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# *Legionella pneumophila* in Cooling Towers: Fluctuations in Counts, Determination of Genetic Variability by Pulsed-Field Gel Electrophoresis (PFGE), and Persistence of PFGE Patterns▽

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**The concentrations of *Legionella pneumophila* in cooling towers may vary considerably over short periods of time, producing significant fluctuations throughout the year. Despite genetic variability, in small geographical areas the same indistinguishable pulsed-field gel electrophoresis patterns may be shared among different cooling towers and persist over time.**

The involvement of cooling towers in some community outbreaks of legionellosis has been demonstrated (3, 6, 8, 9, 11), and it has been suggested that there may be an association between high *Legionella* counts in cooling towers and the occurrence of an outbreak of legionellosis (12). Although not all environmental isolates are associated with human disease, the present guidelines aimed at preventing legionellosis have proposed ranges of risk involving different environmental insults based on *Legionella* counts (4). However, the fluctuations in *Legionella* counts over short periods of time have led some authors to question these risk ranges (1).

Since the recovery of a high inoculum of *Legionella* in a cooling tower is not necessarily related to an outbreak or sporadic cases of legionellosis, genetic studies are necessary to establish the link between environmental and clinical isolates. The "gold standard" technique for genotyping *Legionella* is the analysis of chromosomal restriction patterns by pulsed-field gel electrophoresis (PFGE). Isolation of *Legionella* strains from a cooling tower which are genetically indistinguishable from clinical isolates is considered to be clear evidence of the implication of the tower in an outbreak (1, 9). However, little is known regarding the genetic variability of *Legionella* within the same tower and in cooling towers from areas in the vicinity. Likewise, to our knowledge, no study has determined the persistence of molecular patterns in isolates in cooling towers. All these circumstances have epidemiological and legal consequences when assigning responsibility for an outbreak of legionellosis to a particular installation.

The main objectives of this study were to establish the fluctuations in *Legionella* counts over time and to determine the genetic variability and persistence of PFGE patterns in cooling towers.

Fifteen cooling towers (A to O) were selected within a radius of 3 km. Four of them (J, K, M, and N) shared the same main line for water distribution. The other cooling towers had a different domestic distribution system but were supplied by the same municipal water network. All the cooling towers followed the controls and hygiene measures required by Spanish regulations (RD 865/2003), and we were able to verify that the levels of disinfectant (sodium hypochlorite [0.2 to 0.5 ppm residual] in conjunction with the discontinuous dosage of nonoxidant biocide [dibromonitrilopropionamide]) were in accordance with legislation at the time of sampling. Cooling water samples were cultured for *Legionella* fortnightly over a 1-year period. The samples were concentrated by filtration, and four plates of GVPC agar (Oxoid, Wesel, Germany) were seeded from each sample: (i) direct sample with acid pretreatment and (ii) concentrated sample without pretreatment, (iii) concentrated sample with acid pretreatment, and (iv) concentrated sample with heat pretreatment. The plates were incubated at 36°C for 10 days in a moist $CO_2$-enriched atmosphere. *Legionella* spp. were identified by Gram staining and the pattern of nutritional requirements. An agglutination test (Oxoid) confirmed the identification of the species as *L. pneumophila* and determined the serogroups.

For chromosomal DNA subtyping 10 *Legionella* isolates were selected, whenever possible, for each sample. All isolates were analyzed by PFGE as described previously (10). According to the criteria of Tenover et al. (13), chromosomal PFGE patterns differing in one or more bands were considered distinct in this study.

Table 1 shows the results obtained for *Legionella* counts. *Legionella* spp. were isolated in 13 (86.6%) of the 15 towers with the fluctuations in *Legionella* counts in these towers ranging from <50 CFU/liter (detection limit) to 2,000,000 CFU/liter (Fig. 1). In 75.9% (284/374) of the samples analyzed, the *Legionella* counts were less than 100 CFU/liter, while counts greater than 10,000 CFU/liter were found in 15% (56/374) of the total. No correlation was found between volumetric and operational differences between cooling towers and bacterial counts. *L. pneumophila* serogroup 1 and serogroups 2 to 14 were isolated from 13 and 2 cooling towers, respectively (Table 2). These results confirm and expand those observed by other authors (1) and demonstrate that the concentrations of *L. pneumophila* in cooling towers may vary considerably over

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TABLE 1. Cooling towers and levels of colonization

| Cooling tower | Vol ($m^3$) | Evaporation ($m^3$/h) | No. of samples | | | | |
|---|---|---|---|---|---|---|---|
| | | | Analyzed | Positive | With level (CFU/liter) of colonization: | | |
| | | | | | >100–<1,000 | >1,000–<10,000 | >10,000 |
| A | 5 | 0.83 | 25 | 7 | 1 | 0 | 5 |
| B | 5 | 0.71 | 25 | 21 | 1 | 1 | 19 |
| C | 8 | 0.48 | 25 | 19 | 2 | 8 | 9 |
| D | 8 | 0.48 | 24 | 22 | 4 | 3 | 15 |
| E | 2 | 0.71 | 25 | 0 | 0 | 0 | 0 |
| F | 8 | 0.53 | 26 | 8 | 2 | 2 | 0 |
| G | 2 | 0.09 | 26 | 1 | 0 | 1 | 0 |
| H | 2 | 0.13 | 26 | 1 | 1 | 0 | 0 |
| I | 2 | 0.14 | 26 | 1 | 0 | 0 | 0 |
| J | 20 | 1.97 | 26 | 6 | 1 | 0 | 5 |
| K | 20 | 1.97 | 26 | 6 | 3 | 2 | 1 |
| L | 20 | 1.97 | 25 | 1 | 0 | 0 | 1 |
| M | 20 | 1.97 | 24 | 2 | 1 | 1 | 0 |
| N | 20 | 1.97 | 23 | 1 | 0 | 0 | 1 |
| O | 2 | 0.18 | 22 | 0 | 0 | 0 | 0 |
| Total | | | 374 | 96 | 16 | 18 | 56 |

short time periods, producing significant fluctuations throughout the year. These oscillations reflect the dynamism of these ecosystems in which numerous bacterial species, protozoa, and ciliates make up an adherent biofilm (5). These associations contribute to the amplification of the inoculum of *Legionella* spp. and make them resistant to the biocides used for cleaning and disinfecting these installations (2, 14). The fluctuations observed make it difficult to establish norms and guidelines to specify the minimum sampling periods to maintain a cooling tower under control and to avoid large outbreaks of legionellosis.

Sixteen chromosomal restriction patterns determined by PFGE were detected among the isolates from 13 positive cooling towers. The strains of *L. pneumophila* serogroup 1 exhibited 15 of the 16 different PFGE patterns, while all the strains of serogroups 2 to 14 showed the same PFGE pattern. Five of the 16 PFGE patterns were shared by different cooling towers (Table 2). As expected, shared PFGE patterns were observed in those cooling towers with the same main water supply, but some of the other cooling towers also exhibited shared PFGE patterns. Hypothetically, when a clone of *Legionella* from the municipal water network colonizes a cooling tower, it may undergo an adaptation process depending on its ability to respond to the environmental pressure. The environmental pressures leading to the adaptation response may be similar in

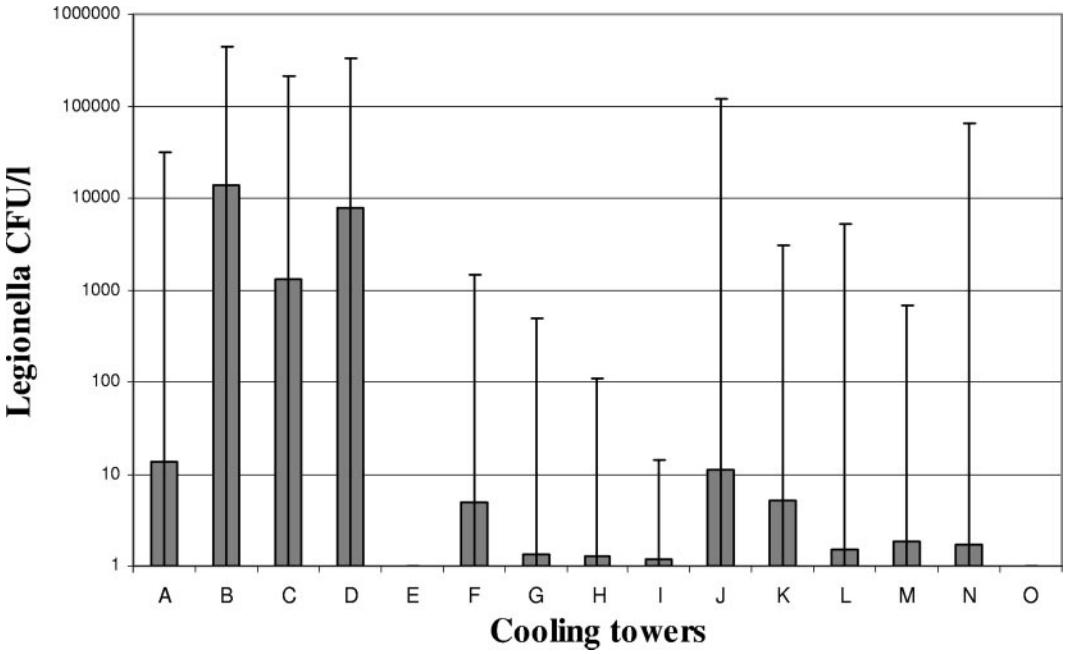


FIG. 1. Geometric mean *Legionella* counts in each cooling tower. Bars represent the standard deviations.

TABLE 2. Serologic identification of the serogroups of *L. pneumophila* strains and PFGE patterns found in each cooling tower

| Cooling tower | Serogroup(s) | PFGE pattern(s) |
|---|---|---|
| A | 1 | I |
| B | 1, 2 to 14 | I, II, III |
| C | 1 | IV, V |
| D | 1 | VI |
| F | 1 | VII |
| G | 1 | VIII |
| H | 1, 2 to 14 | I, II |
| I | 1 | IX |
| J | 1 | X, XI |
| K | 1 | X, XII, XIII |
| L | 1 | XIV |
| M | 1 | XV |
| N | 1 | XIII, XV, XVI |

small areas, which would increase the chance of finding similar PFGE patterns.

On comparing the PFGE patterns of the *Legionella* isolates from cooling towers which were positive more than once, it was found that these patterns persisted in each cooling tower, at least during the study period.

The concept of clonal variability is of great importance for the epidemiological studies performed when a community outbreak of legionellosis is declared (7). The variability among the isolates from different cooling towers facilitates the determination of the source responsible for an outbreak. Nevertheless, in small areas, some cooling towers can share some of their molecular patterns; therefore, a great number of facilities should be investigated when an outbreak is declared. Moreover, the clonal variability observed within the same cooling tower makes it necessary to undertake molecular analysis of a greater number of isolates from each one.

The persistence of PFGE patterns observed may also be a useful tool in epidemiological studies. Media response to a community outbreak of legionellosis may lead the owners of the installations to attempt disinfection with large quantities of disinfectants before samples are collected by the authorities for epidemiological and molecular studies. In these cases, strains of *Legionella* cannot be recovered from all the installations that were positive at the time at which the outbreak was declared. However, the persistence of these strains allows their recovery at a later date, thereby providing a resolution to many of the outbreaks which would otherwise have remained unsolved.

Although these cooling towers were not related to any outbreak, at least during the study, we believe that the results obtained may be extrapolated to other strains which have been related to clinical cases. This study offers a perspective on some epidemiological variables when investigating a community outbreak of Legionnaires' disease in a small geographical area. *Legionella* count variation, genomic variability, shared PFGE patterns, and persistence of *Legionella* PFGE patterns should be taken into account when cooling towers are the facilities under investigation.

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