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Research article

Development of a DGGE method to explore *Legionella* communities

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ABSTRACT

Legionella risk assessment is nowadays based on the presence and concentration of either *Legionella pneumophila or Legionella spp*. Many species of *Legionella* can cause Legionnaires' disease, indeed about half of the known species have been associated with infection. The aim of this work was to develop a method to assess the composition of the *Legionella* species community in an environmental sample in order to have a better understanding of the contamination of the ecosystem by pathogenic strains.

The method is based on the comparison of PCR-DGGE profile of DNA sample with a database consisting in DGGE profiles of *Legionella* species. Such a database includes all pathogenic *Legionella* strains. In order to homogenize and normalize the different DGGE fingerprint, a reference marker has been built and added during DGGE gel analysis. This study gives a valuable advance in the methods available for the understanding of *Legionella* contamination of water environments.

1. Introduction

Legionellae are facultative intracellular gram-negative bacteria present in aquatic environments, such as interstitial water and groundwater (Rowbotham, 1980). Aerosolized water from cooling tower, domestic hot water devices, or nebulizers can also contain *Legionella* bacteria (Krøjgaard et al., 2011; Lee et al., 2010). Inhalation of contaminated water containing *Legionella* cells can lead to legionellosis or Legionnaires' disease, corresponding to an atypical pneumonia that can be fatal. During the last decade, cooling towers have been identified or strongly suspected as the source of community outbreaks of Legionnaires' disease (Sabria et al., 2006; Sala Ferré et al., 2009).

Today, the genus *Legionella* comprises over 60 species (http://www.bacterio.net/legionella.html) Among them, more than 20 were isolated at least once from patients and are considered as pathogens for humans (Table 1) (Benson and Fields, 1998; Fields et al., 2002; Gomez-Valero et al., 2019; Helbig et al., 1995; Percival and Williams, 2014). *L. pneumophila* is the major cause of legionellosis in Europe and in USA, accounting for more than 91% of the cases worldwide (Breiman and Butler, 1998; Reingold et al., 1984; Yáñez et al., 2005). Other species have also been involved in human infections such as *L. longbeachae*, *L. bozemanii*, *L. micdadei*, *L. dumoffii*, *L. feeleii*, *L. wadsworthii* and *L. anisa* (Fang et al., 1989; Reingold et al., 1984). *L. longbeachae*, in particular was responsible for approximately 30% of Legionellosis cases in Australia and New Zealand (Lanser et al., 1990; Ross et al., 1997; Whiley and Bentham, 2011) and nearly 50% in South Australia (Cameron et al., 1991; Yu et al., 2002) and Thailand (Phares et al., 2007). In terms of risk assessment, different national legislations are only based on the monitoring of *Legionella spp* and *L. pneumophila* and the diversity of *Legionella* populations is not considered.

For example in cooling towers in the Netherlands, United Kingdom or France, an alert level is activated from a contamination of 1000 CFU.liter^{-1} of *Legionella spp* (Bartram et al., 2007; Circulaire DPPR/SEI/BAMET/PG/NA, n.d.; Krøjgaard et al., 2011). In this case, preventive and corrective actions are applied, consisting in a treatment of the water system by thermal or chemical disinfection. The use of biocide can cause some environmental problems. Indeed, both antibacterial biocides and metals retrieved in the water of cooling tower can promote a co-selection of resistante strains to biocides and metal but also antibiotic resistance (Pal et al., 2015).

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Table 1. *Legionella* species, *Melting temperature and percentage of GC.*

Species	ATCC	Tm °C[a]	% of GC[b]
L. adelaidensis		80.5	51
L. anisa*	35292	80.8	55.1
L. beliardensis		80.7	55
L. birminghamiensis*	43702	*80.4*	*54.6*
L. bozemanii*	33217	81.2	56.1
L. brunensis		80	53
L. busanensis		80.5	54
L. cherrii		80.1	53
L. cincinnatiensis*	43753	80.2	54.1
L. drancourtii		80.2	52
L. drozanskii		80.3	53
L. dumoffii*	33279	80.4	54.6
L. erythra*	35303	81.5	56.6
L. fairfieldensis		80.3	53
L. fallonii		81	55
L. feeleii sg1*	35072	80.5	54.6
L. geestiana		80.8	54
L. gormanii*	33297	80.5	55.1
L. gratiana	49413	80.2	54.1
L. gresilensis		80.2	53
L. hackeliae sg1*	35250	80.1	54.3
L. impletisoli		80.6	53
L. israelensis		80.9	54
L. jamestowniensis		81.5	55
L. jordanis*	33623	80.9	55.3
L. lansingensis*	49751	80.8	55.3
L. londinensis	49505	81.1	55.8
L. longbeachae*	33462	80.5	54.6
L. lytica		80.9	53
L. maceachernii*	35300	80.4	54.6
L. micdadei*	35218	80.6	55.1
L. moravica		80.6	53
L. nautarum		80.9	53
L. oakridgensis*	33761	80	53.6
L. parisiensis*	35299	80.4	54.6
L. pittsburghensis		79.9	51
L. pneumophila sg6*	33215	80.7	55.1
L. quateirensis		80.9	53
L. quinlivanii	43830	80.9	55.6
L. rowbothamii		79.9	51
L. rubrilucens	35304	81.5	56.6
L. sainthelensis*	35248	80.5	54.6
L. santicrucis	35301	80.2	54.1
L. shakespearei		79.8	51
L. spiritensis	35249	80.9	55.6
L. steigerwaltii	35302	79.8	53.6
L. taurinensis		81.5	54
L. tucsonensis*	49180	81.5	56.6
L. wadsworthii*	33877	80.4	54.6
L. waltersii	51914	***79.7***	***53.1***
L. worsleiensis		80.8	52
L. yabuuchiae		80.8	52

* Pathogenic species.
Species used in this study are indicated in bold.
Tm has been calculated on amplified sequence without the GC clamp.
[a] Tm were calculated with Primo Melt 3.4 software http://www.changbioscience.com/primo/primomel.html.
[b] The of GC% is calculated manually from the part of the amplified sequence without GC clamp.

Furthermore, the composition of the *Legionella* community in water networks was recently well documented (Dilger et al., 2018; Lesnik et al., 2016; Zhang et al., 2017; Peabody et al., 2017). However, the techniques applied to these studies such as metagenomic strategies were incompatible with a monthly monitoring of cooling tower installation in terms of cost, time and expertise required. Among the available techniques, PCR-DGGE (Denaturing Gradient Gel Electrophoresis) method has been considered for a long time, as a suitable technique, being cheap (less than 10 dollars per sample), simple to use, rapidly completed (24 h) and reliable. However the main drawback of this technique is the complexity of the gel analysis. Indeed, gels usually present numerous bands and each band can correspond to several species. The bacteria identification requires extraction and sequencing of the bands leading to a longer and more expensive global method. Thus, PCR-DGGE strategy is mainly described in applications with poor bacterial diversity (Andorrà et al., 2008).

In this study, we propose the DGGE method for a direct first approach (without sequencing) to access the *Legionella* community structure in complex environmental samples. The method is based on the amplification of the sample by a semi-nested PCR leading to the reduction of the number of bands per gel, followed by the sample DGGE gel profile analysis (Huang et al., 2017). The gel profile is compared to a gel profiles database containing all pathogenic *Legionella* species. The comparison is possible through the normalization of the different gels using a home made reference marker. The proposed approach was tested on a cooling tower water sample.

2. Materials and methods

2.1. Legionella species

Twenty eight strains of *Legionella* have been used in this study and are listed in Table 1. Strains were kindly donated by the French Reference Centre for *Legionella* in Lyon. DNA extraction from bacteria was performed using QIAamp DNA minikit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The purified DNA was recovered in 50 µL of EB buffer (Qiagen, Hilden, Germany) and frozen at -20 °C until analysis.

2.2. Water sample

Water samples of cooling tower were collected in 1 L sterile bottles. The samples were filtered through 0.45µm polycarbonate filters. DNA was recovered from filters using Aquadien extraction kit (Biorad, Hercules, CA).

2.3. PCR conditions

2.3.1. Primers used

A semi-nested PCR specific to the *Legionella* strains 16s rRNA gene was used. The first amplification, with primers Leg 225 (5′-AAGATTAGCCTGCGTCCGAT-3) and Leg 858 (5′-GTCAACTTATCGCGTTTGCT-3′), produced a 654bp DNA fragment (Miyamoto et al., 1997).

The second amplification was performed with the primer V3F (5′-ACTCCTACGGGAGGCAGCAG-3′) and V3R spanning a 200 bpfragment of V3 region (5′-TTACCGCGGCTGCTGGCAC-3′). Sequences of these primers derived from W49 and W104 primers described by Wéry et al. (2008).

A GC- Clamp at the 5′end was added to the forward primer to avoid total denaturing of DNA during DGGE migration. This primer was called V3F-GC (5′-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG-ACTCCTACGGGAG GCAGCAG-3′).

2.3.2. Semi-nested PCR conditions

PCR was performed in 0.5 ml tubes using an Eppendorf thermo cycler® (Eppendorf, Le Pecq, France). The Taq polymerase was the

KAPA2G™FastHotStart (Clinisciences, France). The 25 μL PCR mixture contained: 5μL of 5x Kapa2G buffer (with1.5mM $MgCl_2$), 200μM of dNTP, 0.5μM of each primer, 0.5 units of KAPA2G FastHostStart DNA polymerase, and 1 μL of template DNA (1μL of total purified DNA for the first step and 1μL of the purified first-step PCR product at a tenfold dilution for the second amplification).

The first step (with Leg 225 and Leg 858) was performed with an initial denaturation at 95 °C for 2 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 15 s, extension at 72 °C for 30 s, and a final extension at 72 °C for 5 min.

The second PCR step (with V3F-GC and V3-R) was performed with an initial denaturation step at 95 °C for 2 min, followed by 30 cycles of denaturation at 95 °C for 15 s, annealing at 61 °C for 15 s, extension at 72 °C for 2 s and a final extension at 72 °C for 5 min.

2.4. DGGE migration

DGGE was carried out using the CBS-DGGE 2000 system (C.B.S. Scientific Co., Inc, USA). Tweelve μL of PCR product, containing 3μL of 6X loading Dye/SDS solution (Fermentas, Germany) were loaded onto a 1-mm-thick gel. The gel was composed of 10 % bispolyacrylamide (37.5:1) in 1X TAE (40 mM Tris-acetate pH = 7.4, 20mM sodium acetate, 1 mM Na_2-EDTA) forming a 30–60% linear denaturing gradient (100% denaturant solution containing 7M urea (Sigma- Aldrich, France) plus 40% formamide (Sigma- Aldrich, France)). Electrophoresis was performed at constant voltage, 90 V, and temperature, 60 °C for 18 h. Gels were then stained for 1 h in 1.25X TAE containing 1X SYBR Gold (Invitrogen, USA) and photographed.

2.5. Marker production

For marker production, 1μL of the purified first-step PCR product of each species (see section 3.1) was mixed with 14 μL of water. Five microliters of this mixture were used as a template for the second amplification using primers V3F-GC and V3R. The reference marker was loaded at the gel extremitie of each DGGE experiment.

2.6. Sequencing of DGGE bands and sequence analysis

Bands were excised from the DGGE-gel, transferred into 200 μL of elution buffer EB and incubated for 3 h at 37 °C to allow diffusion of DNA. Five microliters of diffusion product were used for PCR amplification with V3F and V3R primers as follow: one cycle at 95 °C for 2 min, followed by 35 cycles at 95 °C for 15 s, 55 °C for 15 s, and 72 °C for 2 s and a final extension cycle at 72 °C for 10 min.

PCR products were purified using PCR and DNA Fragment Purification Kit (Neobiotech, France) and were sequenced by GATC Company (France).

Sequence identification was performed by BLAST (http://blast.ncbi.nlm.nih.gov). Melting temperatures of PCR fragments were determined using Primo Melt 3.4 software http://www.changbioscience.com/primo/primomel.html. Tm were calculated on amplified sequence without the GC clamp.

2.7. Image and statistical analysis

DGGE profiles were obtained under UV illumination with Molecular Imager Gel Doc XR System (Biorad, France). There were analyzed with FPQuest software (BioRad, France). Similarity indexes were calculated for each pattern, and between patterns using the Pearson coefficient (Seksik et al., 2003).

The linear correlation between multiple variables (Scatterplot matrice) was performed using R (software 3.2.2).

2.8. Quantitative PCR

Quantitative PCR was performed on the iCycler IQ apparatus (Biorad, Hercules, CA) using the IQ-Check *Legionella* kit, according to the manufacturer's instructions,. Protocol of quantification by PCR followed the NF T90-471 standard.

3. Results

The method developed for specific identification of *Legionella* species consisted in the production of a DGGE profile of DNA extracted from a water sample, followed by the identification of the bands obtained from the gel migration. This identification was possible thanks to the use of a DGGE marker and the normalized migration distance analysis.

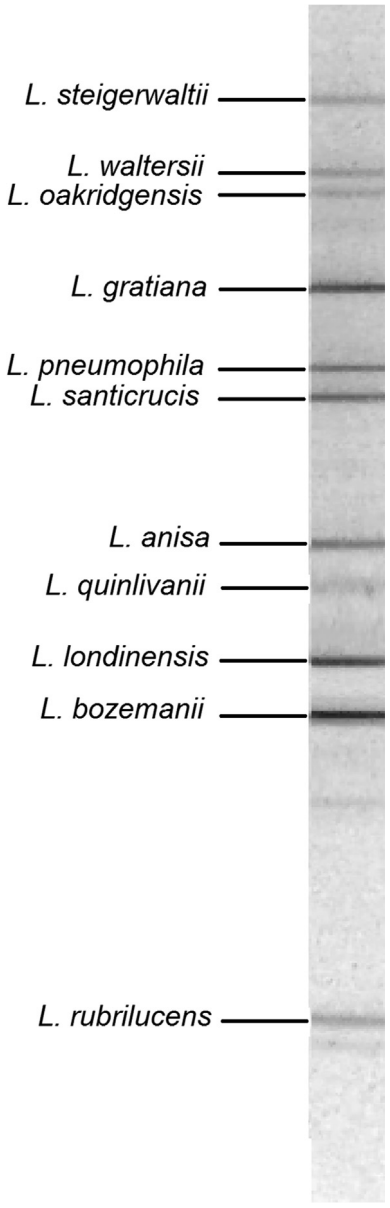


Figure 1. DGGE –reference marker. Reference marker was obtained by PCR using V3F-GC and V3 R primer and as template a mixture of the purified first-step PCR products (Leg 225 and Leg 858 primers) of *L. anisa, L. bozemanii, L. gratiana, L. londinensis, L. oakridgensis, L. pneumophila, L. quinlivanii, L. rubrilucens, L. santicrucis, L. steigerwaltii, L. waltersii.*

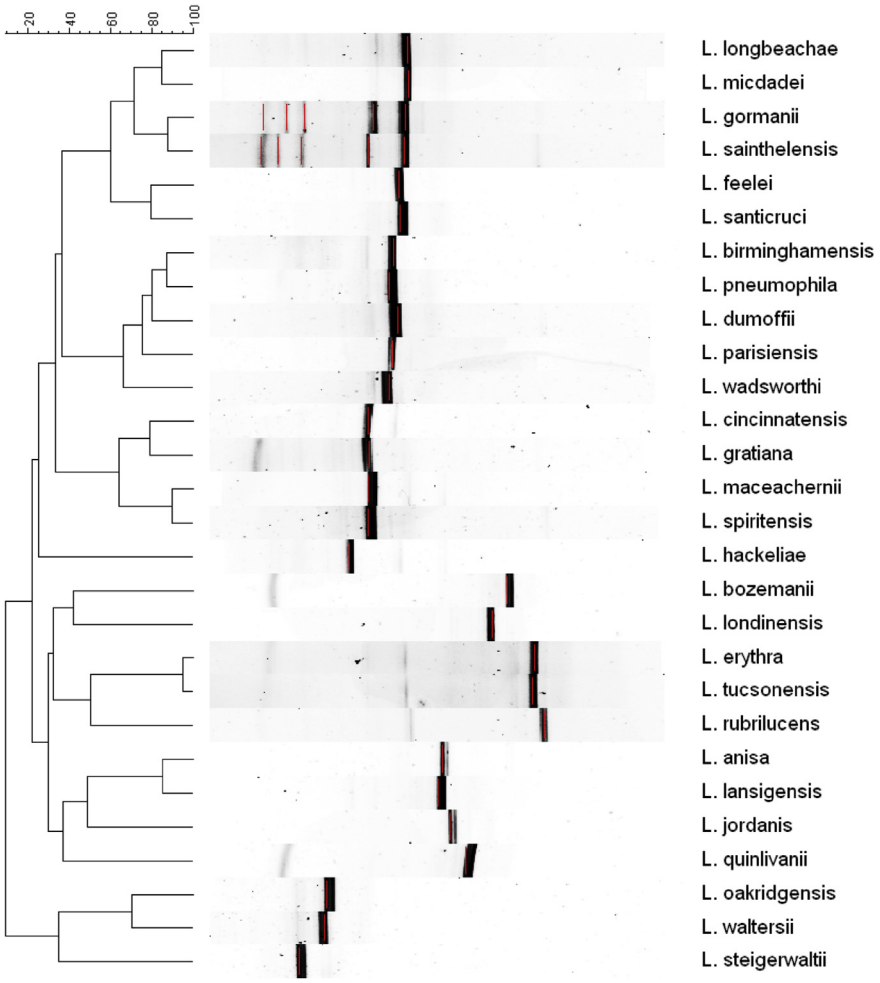


Figure 2. Dendogram showing the normalized band position of *Legionella* reference strains. Hierachical cluster was performed using Pearson coefficient to calculate similarities with the unweighed pair-group method using average linkages (UPGMA).

3.1. Choice of Legionella species for the DGGE marker

Reference marker was obtained by PCR using V3F-GC and V3 R primers on a template consisting in a mixture of the purified first-step PCR products (Leg 225 and Leg 858 primers) of *L. anisa, L. bozemanii, L. gratiana, L. londinensis, L. oakridgensis, L. pneumophila, L. quinlivanii, L. rubrilucens, L. santicrucis, L. steigerwaltii, L. waltersii.*

A reference DGGE marker has been produced in order to align and normalize the DGGE profiles of the samples. This marker is composed of bands originated from a selection of *Legionella* species according to: (i) specific structural properties, such as the melting temperatures (MT) and GC percentage (% of GC). Table 1 shows these properties for 52 *Legionella* strains; (ii) strain pathogenicity. Taking into account a wider range of MT, % of GC and the pathogenicity, the following 11 strains were selected: *L. anisa, L. bozemanii, L. gratiana, L. londinensis, L. oakridgensis, L. pneumophila, L. quinlivanii, L. rubrilucens, L. santicrucis, L. steigerwaltii, L. waltersii* (Figure 1 and S1).

3.2. Constitution of a Legionella DGGE fingerprinting database

The DGGE profiles of 16S rRNA gene fragment from the 28 *Legionella* species were produced, leading to a set of gel migration distances (Figures S1, S2, S3, S4, S5 and S6). These migration distances were then aligned and normalized by comparison with the reference marker positioned at the extremities of each gel. Calculation of similarity between bands profiles was based on Pearson coefficient to calculate similarities with the unweighed pair-group method using average linkages (UPGMA) clustering (Figure 2).

Among the 28 *Legionella* species, two strains exhibited more than one band *(L. gormanii* and *L. sainthelensis*) (Figure 2), that could be explained by an intragenomic sequence heterogeneity among the multiple copies of 16S rRNA gene as previously described by Coenye and Vandamme (2003). Migration distance of each specie was determined and for species exhibiting more than one band, the more intense band was considered for analysis. Relationship between migration distances, melting temperature and GC content of PCR fragment were found using a correlation calculation (Figure 3). Migration distance can be explained at 94% by the Tm parameter and at 92% by percentage in GC parameter.

Two species, *L. ery*thra and *L. tucsonensis,* exhibited the same migration profile and the same Tm. Differences between sequences was based on 4 single nucleotide polymorphisms (SNPs) out of the 20 bp lengh of the fragment, among which 3 are at a 6 bp distance. All mutations were transversion type and concerned 2 purine and 2 pyrimidine bases (Figure 4). Proximity of mutations, and equal GC percentage in the sequence could explain that no difference pattern was observed between these 2 species in DGGE analysis. Finally**,** a database containing all bands was produced (Figure 2).

3.3. Example of application to a real water sample

Legionella diversity was studied in an environmental water sample collected from a cooling tower. DNA was extracted from the water

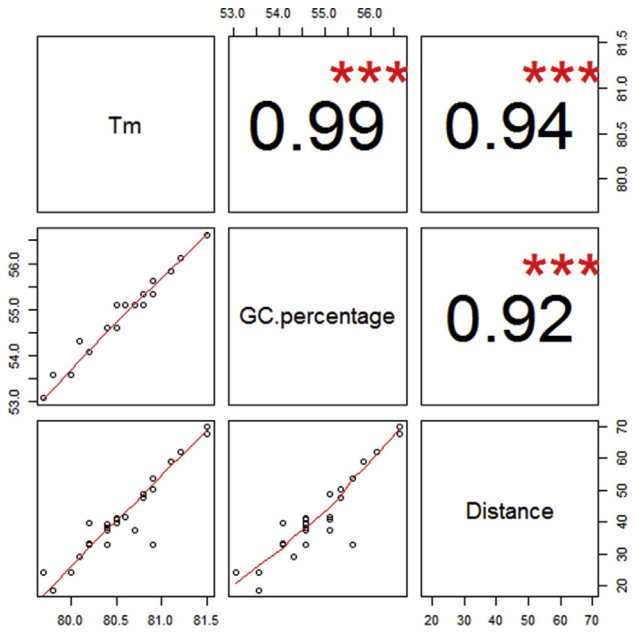
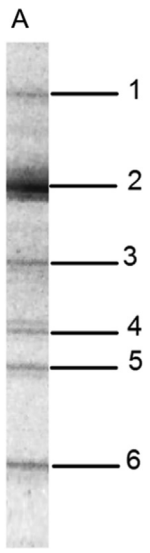


Figure 3. Scatter plot matrix and correlation coefficient between sequence parameters and migration distance. Scatter plot matrix (*lower*) and correlation matrix (*upper*) of melting temperature, percentage of GC content and migration distance of *Legionella species. P value* less than the significance level of 0.001 are noted on scatter plot matrix by the symbol ***. This means that all the correlations are statistically significant.



B

Legionella spp (UG/L)	*Legionella pneumophila* (UG/L)
26 000	LOD < 250

Figure 5. *Legionella* species identification in an environmental sample. (A) DGGE profile (B) real time PCR results.

sample and the PCR-DGGE method applied as previously described (Figure 5A, Supplementary data: Figure S1). In parallel, *Legionella spp* and *L. pneumophila* was quantified by real time PCR method. A concentration level was assessed for *Legionella spp.* (26 000 GU/L) while no *Legionella pneumophila* was detected (Figure 5 B).

Six bands appeared on the PCR-DGGE profile indicating that *Legionella spp* could be detected in the water sample. In addition, the comparison to the database allowed to identify four out of the 6 bands. The two other bands were not present in our database and have been identified as clinical species after sequencing (Table 2). The identification by sequencing of the co-migrant bands with marker bands was also carried out as a control. The sequences obtained were in agreement for 3 out of the 4 bands identified using PCR-DGGE method corresponding to *L. anisa* (GenBank accession number JF720397), *L. santicrucis* (GenBank accession number HF558374) and *L. waltersii* (GenBank accession number JF720408). The forth band was identified as *Legionella spp* 2C50 (GenBank accession number KC352892), an environmental strain, absent from the database, however for this identification, sequencing provide a sequence of only 96 pb in lenght.

4. Discussion

The aim of this work was to develop a gainful method for the detection and identification of *Legionella* community structure in environmental samples, based on a sample semi-nested PCR - DGGE gel comparison with a semi-nested PCR -DGGE profile database. The method allows the identification of specific species without the use of sequencing.

Culture and qPCR are the two main methods used for monitoring the *Legionella* contamination in environmental samples. The PCR methods were developed for the detection and the quantification of *Legionella spp* and *L. pneumophila*. However the PCR method, without additional step of sequencing, is not able to identify simultaneously all the *Legionella* species present in a complex environmental sample. It seems important when detecting *Legionella spp*. that the composition of the *Legionella* community be characterized, especially the twenty or so species well described as associated with infection (Helbig et al., 1995; Benson and Fields, 1998; Fields et al., 2002; Percival and Williams, 2014).

The method presented in this study has the capacity to monitor the pathogenic strains in a sample and to provide additional information to the concentration of *Legionella spp* and *Legionella pneumophila*. Indeed, a fine-tuned analysis is possible with the presented method. Moreover, this work highlights that it is possible to obtain simplified DGGE gel from environmental samples using the semi-nested PCR and to target and follow only one bacterial genus. The major limit of this approach is the

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L.erythra          1 ACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGGGGAACCCT     50
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
L.tucsonensis      1 ACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGGGGAACCCT     50

L.erythra         51 GATCCAGCAATGCCGCGTGTGTGAAGAAGGCCTGAGGGTTGTAAAGCACT    100
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
L.tucsonensis     51 GATCCAGCAATGCCGCGTGTGTGAAGAAGGCCTGAGGGTTGTAAAGCACT    100

L.erythra        101 TTCAGTGGGGAGGAGGGTTGACAGGTTAAGAGCTGGTTGACTGGACGTTA    150
                     ||||||||||||||||||.|||||||||||||.|.|||.|||||||||
L.tucsonensis    101 TTCAGTGGGGAGGAGGGTTGCCAGGTTAAGAGCTAGGTGATTGGACGTTA    150

L.erythra        151 CCCACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAA    196
                     ||||||||||||||||||||||||||||||||||||||||||||||
L.tucsonensis    151 CCCACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAA    196
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Figure 4. Alignment sequence of 16S rRNA gene fragments from *L. erythra* and L. *tucsonensis*. 16S rRNA gene fragments were determined using Pdraw software. Multiple alignment sequence was performed using ClustalW2.

Table 2. Comparison of DGGE analysis with sequencing results.

Bands	Identification by DGGE	Identification by sequencing				
		Legionella species	Accession number (Genbank)	Sequence size (pb)	Query coverage (%-pb)	% identity (%-pb)
1	No identification	*Legionella sp. D4522*	JN381004	157	100–157	100–157
2	*L. waltersii*	*L. waltersii MDC1619*	JF720408	148	100–148	99–146
3	*L. dumoffi*	*Legionella sp. 2C50*	KC352892	96	100–96	100–96
4	*L. santicrucis*	*L. santicrucis SC-63-C7*	HF558374	157	100–157	97–152
5	*L. anisa*	*L. anisa MDC 1224*	JF720397	104	94–98	100–98
6	No identification	*Legionella sp. KR-14*	JN381003	104	94–98	100–98

observation of similar profiles between two non pathogenic species *L. erythra* and *L. tucsonensis* due to the high level of sequence similarity.

Supporting this approach, future developments will be directed at incrementing the number of *Legionella* species profiles contained in the database. We hypothetize that this method could be used also for other pathogens, allowing a better characterization of environmental contamination. Moreover, using the DGGE technique, the monitoring over time or space of the targeted community through several samples can be carried out.

In conclusion, this method, complementary to the existing ones, could be used to follow the dynamics of *Legionella* species in water networks.

Declarations

Author contribution statement

S. Bayle, A. Cadière: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

B. Martinez- Arribas: Performed the experiments.

S. Jarraud, P. Giannoni: Contributed reagents, materials, analysis tools or data.

L. Garrelly, B. Roig: Analyzed and interpreted the data.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

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