Molecular typing of the *Legionella pneumophila* population isolated from several locations in a contaminated water network

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**Abstract** The RAPD (random amplified polymorphic DNA) technique has been developed for the molecular typing of *Legionella* in order to characterise the populations of hot water systems. During this study, 22 primers were tested and the four most informative ones were selected. The optimisation of the PCR conditions allowed the setting up of a powerful discriminative genotyping method. Moreover, the definition of a quality management method allowed definition of the key steps and the number of replicates to ensure reproducibility of the RAPD pattern. The RAPD was used to study the hot water network of a building. *Legionella* colonies (91) were isolated from seven locations and genotyped. The diversity of the population in one sample could vary from one to seven different strains. The study of the traceability showed that, in most of the cases, different populations could be present at different locations of the same network.

**Keywords** Hot water; *Legionella pneumophila*; molecular typing

**Introduction**

Legionellosis has become a major preoccupation in recent years, and health and safety requirements for water consumers have significantly increased. For water suppliers, the control of the sanitary risk is extremely important and demands knowledge of the microbiology of the installations. In this field, it is critical to have expertise on molecular tools that allow establishment of clonality links between *Legionella* strains.

Several molecular typing methods, for example, restriction length fragment polymorphism (RFLP), pulse field gel electrophoresis (PFGE), ribotyping and random amplified polymorphic DNA (RAPD), aim to distinguish strains of microbial species (Smith and Cantor, 1987; Stull *et al*., 1988; Welsh and McClelland, 1990; Williams *et al*., 1990; Romling *et al*., 1992). These techniques are used in many applications, including epidemiology, hospital infection control and environmental microbiology, as well as population and molecular genetics evolution. Most of these techniques are laborious, expensive and not suited for studying a large number of strains. On the other hand, RAPD has received considerable attention as a molecular typing method, due to its simplicity, sensitivity, flexibility and relatively low cost. Compared to the other DNA typing methods, RAPD appears to be one of the most discriminating and sensitive techniques, and has been successfully applied for the characterisation of several organisms (Cave *et al*., 1994; Morais *et al*., 1997; Arias *et al*., 1998; Franklin *et al*., 1999; Vogel *et al*., 2000). Furthermore, the RAPD technique requires only very small quantities of DNA and can be applied to any species from which DNA can be prepared. It does not require prior knowledge of the biochemistry or molecular biology of the species being studied (Welsh and McClelland, 1990). Consequently, RAPD is particularly well adapted to the study of bacteria isolated from the environment.

During this study, the RAPD technique was developed for the molecular typing of *Legionella* and used to study the *Legionella* population isolated from a hot water network of a building located near Paris (France).
Materials and methods

Bacterial strains

Legionella strains were isolated from the hot water network of a building located near Paris (France) using the French standard method (as described in the AFNOR NT 90-431) and then were sub-cultured on BCYE (buffer charcoal yeast extract) medium (AES, Rennes, France) for 2 d at 37°C. Serogroups were examined using a latex Legionella test (Oxoid).

RAPD fingerprinting

The total DNA of Legionella strains was prepared using the lysis solution Instagen Matrix (Bio-Rad, Hercules, CA) according to manufacturer’s instructions, with 2 μL of the solution being used as PCR template. Primers used are detailed in Table 1.

The RAPD PCR reactions were carried out in 25 μL reaction volumes under the conditions shown in Table 2. The temperature cycling program used with a Perkin–Elmer GeneAmp 9600 Themocycler was as follows: four initial cycles of 95°C for 5 min, primer hybridisation temperature for 5 min, 72°C for 5 min, followed by 30 cycles of 94°C for 1 min, primer hybridisation temperature for 1 min and 72°C for 2 min. An extension step at 72°C for 10 min was included at the end of the cycle. Each primer hybridisation temperature is indicated in Table 2.

Reaction products were analysed by electrophoresis on 2% (w/v) agarose 1000 (Life Technology) gel at constant voltage (200 V for 3.5 h) and visualised by ethidium bromide staining (1 μg/mL) (Sigma Chemical, St Louis, Mo.). A molecular size marker 100 pb DNA Ladder (Life Technologies, Gibco BRL, Paisley, United Kingdom) was used for reference. Gels were photographed under UV light with a Gel Doc 2000 (Bio-Rad, Hercules, CA) and analysed using Bionumerics software (Applied Maths, Sint-Martens Laten, Belgium). Each RAPD profile was defined by the presence or absence of fragments at particular position on the gel. Simple matching Dice coefficients were calculated and compared with one another (Dice, 1945). The relatedness of DNA samples was estimated as the proportion of shared fragments between samples. The resulting matrix of pair-wise distances was used to construct a phenogram employing the UPGMA (unweighted pair-group method in arithmetic mean). Band tolerance (maximum tolerance in percent of the curve to match bands) was 1%.

Table 1 Primers used to perform the RAPD technique

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oligo C</td>
<td>5’-AGT CAG CCA C-3’</td>
<td>Bansal and McDonell, 1997</td>
</tr>
<tr>
<td>1254</td>
<td>5’-CCG CAG CCA A-3’</td>
<td>Pacheco et al., 1996, 1997a,b, 1998; Osek, 1998</td>
</tr>
<tr>
<td>ERIC21</td>
<td>5’-AAG TAA GTG ACT GGG GTG AGC G-3’</td>
<td>Van Belkum et al., 1996; Zietz, 2001</td>
</tr>
<tr>
<td>SK2</td>
<td>5’-CGG CGG CGG CGG CGG-3’</td>
<td>Lo Presti et al., 1998, 1999, 2000</td>
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Table 2 PCR conditions for RAPD technique on Legionella

<table>
<thead>
<tr>
<th>PCR conditions</th>
<th>Oligo C</th>
<th>1254</th>
<th>ERIC21</th>
<th>SK2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taq Stoffel Buffer (10x) (Applied Biosystems, Foster City, CA)</td>
<td>10 mM</td>
<td>10 mM</td>
<td>10 mM</td>
<td>10 mM</td>
</tr>
<tr>
<td>MgCl₂ (Applied Biosystems, Foster City, CA)</td>
<td>3 mM</td>
<td>2 mM</td>
<td>2 mM</td>
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<tr>
<td>AmpliTaq® DNA Polymerase, Stoffel Fragment (Applied Biosystems, CA)</td>
<td>2 u</td>
<td>2 u</td>
<td>2 u</td>
<td>2 u</td>
</tr>
<tr>
<td>dNTP (Applied Biosystems, Foster City, CA)</td>
<td>200 μM</td>
<td>100 μM</td>
<td>100 μM</td>
<td>200 μM</td>
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<tr>
<td>Primer (Genset Oligo, France)</td>
<td>0.8 μM</td>
<td>0.5 μM</td>
<td>1.0 μM</td>
<td>1.0 μM</td>
</tr>
<tr>
<td>DNA</td>
<td>10 ng</td>
<td>10 ng</td>
<td>10 ng</td>
<td>10 ng</td>
</tr>
<tr>
<td>Primer hybridisation temperature</td>
<td>36°C</td>
<td>36°C</td>
<td>25°C</td>
<td>30°C</td>
</tr>
</tbody>
</table>
Results and discussion

Development of the RAPD technique

Primers (22) described in the literature were tested on five Legionella strains isolated from the environment (two strains of L. pneumophila sg 1, two L. pneumophila sg 2-14 and one was a Legionella ssp.). The primers were evaluated for the number of PCR fragments generated between 200 bp and 2,000 bp and for the intensity of bands. The four most informative primers were selected: Oligo C, 1254, ERIC2I and SK2. The PCR conditions were optimised in order to allow the best readable patterns. The reproducibility of the technique was assessed for the four primers selected. For each strain, five independent DNA extractions were performed on five isolated colonies. Each DNA preparation was used as the matrix for three independent PCR reactions. In total, 15 independent PCR assays with each primer were performed. The percentage of similarity ranged from 87% to 95%, depending on the primer tested (Figure 1).

Furthermore, in order to overcome the reproducibility problems often found with the use of the RAPD technique and to ensure the validity of RAPD patterns, a quality management method was defined. For each strain, two independent DNA extractions were performed on isolated colonies. Each DNA preparation was used as the matrix for three independent PCR reactions. Six independent PCR assays were loaded on the same gel, and the reproducible patterns were selected to be re-loaded on a terminal gel for the comparison.

Typing of Legionella in the hot water system of a building

The method developed was used to study the Legionella population isolated from the hot water network of a building located near Paris (France). The hot water network was made up of eight separated networks with a production unit that distributed hot water to the flats (apartments) by a closed circuit. Unused hot water returned to the production unit. During this study, 20 samples were taken simultaneously for Legionella analysis. The level of Legionella in the network varied from $10^2$ to $10^5$/L. L. pneumophila, and a total of 178 colonies taken from the plates were frozen. After that, the network was submitted to a disinfection procedure.

The RAPD typing method was used to study the diversity of the ecosystem. To our knowledge this was the first time that the composition of the Legionella population in a hot water network had been studied in order to evaluate whether the ecosystem was composed of a large clonal diversity, or whether, on the contrary, there were only a few Legionella

Figure 1 Reproducibility of the method: RAPD electrophoretic patterns of a L. pneumophila strain isolated from the hot water network obtained after 15 independent PCR
major strains in the system. *Legionella* colonies were isolated from two hot water production units (B and C) and also from five flats (flats 73, 128, 536 and 1,000). Some of these flats were related to the two production units B and C. The number of colonies isolated and the traceability between the flats and the hot water production units are described in Figure 2. The study revealed that some samples had a very homogeneous population, whereas, for other samples, up to seven different types could co-exist (Figure 2). These results were favourable to the position that, in case of an environmental investigation, it was necessary to isolate a large number of colonies to clearly determine the composition of the sample.

In order to establish the clonality links between different isolates taken from the contaminated hot water network at different points of the distribution system, the RAPD types of all isolated strains were compared. The results showed that the population of *Legionella* isolated from the flats was not exactly the same as the one isolated from the water heater. The differences could be attributed to the presence of a biofilm or to the variation of the population balance according to the temperature or other physical conditions of the network. In this case, it was not easy to establish a relation.

Genotyping could be used to improve the surveillance of the water quality by comparing the genotypes of the strains isolated from networks to the ones of strains which are known to be pathogenic and often retrieved in cases of legionellosis. This approach should require a database of the pathogenic strains most often retrieved in a region.

**Conclusions**
The RAPD typing method developed for *Legionella* in this study was very reproducible and provided very useful information on the population in a water network. The results obtained brought some insight into the estimation of the diversity of the population and on the number of colonies to be isolated from the environment in order to evaluate this

![Figure 2](https://iwaponline.com/wst/article-pdf/50/1/281/421346/281.pdf)

**Figure 2** RAPD types of the 91 colonies isolated from the hot water system of the building. (Common types for several samples are presented in bold. SG means serogroup)
diversity. The comparison of the RAPD types of the environmental strains and the collection of pathogenic strains could be a useful way to evaluate the sanitary risk in the network.

References


