 isolates by AR and their relatedness is shown in Figure 1. Eleven different ribogroups characterized by inter-isolate similarity coefficients of $\geqslant 0{\cdot}95$ were identified and 30/63 (47·6%) isolates fell into the three most frequent groups; seven ribogroups were represented by two or fewer isolates (Table 2). The dendrogram of similarity derived from *Sfi*I macrorestriction analysis is shown in Figure 2. By PFGE analysis isolates with patterns that differed by no more than three

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Table 2. *Source and genotyping patterns of 63* Legionella pneumophila *environmental isolates*

Designation	Source	Date of sampling	Sero-group	AR pattern	PFGE pattern
A1	Hospital A	29 Apr. 2000	6	R-1*	P-1*
A2		8 Oct. 2000	6	R-1	P-1
A3, A4, A5, A6		24 Jan. 2001	6	R-1	P-1
A7		27 July 2001	6	R-1	P-1
A8		8 Aug. 2001	6	R-1	P-1
A9		20 Aug. 2001	3	R-1	P-1
A10		4 Sep. 2001	3	R-1	P-1
A11		25 Sep. 2001	9	R-1	P-1
A12, A13, A14, A15, A16		25 Sep. 2001	6	R-1	P-1
A17		29 Oct. 2001	6	R-1	P-1
A18, A19, A20, A21		24 Jan. 2002	6	R-1	P-1
A22		24 Jan. 2002	8	R-1	P-1
A23		24 Apr. 2002	6	R-1	P-1
A24		24 Jan. 2002	6	R-1	P-2
G1, G2	Nursing home G	7 Dec. 1999	10	R-1	P-3
J1	Hospital J	1 Mar. 2000	6	R-1	P-4
D1	Hospital D	10 Nov. 1999	8	R-1	P-5
G3	Nursing home G	7 Dec. 1999	1	R-2	P-6
J2	Hospital J	1 Mar. 2000	1	R-2	P-6
A25	Hospital A	8 Oct. 2000	1	R-2	P-6
A26		7 Dec. 2001	1	R-2	P-6
L1, L2	Hotel L	22 Feb. 2001	1	R-2	P-6
A27	Hospital A	24 Jan. 2002	1	R-2	P-6
A28		24 Jan. 2002	1	R-2	P-7
A29		8 Oct. 2000	5	R-3	P-8
A30		8 Aug. 2001	5	R-3	P-8
A31, A32		4 Sep. 2001	5	R-3	P-8
A33		25 Sep. 2001	5	R-3	P-8
E1	Nursing home E	24 Nov. 1999	10	R-3	P-9
I1	Hotel I	22 Jan. 2000	3	R-3	P-10
A34	Hospital A	21 Aug. 2001	6	R-4	P-11
A35		20 Aug. 2001	6	R-4	P-12
A36		24 Jan. 2002	1	R-5	P-13
B1	Hospital B	5 Oct. 1999	1	R-6	P-14
C1	Hospital C	3 Nov. 1999	8	R-7	P-15
H1	Hospital H	2 Oct. 2000	12	R-7	P-16
C2	Hospital C	3 Nov. 1999	6	R-8	P-17
C3, C4		3 Nov. 1999	12	R-8	P-17
K1	Nursing home K	15 Mar. 2000	8	R-9	P-18
K2		15 Mar. 2000	8	R-9	P-19
D2	Hospital D	24 Nov. 1999	9	R-10	P-20
D3		24 Nov. 1999	1	R-10	P-21
F1	Nursing home F	1 Dec. 1999	6	R-11	P-22
L3, L4	Hotel L	22 Feb. 2001	13	R-11	P-23
L5, L6, L7, L8		7 Nov. 2001	13	R-11	P-23

AR, Automated ribotyping; PFGE, pulsed field gel electrophoresis.
* R, ribotype; P, pulsotype.

bands all had similarity coefficients $\geqslant 0{\cdot}80$, and this cut-off was, therefore, used to define a given pulsotype. Twenty-three pulsotypes were distinguished, 16 of which were represented by single isolates only; pattern P-1 accounted for 13 isolates and only two other pulsotypes (P-6 and P-8) contained four or more isolates (Table 2). Forty-six isolates (73%) gave concordant results by AR and PFGE. For example,

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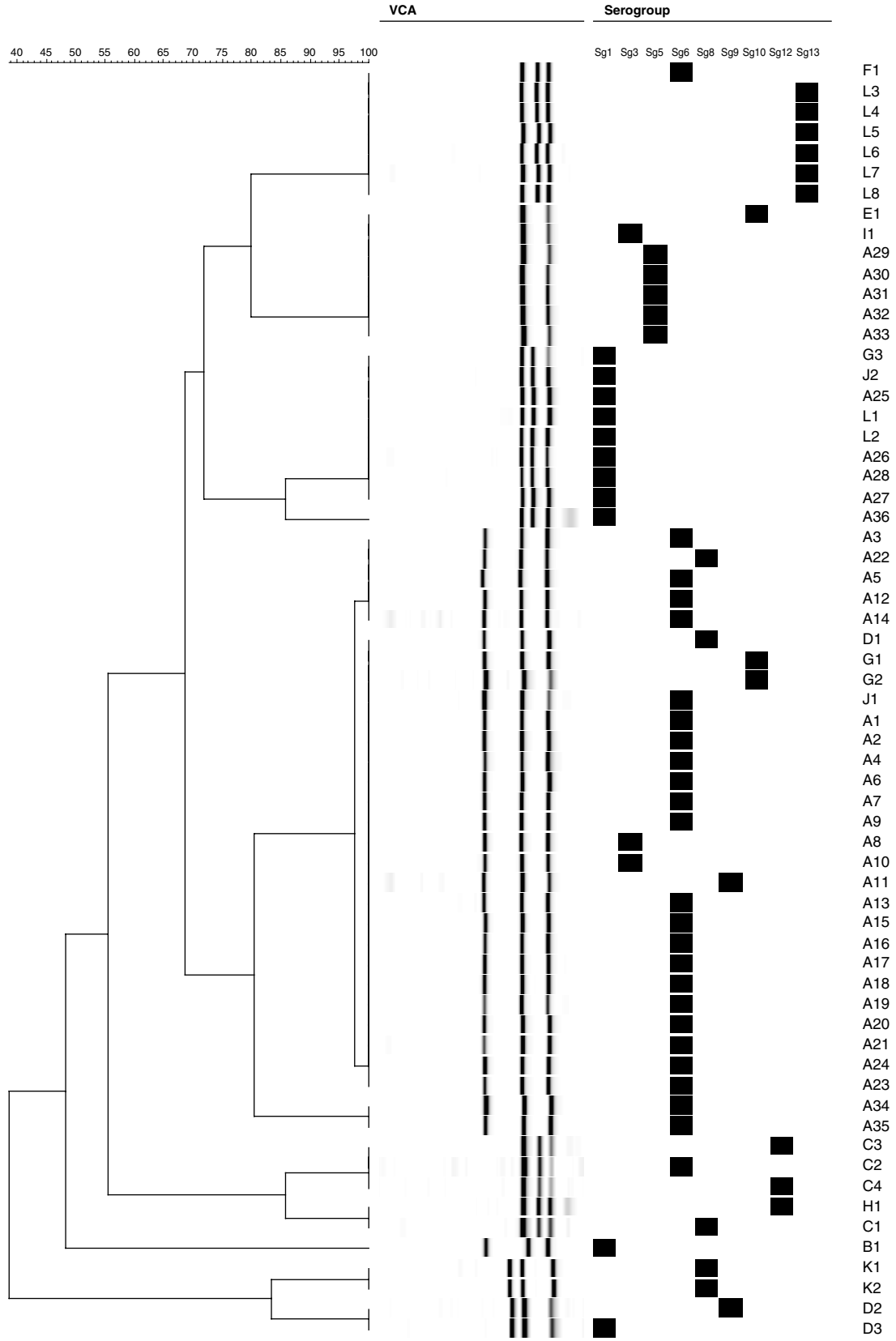

Fig. 1. Dendrogram showing similarities between 63 environmental isolates of *L. pneumophila* based on automated *Eco*RI ribotyping. Isolates with Dice similarity coefficients $\geqslant 0{\cdot}95$ are considered identical (see Materials and methods section). A black box indicates the respective serogroup for each isolate.

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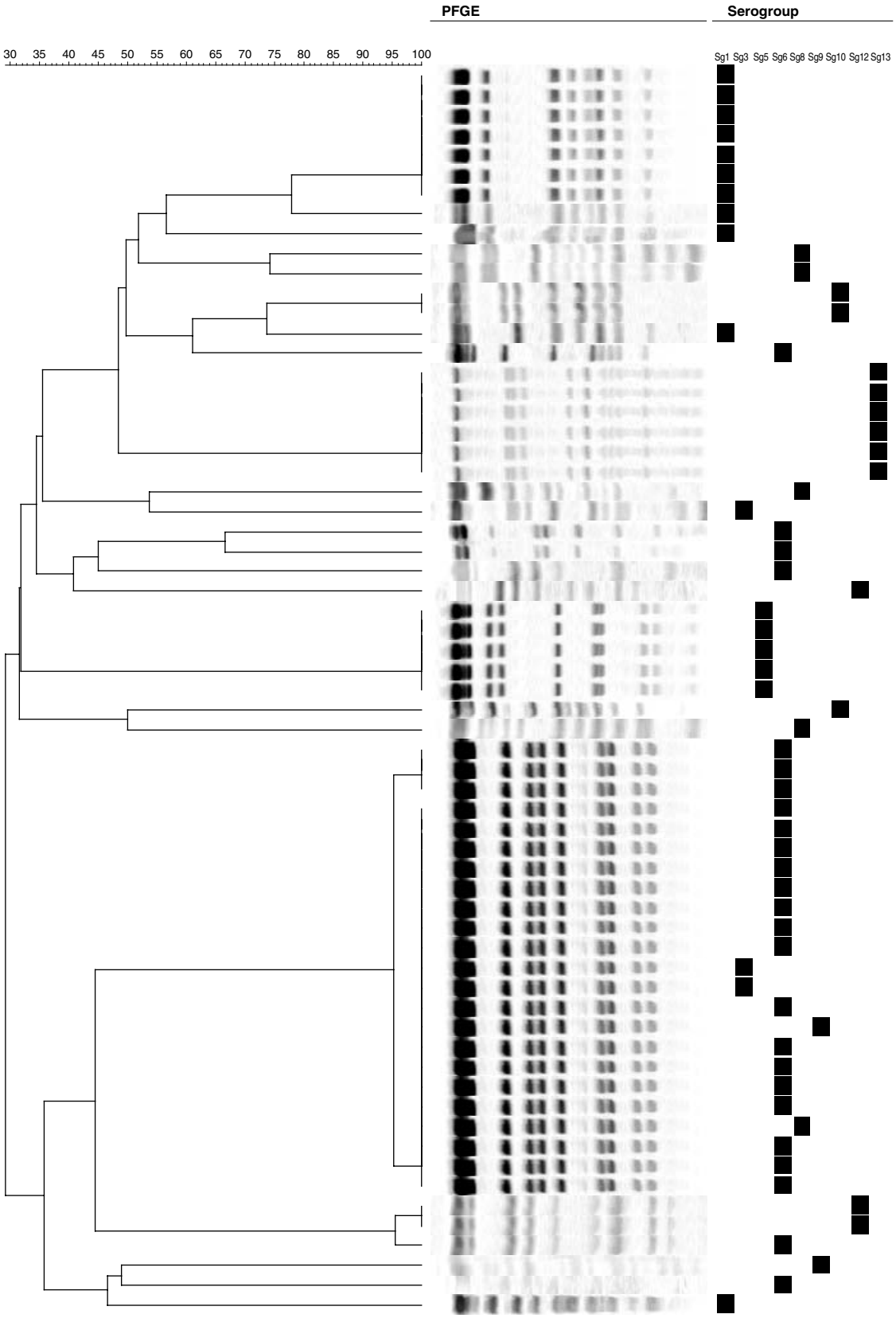

Fig. 2. Dendrogram showing similarities between 63 environmental isolates of *L. pneumophila* based on PGFE. Isolates with Dice similarity coefficients >0·80 are considered identical (see Materials and methods section). A black box indicates the respective serogroup for each isolate.

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23 out of 27 isolates assigned to AR pattern 1 were also 'identical' (i.e. similarity coefficients of $\geqslant 0{\cdot}80$) by PGFE (pulsotype 1) and combination of pulsotypes with ribotypes did not discriminate further among the 63 isolates (Table 2) and all discrepancies between the two methods could be attributed to the higher discriminatory power of PFGE. Indeed, PFGE was able to distinguish two or more subgroups within eight of the ribotypes identified by AR (e.g. five groups among the 27 isolates of ribotype 1, two among the eight isolates of ribotype 2, etc., Table 2).

Genotyping, therefore, confirmed a high degree of genetic heterogeneity among the isolates. Most of the water sources in buildings were colonized by different strains and in some establishments different pulsotypes were recovered in the period of surveillance as evidenced by the finding of eight pulsotypes in Hospital A (Table 2). Two buildings (Hospital A and Hotel L) were persistently colonized by identical strains throughout the study period. However, apparently identical strains were identified in different buildings as seven isolates of pulsotype 6 were recovered in four distinct establishments at different time periods (Hospitals A and J, Nursing home G and Hotel L; Table 2).

Relationship between serogroups and genotypes

With the exception of *L. pneumophila* Sg5 and Sg13, all the serogroups appeared to be heterogeneous, with representatives found in multiple pulsotypes (Table 2). It is noteworthy that, in some cases, genotyping revealed isolates of different serogroups to be representative of a single genetic type. Indeed, the large cluster of 23 isolates recovered in Hospital A (R-1, P-1) included four different serogroups (3, 6, 8 and 9). A similar situation was demonstrated in Hospital C, where three strains representing two different serogroups (6 and 12) were identical in genotype (Table 2).

DISCUSSION

In this study we monitored 12 buildings over 3 years located in an area of northern Italy for the presence of Legionellaceae. Despite the fact that the circulating water temperature in their network distribution systems was always maintained above 55 °C, *L. pneumophila* isolates were recovered at least once from all of the buildings. However, with exception of one establishment (Hospital A), the *L. pneumophila* count did not exceed 10^3 c.f.u./l and ranged from 20–900 c.f.u./l. Furthermore no case of Legionnaires' disease occurred in the hospitals or in the other buildings during the study period. In Hospital A, in July 2001, the *L. pneumophila* count reached an alert level ($1{\cdot}6 \times 10^4$ c.f.u./l) and this was probably due to a temporary drop in water temperature below 55 °C, but other unknown factors may have contributed. In this case a 'superheat and flush' decontamination protocol was applied but subsequent surveillance cultures revealed recurrent contamination but with a lower bacterial count, which is consistent with reports by other investigators [26].

The 63 *L. pneumophila* isolates displayed a high degree of serotype heterogeneity. Nine different serogroups were identified the most prevalent being Sg6 (40%). This finding together with the low prevalence of *L. pneumophila* Sg1 (17%), is in agreement with the results of other studies performed in environmental settings [18, 27]. Nevertheless, rare serogroups such as Sg12 and Sg13 were also isolated from the water samples.

AR with *Eco*RI restriction enzyme generated 11 different ribogroups among our 63 isolates and this technique was less discriminatory than PFGE which distinguished 23 pulsotypes. As expected, the isolates were genetically heterogeneous and different clonal groups were recovered in the 12 buildings over the study. Indeed, seven isolates assigned to pulsotype 6, all of which belonged to serogroup 1, were recovered from four buildings (Hospitals A and J, Nursing home G and Hotel L) which, with the exception of J and G, are supplied by different ground waters and located in distant areas (>50 km radius) of the province. Other investigators have also recorded identical PFGE patterns in apparently unrelated isolates of *L. pneumophila* [7, 28–30]. This finding led Lawrence et al. [28] to suggest that some clones may be better adapted than others to environmental survival, while others speculated that widespread geographical dissemination of Legionellacae may occur via rain or wind transportation [7]. While we concur with this view a degree of caution must be exercised in interpreting the genotyping data and this should be always combined with sound epidemiological data in order to draw any definitive conclusion [30, 31].

We compared the results of genotyping isolates by AR and PFGE methods. The AR method has been shown recently to provide good species-level discrimination among American Type Culture Collection (ATCC) *Legionella* spp. isolates but its ability to distinguish between strains of *L. pneumophila* was

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much less impressive [14]. From our experience the discrimination achieved by PFGE was clearly superior to AR even though it was markedly more time-consuming (3 days per isolate *vs*. 8 h with AR). PFGE differentiated two or more subgroups within eight of the ribotypes (Table 2) and it revealed 17 isolates with unique profiles while AR defined only four strains as unique. However, despite this inherent lack of discriminatory power AR might offer a reasonably reliable time-saving alternative to PFGE in situations such as those requiring rapid processing of numerous isolates as it gave an acceptably high level of concordance (73%) with the latter. The reproducibility of the AR method inherent in its 'black box' technology could also be useful where cross-laboratory studies are undertaken or where skilled personnel are not available.

Although 36% and 16% of hospital and community-acquired Legionnaires' disease respectively are caused by *L. pneumophila* non-Sg1 [32], there have been relatively few reports of epidemiological subtyping of other serogroups [2, 3, 7, 9, 15–18]. In our study, nine different *L. pneumophila* serogroups were genotyped and in some cases, isolates of different serogroups shared an identical genotype. For example, the cluster of 23 isolates from Hospital A of the same genotype contained strains of four different serogroups. A similar situation was evident in Hospital C, where three strains representing two different serogroups were genetically identical. It is clear that a *L. pneumophila* serogroup can be genetically heterogeneous, but few studies report that isolates of different serogroups can share a common genetic pattern [9, 14, 15, 17, 33]. It has been suggested for *L. pneumophila* Sg1 that point mutations in genes involved in the synthesis of lipopolysaccharide do not affect the overall strain genotype as revealed by DNA profiling or sequence-based typing methods [34].

In conclusion, our study shows that accurate temperature maintenance in the water distribution network can help to contain the contamination of *L. pneumophila* in water reservoirs in buildings, given that complete eradication of contamination appears to be an unrealistic goal. For genotyping the AR technique provides a fairly reliable approximation of PFGE genotyping results although the discriminatory power of the latter is clearly greater. The poor correlation between serogrouping and genotyping data and the diffusion of identical *L. pneumophila* clones in very distant areas of a geographical region warrant further investigation.

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