Molecular evidence for the ubiquitous presence of *Legionella* species in Dutch tap water installations

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ABSTRACT

Our aim was to investigate the occurrence and identity of *Legionella* spp. in Dutch tap water installations using culture, real-time PCR and sequence analysis. The PCR assays used were a 16S rRNA gene based PCR with both a *Legionella* species specific probe and a *L. pneumophila* specific probe and a *L. pneumophila*-specific PCR based on the sequence of the *mip* gene. A total of 357 water samples from 250 locations in The Netherlands was investigated. The detection rates of *Legionella* spp. were 2,2% (8 of 357) by culture, and 87,1% (311 of 357) by PCR. The majority of samples was found to contain *Legionella* species other than *L. pneumophila.* These comprised of Legionella Like Amoebal Pathogens (LLAPs), *L. busanensis*, *L. worsliensis* and others. Fourteen (3,9%) samples were positive for *L. pneumophila* by either culture, 16S rRNA based PCR and/or *mip* based PCR. It is apparent from this study that *Legionella* spp. DNA is ubiquitous in Dutch potable water samples. Our findings further suggest that LLAPs and viable but nonculturable (VBNC) Legionella represent a large proportion of the population in man-made environments.

Key words | detection, Legionella, *Legionella pneumophila*, LLAPs, PCR, water

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INTRODUCTION

Legionellae are intracellular parasites of freshwater protozoa and use a similar mechanism to multiply within mammalian cells. These bacteria cause respiratory disease in humans when a susceptible host inhales aerosolized water containing the bacteria or aspirates water containing the bacteria (Fields *et al.* 2002). Most cases of legionellosis can be traced to human-made aquatic environments where the water temperature is higher than ambient temperature (Stout & Yu 1997). In these thermally altered aquatic environments the balance between protozoa and bacteria can shift, resulting in rapid multiplication of Legionellae, which in turn can translate into human disease. In The Netherlands in 1999 a large outbreak of Legionnaires' disease at a flower show occurred, with 188 patients of which 23 patients died (Den Boer *et al.* 1999). This demonstrated the potential public health risk of the presence of *Legionella pneumophila* in water, especially when usage leads to aerosols. In order to prevent such outbreaks, the Dutch government tightened up regulations about admissible

concentrations of *Legionella* spp. in Dutch water installations in public buildings. A variety of methods, including guinea pig inoculation, fluorescent-antibody (FA) techniques, culture techniques, FISH, and PCR-based assays, have been applied to detect *L. pneumophila* in environmental samples (Wullings & van der Kooij 2006). Culture methods enable the quantitative detection of culture-able bacteria and isolation of strains is commonly used but has a number of limitations. Culture requires a long incubation period, and the recovery is often reduced by antibiotics and sample treatment. Studies have demonstrated that culture-based estimates of the concentration of *L. pneumophila* in concentrated water samples are imprecise and of low sensitivity (Boulanger & Edelstein 1995). Recovery of *Legionella* spp. from water samples is often hampered by overgrowth of colonies of rapidly growing non-*Legionella* background. Several non-*pneumophila Legionella* spp. grow poorly on BCYE agar (Lee *et al.* 1993).

Recently, new methods for detection of *Legionella* spp. in water have been developed to overcome the limitations of culture. PCR methodology has been used based on the 16S rRNA gene (Yamamoto *et al.* 1993; Miyamoto *et al.* 1997; Wellinghausen *et al.* 2001), 5S rRNA gene (Koide *et al.* 1993; Maiwald *et al.* 1994; Villari *et al.* 1998), or on the macrophage infectivity potentiater (*mip*) gene (Koide *et al.* 1993; Villari *et al.* 1998; Wellinghausen *et al.* 2001; Levi *et al.* 2003). PCR techniques have the advantage of fast acquisition of results, detection of non-culturable legionellae, and easier handling of large numbers of samples. Our aim was to investigate the occurrence and identity of *Legionella* spp. in Dutch tap water installations using culture, real-time PCR and sequence analysis.

MATERIAL AND METHODS

Bacterial strains

In order to test in vitro sensitivity and specificity a list of bacterial strains was used as shown in Table 1.

Water samples

A total of 357 water samples were collected from 250 public buildings (mainly company buildings and schools) at different locations throughout The Netherlands between August and December 2001. 352 samples were obtained from potable water (346 cold water and 6 hot water) taps. One sample was obtained from a cooling tower, and 4 water samples were collected from large aerosol generating devices (e.g. air-conditioning). Water samples were collected in sterile 1,000 ml polyethylene bottles and samples were delivered to the laboratory within one day. For potable water samples, the first 1,000 ml of water from tapwater faucets was drained into a bottle. This first draw sample was not used for analysis. The water was then allowed to run for approximately 60 seconds, and the second draw of 1,000 ml of water was used for microbiological analysis. For non-potable samples from such sources as cooling towers, 250–500 ml water from the bottom or side of the vessel or reservoir was collected. No disinfectant treatment was used. After use, the bottles were rinsed with sterile water and

autoclaved at 121°C for 20 minutes. The temperature during transport was between 10–20°C. 500 ml samples were filtered through 0.2 μm polycarbonate membranes (Merck, Darmstadt, Germany), and the filtrate was resuspended in 1 ml of sterile distilled water.

Culture

Both 0.1 ml of the filtrate was plated on a buffered charcoal yeast extract agar (BCYE) and on a BCYE agar with glycine, vancomycine, polymixine and cycloheximide (GVPC, Oxoid, Haarlem, The Netherlands) and incubated for 7 days at 35°C in a humidified atmosphere. Colonies that grew on BCYE agar but failed to grow on blood agar were expected to be *Legionella* spp. Further identification was carried out by determination of the *L. pneumophila* serogroups using a slide agglutination test (Microscreen®, Microgen bioproducts, Camberly Surrey, United Kingdom). A distinction was made between *L. pneumophila* serogroup 1 and *Legionella pneumophila* serogroup 2–14.

Sample preparation for PCR

To minimize inhibition of PCR amplification, 200 μl of the filtrate was processed with MagNA Pure®, using the Total Nucleic Acid Kit (Roche Diagnostics) with an elution volume of 50 μl. 5 μl of the eluate was used as template in the PCR.

PCR assays

For the detection of Legionella in water samples two separate assays were used. The primers of the first assay were based on the 16S rRNA gene as was described previously (van der Zee *et al.* 2002). In short, primers *Leg1* (forward 5′-TACCTACCCTTGACATACAGTG-3′) and *Leg2* (reverse 5′-CTTCCTCCGGTTTGTCAC-3′) were used to obtain a 200 bp amplicon. Real-time detection was done with a Legionella genus specific fluorescent probe (LSPP: VIC-5′-GGTTGCGTCGTTACG-3′) conjugated to a minor groove binder (MGB). A *L. pneumophila* specific fluorescent MGB probe (LPN: FAM-5′-GAGTCCCCAC-CATCACATG-3′) was used on the complementary DNA strand.

Table 1 │ Legionella strains investigated by 16S rRNA and *mip* PCR assays

Species and serogroup	Strain or reference
L. pneumophila	
1. (Philadelphia 1)	ATCC33152
2. (Togus 1)	ATCC33154
3. (Bloomington2)	ATCC33155
4. (Los Angeles 1)	ATCC33156
5. (Dallas 1E)	ATCC33216
6. (Chicago 2)	ATCC33215
7. (Chicago 8)	ATCC33823
8. (Concord 3)	ATCC35096
9. (IN 23)	ATCC35289
10. (Leiden 1)	ATCC43283
11. (797/PA/H)	ATCC43130
12. (570-CO-H)	ATCC43290
13. (82A3105)	ATCC43736
14. (1169-MN)	ATCC43703
Legionella spp.	
L. anisa	ATCC35292
L. birminghamensis	ATCC43702
L. bozemanii-1	ATCC33217
L. bozemanii-2	ATCC35545
L. brunensis	ATCC 43878
L. cherii	ATCC35252
L. dumoffii	ATCC35850
L. erythra	ATCC35303
L. feeleii-1	ATCC35072
L. feeleii-2	ATCC35849
L. gormanii	ATCC33297
L. hackeliae-1	ATCC35250

Table 1 │ (continued)

Species and serogroup	Strain or reference
L. israelensis	ATCC43119
L. jamestowniensis	ATCC35298
L. jordanis	ATCC33623
L. longbeachae-1	ATCC33462
L. longbeachae-2	ATCC33484
L. maceachernii	ATCC35300
L. micdadei	ATCC33218
L. moravica	ATCC43877
L. oakridgensis	ATCC33761
L. parisiensis	ATCC35299
L. rubrilucens	ATCC35304
L. sainthelensis	ATCC35248
L. santicrusis	ATCC35301
L. spiritensis	ATCC35249
L. steigerwaltii	ATCC35302
L. tucsonensis	ATCC49180
L. wadsworthii	ATCC33877
Non-*Legionella* bacteria	
Streptococcus pneumoniae	ATCC49619
Bordetella pertussis	Tohama strain
Bordetella parapertussis	B24
Mycoplasma pneumoniae	ATCC15293
Chlamydia pneumoniae	ATCCVR1355
Acinetobacter baumannii	
Pseudomonas aeruginosa	ATCC27853
Lactobacillus caseï.	

So as not to miss *L. pneumophila* in mixed legionella populations in water, we developed a *L. pneumophila*-specific PCR based on the sequences of the *mip* gene in addition. Primers Mip-F1 (forward 5′-GCCAAGTGGTTTG-CAATACAAA-'3) and MipR1 (reverse 5′-CTCGACAGT-GACTGTATCCGATTT-'3) were chosen with PrimerExpress (ABI), amplifying a 80 bp fragment. Real-time detection was done with TaqMan probe Lpn-mip (FAM-5′-TAAT-CAATGCTGGAAATGGTGTTAAACCCG-3′-TAMRA).

Controls

In both assays negative controls were included. Sensitivity controls consisted of 10-fold dilutions of *L. pneumophila* DNA ranging from 1,000 fg to 10 fg (approximately 230 to 2,3 genome equivalents). *L. bozemanii* has one mismatch with *L.pneumophila* specific probe and is used to guard the border of discrimination between *L. pneumophila* and non-*pneumophila* species. 1,000 g of *L. bozemanii* DNA served as control in the 16S rRNA based PCR for discrimination between *L. pneumophila* and other Legionella species. As internal control Phocine Herpes Virus (PhHV) was added to the samples to monitor processing as well as PCR inhibition, and detection of PhHV was included in the *mip* PCR. Primers PhHV-F1 and PhHv-R1 amplified a 80 bp amplicon that was detected in real-time with TaqMan probe PhHV-1 (VIC-5′-TTTTTTATGTGTCCGCCACCATCTG-GATC-'3-TAMRA). All primers and probes were synthesized by Applied Biosystems (ABI, Nieuwerkerk a/d IJssel, The Netherlands).

PCR conditions

The PCR mixture for the 16S rRNA PCR assay contained 5 μl of sample DNA, 10 pmol of each primer Leg 1 and Leg 2, 3 pmol of each of probes Lsp-1 and Lpn-1, 12.5 μl *TaqMan* Universal Master Mix (ABI, Nieuwerkerk a/d IJssel, The Netherlands), 1 μl bovine serum albumin (BSA;10 mg/ml) per sample (New England Biolabs, Beverly, USA) and PCR grade sterile water to a final volume of 25 μl. The PCR mixture for the *mip* gene based PCR contained 5 μl of sample DNA, 5 pmol of primer MIP-F2, 7.5 pmol of primer MIP-R2, 3.75 pmol of probe MIP-2, 10 pmol of each primer PhHv-R1 and PhHv-F1,

3.75 pmol of probe PhHv-1, 12.5 μl *TaqMan* Universal Master Mix (ABI, Nieuwerkerk a/d IJssel, The Netherlands), 1 μl BSA (10 mg/ml) per sample (New England Biolabs, Beverly, USA) and PCR grade sterile water to a final volume of 25 μl. Real–time PCR was performed on a Abiprism®7900HT Sequence Detection System (Applied Biosystems, Foster City, California, USA). DNA was amplified according to the following parameters: after 2 min incubation at 50°C and 10 min denaturation at 95°C, amplification consisted of 50 cycles of 15 sec of denaturation at 95°C followed by 1 min of annealing and extension at 60°C. Results were expressed as threshold cycle values (Ct), corresponding to the cycle at which PCR enters the exponential phase. If no increase in fluorescent signal is observed after 50 cycles, the sample is assumed to be negative.

Identification of non-*pneumophila* Legionella spp

Identification of 90 non-*pneumophila* Legionella PCR positives (6 culture positives and 84 PCR positives) was done by PCR reamplification and sequence analysis of the 200 bp 16S rRNA gene fragment (Base Clear, Leiden, The Netherlands). The sequences obtained were compared with those submitted to the GenBank database by using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/).

RESULTS

Specificities of PCR assays

The specificities of both the 16S rRNA gene based PCR and the *mip* gene based PCR were investigated using a panel of human-pathogenic and environmental Legionella listed in Materials and Methods. Also a panel of non-Legionella bacteria was investigated. With the 16S rRNA gene based PCR only *Legionella* species were detected, and the probes correctly discriminated between *Legionella pneumophila* and non-*pneumophila* Legionella species. With the *mip* gene based PCR all serogroups of *L. pneumophila* were detected. No PCR positives were found among non-Legionella bacteria. A 100% specificity of each PCR assay was observed.

Sensitivities of PCR assays

The sensitivity of the 16S rRNA based PCR was highest with a lower detection limit of 0.1 CFU and 10 fg of chromosomal DNA. As the genome of one Legionella consists of approximately 4.3 fg DNA (Wellinghausen *et al.* 2001), we can calculate an estimated equivalent of 200 legionellae/l. In the *mip* gene based PCR assay, the lower detection limit was 100 fg, the theoretical equivalent of approximately 2,000 legionellae/l.

Evaluation of results on water samples using culture and real-time PCR

In the analysis of the water samples, the detection rates of *Legionella* spp. were 87.1% (311 of 357) by PCR and 2.2% (8 of 357) by the culture method (Table 2). A total of 14 water samples (3.9%) was positive for *L. pneumophila*. Of these 14 samples one was positive in both PCR (*mip-* and 16S rRNA gene based assays) and culture (serogroup 1, 190 CFU/L), 7 samples in *mip-* and 16S rRNA gene based

assay but not in culture, and 4 samples only in 16S rRNA PCR. The mean Ct value of these 4 samples was lower compared to *mip* gene based PCR positive samples, reflecting the higher sensitivity of the 16S rRNA PCR. One *L. pneumophila* positive sample was positive in culture (serogroup 2–14, 110 CFU/L) but reacted with the genus specific probe in the 16S rRNA based PCR. A total of 298 water samples (83.5%) was positive for *L. non-pneumophila* spp. Of these 298 samples 6 were positive in both 16S rRNA based PCR and culture (average 556 CFU/L, range 50–2,600 CFU/L), 291 positive only in 16S rRNA based PCR and one sample tested positive in both *mip-* and 16S rRNA based PCR. We found eight (2.2%) water samples contained inhibitors of PCR amplification. The sample obtained from a cooling tower was positive for *L. non-pneumophila* spp. in PCR, but negative in culture. Four water samples were collected from large aerosol generating devices; two culture negative samples contained inhibitors of PCR amplification, one sample was *L. non-pneumophila* spp. positive in PCR but negative in culture, and one sample was negative in both PCR and culture.

Table 2 │ Results of *Legionalla* spp. detection by 16S rRNA based PCR assay, *mip* based PCR assay, and culture. Lspp; *Legionella* non-*pneumophila* spp., Lpneu; *L. pneumophila*, Ct; cycle treshold value, +; positive result, −; negative result

16S rRNA PCR

Lspp.((mean) Ct)	Lpneu ((mean) Ct)	*mip* PCR ((mean) Ct)	Culture	Number of samples (%)	Conclusion
+(29)	+(33)	+(36)	+	1 (0.3%)	*L. pneumophila*
+(30)	+(32)	+(35)	−	7 (2.0)	*L. pneumophila*
+(32)	+(34)	−	−	4 (1.1%)	*L. pneumophila*
+(29)	−	−	+	1 (0.3%)	*L. pneumophila*
+(28)	−	−	+	6 (1.7%)	*L. non-pneumophila*
+(33)	−	+(37)	−	1 (0.3%)	*L. (non)-pneumophila*
+(30)	−	−	−	291 (81.5%)	*L. non-pneumophila*
−	−	−	−	38 (10.6%)	negative
Inhibited				8 (2.2%)	
				357 (100%)	Total

Sequence analysis results

The 84 samples originating from 51 locations that were positive for *Legionella* non-*pneumophila* spp. in the 16S rRNA PCR were analysed by sequencing (Table 3). 31 aligned amplification products showed 98%-100% match with Legionella Like Amoebal Pathogens (LLAPs), 27 with *L. busanensis,* and 11 with *L. worsliensis.* Two sequences showed homology to uncultured bacteria WCHA-76, and GOUTA10, which are like Legionella members of the *Proteobacterium* family. Cultured strains were identified with PCR and sequence analysis as *L. dumoffii* (1), *L. donaldsonii* (1), *L. anisa* (1), *L. erythra* (1) and *L. worsliensis* (2).

DISCUSSION

Theoretically, PCR would present the method of choice for the detection of *Legionella* species in water, as has been suggested before (Wellinghausen *et al.* 2001). PCR is equally sensitive for all Legionella, and facilitates handling of a large

Table 3 | Results of identification by sequence analysis of the 200 bp 16S rRNA gene-fragment

Species (% match)	Genbank accession nr.	Mutation	Position	N
LLAP 1 (100%)	X97355			22
LLAP 1 (99%)	X97355	G → A	1,112	5
LLAP 14 (100%)	U66104			4
L. adelaidensis (99%)	Z49716	T → C	1,112	1
L. birminghamiensis (100%)	Z49717			1
L. busanensis (100%)	AF424887			8
L. busanensis (99%)	AF424887	G → A	1,119	19
L. erythra (100%)	Z32638			1
L. macheachernii (98%)	AF227161	C → A	1,017	2
		G → A	1,112	
L. parisiensis (99%)	Z49731	G → A	1,068	1
L. parisiensis (98%)	U59697	G → A	1,110	1
		G → C	1,137	
L. shakespearei (100%)	Z49736			1
L. worsliensis (100%)	Z49739			9
L. worsliensis (99%)	Z49739	T → C	1,112	2
WCHA1–76 (100%)	AF050529			2
GOUTA10 (100–97%)	AY050582			5
Total				84

number of samples. The major difference between culture and PCR found in our study may be due to a number of reasons. First, recovery rates obtained by culture are usually noticeably less than 100%, especially in potable waters (Villari *et al.* 1998). Culture requires a panel of differential and selective media, and has a relatively low sensitivity, ranging from between 10 to 80% in clinical materials (Murdoch 2003). Growth for *Legionella pneumophila* is better on standard media used for primary isolation than for *Legionella* non-*pneumophila* spp. (Lee *et al.* 1993). It has also been recognized that filtration or centrifugation of large water sample volumes results in a loss of up to 90% of culturable Legionella (Wellinghausen *et al.* 2001; Levi *et al.* 2003). The significance of the large percentage of PCR positive water samples remains unclear. Legionellae detected by PCR may also present non-viable cells or *Legionella* DNA which is not infectious to humans. Even amplification and detection of non-legionella DNA cannot be completely excluded, since alignment of the target sequences of the primers can never exclude all environmentally occurring, known and unknown, bacteria. Therefore, the high PCR signals should be critically interpreted.

In our study we found only six samples positive for *Legionella* non-*pneumophila* spp. by culture. The recovery rate seems to correspond with PCR quantification since culture positives showed lower Ct values compared to culture negatives, although the differences are difficult to interpret because of the low numbers of culture positive samples. In this study, 31 aligned amplification products showed 98%–100% match with Legionella Like Amoebal Pathogens (LLAPs). LLAPs may be human pathogens, but proving this is difficult because they cannot be detected by conventional culture techniques used for Legionella (Marrie *et al.*, 2001). Moreover, several authors have reported that Legionella bacteria are able to enter a viable but non-culturable (VNBC) state, which may account for the fact that Legionella cannot be cultured from cooling towers suspected to be the source of Legionellosis (Steinert *et al.* 1997).

One *L. pneumophila* positive sample was positive in culture (serogroup 2–14, 110 CFU/L) but reacted with the genus specific probe in the 16S rRNA based PCR. This finding can be explained by the presence of mixed Legionella populations in water, and has been suggested previously (Levi *et al.* 2003). A drawback of the dual-color

two probe 16S based PCR is that with mixed Legionella populations in the same sample, amplification is favoured towards the predominant species. We found that when the ratio of *Legionella* non-*pneumophila* spp. / *L. pneumophila* exceeds 100 in the PCR reaction, *L. pneumophila* DNA was not amplified to a detectable level. Another example of a mixed Legionella population may be presented by one sample that tested positive in the *mip* gene based PCR (and is therefore considered *L. pneumophila* positive) and was positive with only the Legionella genus probe in the 16S rRNA gene based PCR. Minimization of inhibition with bovine serum albumin (BSA) has been successfully used in previous studies (Kreader 1996; Levi *et al.* 2003). We found eight (2.2%) water samples contained inhibitors of PCR amplification. Without the addition of BSA, up to 25% of water-samples tested inhibitory in our laboratory (data not shown).

Our results are in concordance with those found by Wullings & van der Kooij (2006). They studied the occurrence of *Legionella* spp. in water collected from 82 groundwater supplies and 16 surface water supplies, covering 67% of the total drinking water production in the Netherlands. Legionella was detected with 16S rRNA based quantitative real-time PCR in all samples of treated water, and no Legionella was found with the culture method. A phylogenetic analysis of 16S rRNA gene sequences of 202 clones, obtained from a selection of samples, showed a high similarity with *Legionella* sequences in the GenBank database. A total of 40 (33%) of the 16S rRNA gene sequences obtained from treated water were identified as described *Legionella* species and types, including *L. bozemanii*, *L. worsliensis*, LLAPs, *L. quateirensis*, *L. waltersii*, and *L. pneumophila*. 16S rRNA gene sequences with a similarity of below 97% from described species were positioned all over the phylogenetic tree of *Legionella*. The authors conclude that a large diversity of yet-uncultured Legionellae are common members of the microbial communities in ground and surface water supplies.

Although an association between the extent of *Legionella* contamination in water (using culture, expressed as CFU/l) and the occurrence of legionellosis has been described, an association with the exact concentration of Legionella remains unclear. Quantification of Legionella recovered from a single water outlet has been shown to

have no relevance to occurrence of the disease: increased risk appeared associated with the extent of colonisation with *Legionella pneumophila* (Best *et al.* 1983; Kool *et al.* 1999). However, this finding may also be due to the fact that estimates of the concentration of Legionella in concentrated water samples, when using culture, are imprecise and of low sensitivity. The role of non-*pneumophila Legionella* species as human pathogens is largely unknown mainly due to currently used diagnostic methods and because submission of sputum samples for *Legionella* culture is not a standard practice in the evaluation of community-acquired pneumonia (CAP). Furthermore, the *Legionella* urinary antigen test, which is widely used as a diagnostic screening test for *Legionella* infection, does not detect infection due to *Legionella* species, other than that due to *L. pneumophila* serogroup 1 (Fields *et al.* 2002). Infections seem to be very rare, and are reported mainly in severely immunocompromised patients. It is not clear whether these non-*pneumophila Legionella* species are inherently less pathogenic than *L. pneumophila.* Many species are pathogenic for amoebae, share virulence genes with *L. pneumophila*, and multiplication within amoebae has been shown to be linked to pathogenicity (Muder & Yu 2002). The role of LLAPs as human pathogens is largely unknown. Rowbotham (1993) isolated LLAP 1 from the sputum of an 82-year-old woman with persistent pneumonia by cocultivation with *Acanthamoeba polyphaga*. The patient demonstrated a fourfold rise in antibody titer to the bacteria from the infected amoebae. He thereafter screened over 5,000 serum samples submitted for *Legionella* antibody testing, and found that 10 patients met criteria for infection with LLAPs (Adeleke *et al.* 1996). Our findings suggest that LLAPs and VBNC Legionella could represent a large proportion of the population in man-made environments and may, although not likely, constitute an unrecognized reservoir for disease.

CONCLUSIONS

Legionella spp. DNA is ubiquitous in Dutch tap water installations and uncultured *Legionella* spp. are part of the indigenous microbial community. The unique growth requirements of Legionella, the ability to enter a VBNC state and the association of LLAPs with amoebae complicates the detection of Legionella in potable water using standard microbiological techniques. Elucidation of the properties of these organisms is needed to assess their potential public health significance and explain the conditions favouring their growth in aquatic environments. Therefore, prospective studies using culture and validated PCR methodologies are necessary to determine the exact role of non-*pneumophila Legionella* species as a human pathogen.

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