Persistence of *Legionella* in hospital water supplies and nosocomial Legionnaires’ disease

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**Abstract**

The molecular epidemiology of clinical and environmental *Legionella* species isolates was studied in seven hospitals from 1989 to 2006. The number of environmental pulsed field gel electrophoresis (PFGE) patterns ranged from one to nine according to the hospital. Genomic PFGE pattern persistence was observed in 71% of the hospitals, even after 17 years in some hospitals, and the relationship between environmental and clinical isolates was established. The isolates associated with hospital-acquired Legionnaires’ disease corresponded to the persistent environmental PFGE patterns of *Legionella pneumophila* in potable water supplies.

**Introduction**

*Legionella* has frequently been recovered from potable water systems in hospitals. The colonization of hospital water has been linked to cases of hospital-acquired Legionnaires’ disease (Sabria et al., 2002). Studies of molecular typing by pulsed-field gel electrophoresis (PFGE) have been demonstrated to be useful in the investigation of nosocomial *Legionella* infections (Luck et al., 1998; Fry et al., 2000). Likewise, molecular studies of environmental *Legionella* isolates suggest that these bacteria have great molecular subtyping diversity (Chang et al., 1996; Fry et al., 2000; Sabria et al., 2001). Because of this genomic diversity and their wide distribution in aquatic environments, it is very common to isolate more than one clone of *Legionella* species in the water distribution systems of hospitals (Luck et al., 1998; Sabria et al., 2001). In a previous study, it was observed that 85% of the hospitals tested in Catalonia (north-east Spain) were colonized by *Legionella*, and every hospital presented its own PFGE patterns not shared by the other centers (Sabria et al., 2001).

Despite the persistence of PFGE patterns of *Legionella* in the water distribution systems of selected hospitals (Chang et al., 1996; Grattard et al., 1996; Marrie et al., 1999; Rangel-Frausto et al., 1999; Fry et al., 2000; Darelid et al., 2004), the relationship between these persistent subtypes and nosocomial Legionellosis has not been widely investigated (Visca et al., 1999; Oberdofer et al., 2007). In this study the genetic variability and stability of PFGE patterns of *Legionella pneumophila* isolates in the water distribution systems from seven hospitals are described. In addition, the relationship between the PFGE patterns exhibited by the environmental and clinical *L. pneumophila* isolates in five hospitals that reported cases of hospital-acquired Legionnaires’ disease is presented.

**Materials and methods**

During an 18-year period (1989–2006) the authors’ *Legionella* Laboratory has analyzed many clinical and environmental isolates of *Legionella* for molecular typing. The *Legionella* PFGE data belonging to the isolates in this culture...
collection from seven hospitals were reviewed, which comprised 222 environmental (range: 7–92 isolates/hospital) and 28 nosocomial clinical isolates (range: 1–13 isolates/hospital). The isolation period ranged from 6 to 17 years for each hospital. The clinical isolates were derived from five of the seven hospitals, with each isolate corresponding to a single patient.

Genotyping

For chromosomal DNA subtyping (PFGE), genomic DNA was prepared as described previously with some modifications (Sabria et al., 2001). Fragments of DNA were separated in a 1% agarose gel prepared and run in 0.5 × Tris-borate-EDTA buffer (pH 8.3) in a contour-clamped homogeneous field apparatus (CHEF DR II system; Bio-Rad, Ivry sur Seine, France) with a constant voltage of 5 V cm⁻¹ and increasing pulse times (5.6–50.6 s) at 14 °C for 24 h. The lambda ladder PFGE marker (New England Biolabs) was included as a molecular weight marker.

Band pattern analysis was carried out by the unweighted pair group method using arithmetic averages (UPMGA) with the Finger Printing II software (Bio-Rad, Irvin, France). Isolates with a PFGE pattern that differed by ≥1 band were considered to belong to different PFGE genotypes and were designated with capital letters.

Results

Environmental genomic variability

All environmental isolates analyzed were L. pneumophila, except in one hospital (hospital V) where Legionella non-pneumophila was also typed. PFGE showed a high degree of genomic variability among the environmental isolates of Legionella. The number of environmental PFGE patterns varied according to the hospital, ranging from one to nine indistinguishable PFGE patterns (Tables 1 and 3). Each hospital produced its own environmental PFGE patterns, and they were not shared with the other centers.

Environmental persistence

The persistence of the different PFGE patterns recovered from the water distribution systems of the seven hospitals is shown in Tables 1 and 3. The environmental positive sampling intervals in each hospital ranged from 6 (hospital VII) to 16 years (hospital I), except in hospital VI where a second sample was not available.

Table 1. Distribution of environmental isolates PFGE patterns

<table>
<thead>
<tr>
<th>Hospital</th>
<th>Study period</th>
<th>Legionella species</th>
<th>PFGE pattern</th>
<th>Environmental persistence (Years)</th>
<th>Year of isolation (n)*</th>
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<td>L. pneumophila. nonsg. 1</td>
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n, number of isolates that present the PFGE patterns.
Clinical genomic variability

All the clinical isolates analyzed belonged to *Legionella pneumophila* species (Tables 2 and 3). *Legionella* infection in each hospital was associated with a unique PFGE pattern, except in hospital IV where two different PFGE patterns were recovered from two patients (patient 1, PFGE pattern N and patient 2, PFGE pattern P; both PFGE patterns corresponding to distinct serogroups of *L. pneumophila*).

Environmental and clinical correlation

In five hospitals, the isolates associated with hospital-acquired Legionnaires’ disease exhibited the PFGE pattern corresponding to the environmental *Legionella* PFGE pattern persisting longest in the water distribution system (Table 3). The other *Legionella* PFGE patterns found in the water supply systems did not cause any documented infectious episode. In hospitals I and VI the isolates associated with infection in 1989 and 2003, respectively (no environmental samples were available at this time in these hospitals), were later related to the environmental isolates, suggesting persistence of the environmental subtypes for 18 years (hospital I) and 8 years (hospital VI), respectively.

Discussion

*Legionella pneumophila* colonizes water pipes in a large number of facilities. In this study the large genomic variability among environmental isolates of *L. pneumophila* from the water distribution systems of hospitals was demonstrated. The fact that each hospital exhibited its own PGFE patterns not shared with other PGFE patterns from other hospitals confirmed a previous observation (Sabria et al., 2001). Moreover, despite the variability of genetic subtypes observed during different sampling periods, some PFGE patterns persisted longer than 17 years. It is noteworthy that most of the isolates associated with the infectious episodes corresponded to the PFGE pattern persisting longest in the hospital water environment.

The large PFGE genomic variability demonstrated in this study has not been observed by other authors. Using amplified fragment length polymorphism (AFLP), other researchers (Darelid et al., 2004) identified the same AFLP pattern in three out of six Swedish hospitals located within an area of 100 km. Lawrence et al. (1999) reported the wide distribution of a particular PFGE pattern within the Paris area, despite the similarity criteria used by these authors being less discriminatory than that used in the present study.
The factors causing greater or lesser genetic diversity in some areas are unknown. Moreover, the factors related to the presence of one or several clones, or those that influence the appearance or disappearance of specific subtypes, remain to be explained, as does the persistence of some clones in the environment. Some authors have attributed the changes in \textit{L. pneumophila} isolates typed (appearance/disappearance) to the disinfection measures used (Struelens et al., 1992; Darelid et al., 2004; Perola et al., 2005; Triassi et al., 2006).

The quality of the water or continuous disinfection procedures of the potable water supplies in hospitals may influence the observed genomic variability. Different disinfection methods such as superheat-and-flush, hyperchlorination and copper–silver ionization were used in the hospitals studied during the study period, with a concomitant increase in the variation of PFGE patterns observed. In spite of all these different methods, the molecular PFGE patterns related to the clinical cases were maintained throughout the study period.

It is assumed that \textit{Legionella} arrives in potable water distribution systems from the mains of the cold water network at concentrations undetectable by routine laboratory methods. Once the system has been colonized in the presence of favourable conditions the bacteria may live free in the planktonic phase or as an intracellular parasite of protozoa within the complex microbial structure of the biofilm. The presence of a biofilm throughout the water distribution system facilitates the growth of \textit{Legionella} and interferes with the environmental stressful conditions. Therefore, it is possible for different PFGE patterns of \textit{Legionella} to cohabit over time in the water distribution systems, albeit at almost undetectable levels.

A change in the environment, such as may occur on application of disinfection measures, causes the ecological niche to destabilize and the inocula of the predominant subtypes in the water to diminish, allowing other subtypes, previously in the minority, to overgrow. The fact that the disinfection measures used were more effective in the planktonic phase than in the biofilm and could not eradicate the microorganisms present in the water distribution systems could be the reason for the persistence of colonization.

The acquisition of mechanisms of resistance in \textit{Legionella} to disinfection measures such as hyperchlorination or copper/silver ionization has been reported in a few studies (Kuchta et al., 1985; Mietzner et al., 2005). On the contrary, the associations protozoa–\textit{Legionella} and \textit{Legionella}-biofilm have been demonstrated to be more resistant to disinfection measures either by the ability of \textit{Legionella} to grow within a protozoa or enter in a viable but not-culturable (VBNC) status (Kilvington et al., 1990; Hwang et al., 2006). Thus, when starvation conditions are reduced, the \textit{Legionella} again colonizes the water system.

It remains unknown as to which factors cause some environmental subtypes to produce infection where as other subtypes do not. In this study, clinical isolates from the same patients showed the same PFGE pattern (data not shown), whose high prevalence in each water distribution system and persistence over time showed a better adaptation to the ecological niche than other \textit{Legionella} subtypes. Whether it is the greater virulence of these subtypes or the simple fact of being in higher numbers and persisting longer that leads to their association with clinical cases remains under debate.

It is known that environmental \textit{Legionella} isolates can express different degrees of virulence, even though their relationship with the appearance of episodes of infection is controversial (Bollin et al., 1985; Luck et al., 1994; Marrie et al., 1999). The authors have observed that hospitals colonized by \textit{L. pneumophila} strains with greater cytopathogenicity have a trend to present a larger number of cases of hospital-acquired Legionnaires’ disease (García-Nuñez et al., 2000).

Because molecular typing is performed on a random number of environmental isolates, the possibility of other subtypes coexisting within the environment of those selected cannot be ruled out. In the hospitals studied, an average of 9.3 environmental isolates were analyzed per year. Therefore, if more isolates had been available in some samplings, it is likely that a higher frequency of hospitals containing more PFGE patterns or demonstrating genomic persistence would have been observed. In a long-term retrospective study such as the present one, it has been taken into account that since 2001 Spanish regulations require environmental surveillance in hospitals. Before this law, surveillance was performed according to the criteria of each hospital and the degree of awareness about the subject. Another limitation may be the variations in the surveillance systems and methods of \textit{Legionella} detection from hospital to hospital, thereby not guaranteeing that all nosocomial pneumonias were tested for \textit{Legionella} during the study period. Likewise, the low productivity of sputum cultures and the fact that this was not a study of active legionellosis surveillance made it difficult to obtain more clinical isolates to evaluate.

In conclusion, this study demonstrated a high genomic variability of \textit{Legionella} subtypes in the water distribution system of seven hospitals, and the persistence over time of those \textit{Legionella} subtypes associated with most of the \textit{Legionella} infection episodes. These observations may justify a periodic genotype analysis of environmental isolates to determine the prevalence of more colonizing or predominant \textit{Legionella} subtypes as well as the impact of disinfection measures on them.

\section{Acknowledgements}

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References


