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Quantitative Detection of *Legionella pneumophila* in Water Samples by Immunomagnetic Purification and Real-Time PCR Amplification of the *dotA* Gene

M. A. Yáñez, C. Carrasco-Serrano, V. M. Barberá, and V. Catalán*

Labaqua, Alicante, Spain

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A new real-time PCR assay was developed and validated in combination with an immunomagnetic separation system for the quantitative determination of *Legionella pneumophila* in water samples. Primers that amplify simultaneously an 80-bp fragment of the *dotA* gene from *L. pneumophila* and a recombinant fragment including a specific sequence of the *gyrB* gene from *Aeromonas hydrophila*, added as an internal positive control, were used. The specificity, limit of detection, limit of quantification, repetitivity, reproducibility, and accuracy of the method were calculated, and the values obtained confirmed the applicability of the method for the quantitative detection of *L. pneumophila*. Moreover, the efficiency of immunomagnetic separation in the recovery of *L. pneumophila* from different kinds of water was evaluated. The recovery rates decreased as the water contamination increased (ranging from 59.9% for distilled water to 36% for cooling tower water), and the reproducibility also decreased in parallel to water complexity. The feasibility of the method was evaluated by cell culture and real-time PCR analysis of 60 samples in parallel. All the samples found to be positive by cell culture were also positive by real-time PCR, while only eight samples were found to be positive only by PCR. Finally, the correlation of both methods showed that the number of cells calculated by PCR was 20-fold higher than the culture values. In conclusion, the real-time PCR method combined with immunomagnetic separation provides a sensitive, specific, and accurate method for the rapid quantification of *L. pneumophila* in water samples. However, the recovery efficiency of immunomagnetic separation should be considered in complex samples.

Legionella is one of the main causative agents of severe atypical pneumonias, particularly among people with impaired immune systems. Although the genus *Legionella* comprises more than 40 species with 64 serogroups (6), *L. pneumophila* is the most common pathogenic species, accounting for more than 90% of legionellosis cases. Present in soil and natural aquatic environments (12), legionellae sometimes survives as an intracellular parasite of amoebae and ciliates (7). Legionellae have also found a niche in several man-made aquatic environments such as potable water systems, cooling towers, and wastewater systems (9, 10). Consequently, the possible presence of legionellae in bioaerosols generated from soil or aquatic environments poses a significant hazard for human health (34).

Outbreaks of *L. pneumophila* occur throughout the world (39), impacting public health as well as various industrial, tourist, and social activities. For these reasons some countries specifically regulate the surveillance and control of *L. pneumophila* in water regularly and assess its presence by culture on a selective medium (25). However, this monitoring technique is time-consuming due to the slow growth rate of the bacterium, the inability to detect viable noncultivable bacteria, and the difficulty in isolating legionellae in samples contaminated with high levels of other microbiota. To avoid these problems, nucleic acid amplification techniques, mainly PCR, have been described as useful tools for the detection of *L. pneumophila* in clinical and environmental samples. Several PCR-based methods for the detection of *L. pneumophila* DNA have been described, but most of them are based on the amplification of the macrophage infectivity potentiator (*mip*) gene (4, 9) or the 16S or 5S rRNA gene (17, 36, 45).

In this work we proposed amplification of an alternative *L. pneumophila* gene, the defective organelle trafficking (*dotA*) gene. This gene is involved in *L. pneumophila* virulence and is regarded as a pathogenicity island, such as *cagA* in *Helicobacter pylori*, *hly* in uropathogenic *Escherichia coli*, or the *vir* complex in *Agrobacterium tumefaciens* (3). In this way, *dotA* and *mip* are part of the mechanism that mediates the initial invasion of eukaryotic cells and the subsequent intracellular survival and multiplication (8). The *dotA* gene product also regulates trafficking of the *L. pneumophila* phagosome, playing a fundamental role in regulating initial phagosome trafficking decisions either during or immediately after macrophage uptake (38). *L. pneumophila* strains that possess a mutation in *dotA* cannot replicate intracellularly because they are unable to alter the endocytic pathway of macrophages (21).

Despite the advantages of conventional PCR, two main obstacles remain. One is the presence of PCR inhibitors, such as humic and fulvic acids and metals, in environmental samples that can produce false-negative results. The second is that conventional PCR is a qualitative assay, informing only of the presence or absence of the microorganism. Various methods have been described that permit procurement of pure DNA lacking PCR inhibitors. These methods include rapid gel filtration to remove humic substances (1), filtration through chelating ion exchange resins to eliminate metal ions (19, 43), addition of polyvinylpyrrolidone to remove polyphenols (23), and cesium chloride density centrifugation to improve general

* Corresponding author. Mailing address: Labaqua, Pol. Ind. Las Atalayas, c/Del Dracma, 16-18, 03114 Alicante, Spain. Phone: 34.96510.60.70. Fax: 34.96510.60.80. E-mail: vicente.catalan@labaqua.es.

DNA purity (23). The advantages and drawbacks of all of these methods have been reported (1).

By contrast, purification of the intact cell rather than purification of the DNA provides another strategy for eliminating PCR inhibitors. Thus, the use of immunomagnetic separation methodologies, which permit DNA isolation with a minimum of inhibitors (32), has been developed. Immunomagnetic separation relies on the interaction between antibodies attached to paramagnetic beads and cell surface antigens, permitting separation of specific cells by placing a bead-cell suspension in a strong magnetic field. In this way, immunomagnetic separation provides a simple but powerful method for extracting the desired microorganism from heterogeneous bacterial suspensions, such as those encountered in food, clinical, and environmental samples (11).

The recent advent of fluorescent probe-based PCR technology (real-time PCR) has led to the development of a quantitative assay, which was lacking in conventional PCR. With real-time quantitative PCR, signal fluorescence that is released during amplification is proportional to the amount of product generated, and the initial copy number can be estimated from the exponential phase of product accumulation by comparison to a standard curve.

With the above knowledge, the aims of this work were to evaluate an immunomagnetic separation method for the purification of *L. pneumophila* from water samples and to develop and validate a quantitative *L. pneumophila* PCR method based on amplification of the *dotA* gene.

MATERIALS AND METHODS

Bacterial strains and cultivation. The *Legionella* strains and the other bacterial species used in this study are described in Table 1. *Legionella* strains were grown on buffered charcoal-yeast extract containing 0.1% α-ketoglutarate, adjusted to pH 6.9 with KOH, and supplemented (per liter) with 0.4 g L-cysteine and 0.25 g ferric pyrophosphate (BCYE). For the isolation of legionellae from environmental samples, GVPC medium was used. This medium is identical to BCYE except 3 g glycine, 1 mg vancomycin, 50,000 IU polymyxin B, and 80 mg cycloheximide are added to 1 liter of BCYE medium. Inoculated plates were incubated at 37°C in a humidified atmosphere containing 5% carbon dioxide.

Escherichia coli was cultivated on Tergitol-7 agar (Oxoid), *Salmonella enterica* serovar Typhimurium on Rambach agar (Merck), *Mycobacterium tuberculosis* on Seven H11 agar (Becton Dickinson), *Clostridium perfringens* on thioglycolate broth, and *Enterococcus faecium* on Slanetz and Bartley medium (Oxoid). All inoculated plates were incubated at 37°C for the period of time appropriate for each species.

Samples. To prepare spiked samples, legionellae were grown for 3 days on BCYE medium, cells were harvested, and a peptone-water suspension was prepared. This suspension was serially diluted 10-fold in peptone water. Next, 2 ml of dilutions from 10^{-5} to 10^{-9} were added to 1 liter of each water matrix to obtain the contaminated samples.

In addition, we also analyzed 25 samples from potable hot water systems belonging to different hotels in Alicante, Spain, and 35 water samples from different cooling tower systems.

Immunomagnetic bead preparation. An anti-*Legionella pneumophila* polyclonal antibody (OBT0943; Oxford Biotechnology) was coupled to superparamagnetic beads (Dynabeads M-280 Tosylactivated; Dynal Biotech) by incubating 3 μg of antibody with 10^7 Dynabeads for 24 h at 37°C with slow tilt rotation. After washing with phosphate-buffered saline, pH 7.4, containing 0.1% bovine serum albumin, the suspension reached a final concentration of 4×10^8 particles per ml of wash buffer.

Sample processing and immunomagnetic purification. Water samples were concentrated by filtration through 0.4-μm-pore-size, 47-mm-diameter polycarbonate membranes, which were placed in 10 ml of sterile deionized water in a screw-cap tube. To release the cells from the membrane, the tube was vortexed for 3 min, and the 10 ml of solution was further concentrated to 1 ml using Amicon Ultra-15 filters (Millipore, Molsheim, France). Each concentrated sample was added to a microcentrifuge tube containing 50 μl of bacterium-binding beads to reach a final concentration of 2×10^7 bacterium-binding beads per ml of sample. Samples were then incubated for 60 min at room temperature with gentle agitation. Bacteria bound to magnetic beads were drawn to the wall of the microcentrifuge tube by a magnetic particle concentrator (Dynal MPC-M; Dynal). Finally, the supernatant was carefully removed, with a micropipette, without disrupting the bacterium-bead complexes.

DNA isolation from environmental samples and pure cultures. The processed water samples and bacterial colonies from pure cultures were resuspended in 200 μl of 20% Chelex 100 resin (Bio-Rad Laboratories, Richmond, CA). DNA was then extracted by three freeze-thaw cycles (−75°C for 10 min and 94°C for 10 min), and cellular debris was removed by pelleting at $10{,}000 \times g$ for 1 min. The quantity of genomic DNA was measured by spectrophotometry at 260 nm in triplicate, and DNA purity was checked using the A_{260}/A_{280} ratio (29).

The number of copies of the *dotA* gene in purified DNA samples was calculated by assuming an average molecular mass of 660 Da for 1 bp of double-stranded DNA (PCR Applications Manual, 2nd ed., Roche Diagnostics GmbH, Mannheim, Germany, 1999). The calculation was performed with the following equation: number of copies = quantity of DNA (fg)/mean mass of the *L. pneumophila* genome. The mean mass of the *L. pneumophila* genome was calculated from the mean size of the genome, which is assumed to be 3.9 Mb (5).

Amplification conditions and calculation of efficiencies. To design oligonucleotides specific for *L. pneumophila*, a multiple alignment of *dotA* sequences deposited in the GenBank database (accession numbers AY36018 to AY36035 [21], AF095231 to AF095235, AF440205 to AF440215, and AY280173 to AY280338) was performed using CLUSTAL X software (42). The sequences of the output regions were searched against GenBank sequences with the BLAST family program package (28) to ensure the specificity of primers and probes. Finally, primers and probes were analyzed for the requirements imposed by real-time quantitative PCR using Primer Express (version 2.0) of Applied Biosystems. When an optimal design was found (Table 1), primers were synthesized commercially (Applied Biosystems).

The amplification reactions were performed in optical microplates using a total volume of 25 μl. The reaction mixtures contained 1× TaqMan universal PCR master mix (PCR buffer, deoxynucleoside triphosphates, AmpliTaq Gold polymerase, internal reference signal 6-carboxy-x-rhodamine [ROX], Amp Erase uracil *N*-glycosylase [UNG], $MgCl_2$; Applied Biosystems, Foster City, CA), 300 nM of each *L. pneumophila*-specific oligonucleotide primer, and 250 nM TaqMan Minor Grove Binding (MGB) *L. pneumophila*-specific probe labeled with 6-carboxy fluorescein (FAM). An MGB probe is a modification of a TaqMan probe that forms hyperstabilized duplexes with cDNA (2). It is specifically recommended when there are few regions available for probe selection and it is impossible to get a high enough melting temperature (T_m) to match the selected primers. For instance, a 12-mer probe with an MGB group has a melting temperature identical to that of a 27-mer DNA probe lacking an MGB group (22).

To detect PCR inhibitors, a hybrid internal control was constructed that could be amplified simultaneously with the target DNA by using the same primers set. The control sequence contained a fragment from *gyrB* of *Aeromonas hydrophila* (Spanish Type Culture Collection, CECT 839) that was linked at both ends with the same sequences as the *dotA*F and *dotA*R primers. Two hybrid primers, *dotA*F*gyrB* (*dotA*F sequence CAAGGCGTTCGTCGAATACC) (positions 562 to 583) and *dotA*R*gyrB* (*dotA*R sequence GCTGCGGAATGTTGTTGGT) (positions 776 to 757) were synthesized, and DNA from *A. hydrophila* was amplified as described previously (35). The resultant amplicon was a fragment containing 161 bp of the *A. hydrophila gyrB* gene, flanked at both ends by two sequences of the *L. pneumophila dotA* gene (primers *dotA*F and *dotA*R). To use this hybrid DNA in real-time PCR, a TaqMan probe was designed in the *gyrB* region and labeled with VIC (PE Biosystems) (Table 3).

Amplification reactions were performed using an ABI Prism 7000 sequence detector (Applied Biosystems). The thermal profile was 2 min at 50°C (activation of the UNG) and 10 min at 95°C (activation of the AmpliTaq Gold DNA polymerase), followed by 15 s at 95°C and 1 min at 60°C for 42 cycles.

The ABI Prism 7000 detection software permits quantification of PCR products in real time, as revealed by the increase of fluorescence signal by 5′-nuclease activity during the amplification process. The threshold cycle (C_t), the cycle at which the fluorescence in the sample increases above a defined threshold, is inversely proportional to the starting amount of nucleic acid. The threshold (C_t) for each standard was plotted against the $\log_{10}$ of the starting DNA quantity to generate a standard curve.

The amplification efficiency (E) was estimated by using the slope of the standard curve and the formula $E = (10^{-1/\text{slope}})$. A reaction with 100% efficiency will generate a slope of −3.32 and has an efficiency of 2.

TABLE 1. Bacterial strains used in this study

Species	Strain identification[a]	Serogroup	Source
L. pneumophila	PHLS. (A) April 2001		External quality assesment
	PHLS. (B) April 2001		External quality assesment
	PHLS. (C) April 2001		External quality assesment
	L401	1	Environment
	L403		Environment
	L410	1	Environment
	L437	1	Environment
	L446		Environment
	L500	1	Environment
	L510	2-14	Environment
	L511	1	Environment
	L512	2-14	Environment
	L514	1	Environment
	L533	1	Environment
	L534	2-14	Environment
	L558	2-14	Environment
	L560	2-14	Environment
	L561	2-14	Environment
	L565	2-14	Environment
	L568	2-14	Environment
	L594	2-14	Environment
	L674	1	Environment
	L922	1 or 9	Environment
	L923	1 or 9	Environment
	L924	1 or 9	Environment
	L926	1 or 9	Environment
	L1587	1	Environment
	L1588	2-14	Environment
	L1598	2-14	Environment
	L1599	2-14	Environment
	L1635	1	Environment
	L1636	1	Environment
	L1638	2-14	Environment
	L1642	1	Environment
	L1657	1	Environment
	L131	1	Environment
	L1130	4	Environment
	L1298	6	Environment
	L1253	2,3	Environment
	L226	10	Environment
	L53	12	Environment
	L63	14	Environment
	L1809	8	Environment
	L1572	6	Environment
	L1528	3	Environment
	L1527	3	Environment
	L1413	9	Environment
	NCTC 11406	6	Collection
	NCTC 11230	2	Collection
	NCTC 11192	1	Collection
Legionella sp.	L782		Environment
Legionella sp.	L593		Environment
Legionella sp.	L1568		Environment
Legionella sp.	L1592		Environment
Legionella sp.	L1586		Environment
L. bozemanii	NCTC 11368		Collection
L. bozemanii	NCTC 11360		Collection
L. gravella feeli	NCTC 12022		Collection
L. jordanis	NCTC 11533		Collection
L. micdadei	NCTC 11371		Collection
L. gormanii	NCTC 11401		Collection
L. dumoffii	NCTC 11370		Collection
Escherichia coli	CECT 434		Collection
Vibrio cholerae	CECT 557		Collection
Enterococcus faecium	CECT 410		Collection
Salmonella enterica	NCTC 12848		Collection
Mycobacterium tuberculosis	NCTC 7417		Collection
Clostridium perfringens	CECT 376		Collection
Aeromonas hydrophila	CECT 839		Collection

[a] CECT, Spanish Type Culture Collection, Valencia, Spain; NCTC, National Collection of Type Cultures, Central Public Health Laboratory, London, United Kingdom; PHLS, Public Health Laboratory Service, London, United Kingdom.

Statistical analysis. Applied Biosystems real-time quantitative PCR data were analyzed by the Applied SDS software version 1.1 (Applied Biosystems). Statistical analysis was performed using Statgraphics Plus version 5 (Manugistics).

RESULTS

Purification of *L. pneumophila* by immunomagnetic separation. To test the ability of immunomagnetic separation to recover different strains of legionellae from water, different distilled water samples were spiked with 50 different strains of *L. pneumophila* belonging to the serogroups described in Table 1. *L. pneumophila* was then concentrated by filtration using polycarbonate membranes, further concentrated with Amicon Ultra-15 filters, and isolated by immunomagnetic separation as indicated above. All the strains tested were retained by the immunomagnetic beads, demonstrating the ability of the polyclonal antibody used to isolate all the serogroups studied.

Since the method of purification involved the isolation of intact cells by an immunomagnetic separation procedure, the percentage of *L. pneumophila* cells recovered from clean water, such as distilled and potable water, was determined by culture isolation. Known concentrations of *L. pneumophila* (NCTC 11192) were used to spike distilled water and potable water, and legionellae were then concentrated and purified by immunomagnetic separation as described above. We counted the number of colonies isolated from the supernatant and from the bacterium-binding beads during the immunomagnetic purification. Because the abundant growth of microbiota found in cooling tower samples hampers the isolation of legionellae by culture methods, the efficiency of the purification method could not be tested in the same way. Water samples containing high levels of microbiota can be treated with acid or heat to reduce microbiota, but *Legionella* concentrations are also reduced. To overcome this hurdle, we used real-time PCR to determine the efficiency of *Legionella* purification from cooling tower samples.

The efficiency of immunomagnetic bead isolation of *L. pneumophila* from spiked distilled water was higher than that of spiked potable water, which was higher than that of cooling tower water (Table 2). The average recovery rate for distilled water was 59.9%, for potable water 42.0%, and for cooling tower water 36.0%. The recovery rates decreased as the complexity, i.e., the number of components, of the water increased. In parallel, the reproducibility of the recovery rates also decreased. In the case of distilled water, the values of recovery were very similar for all samples tested (standard deviation, 8%). By contrast, the recovery rates for potable water ranged from 74.1% to 16% (standard deviation, 17.7%), and for cooling tower water from 89% to 7.7% (standard deviation, 32.8%).

TABLE 2. Recovery of *L. pneumophila* by immunomagnetic purification from distilled and potable water (CFU) and cooling tower water (number of copies)

Sample	CFU or no. of copies		Recovery (%)
	Supernatant	Bacterium-beads	
Distilled water	80×10^6	12×10^7	60.0
	70×10^6	12×10^7	63.0
	40×10^6	40×10^6	71.0
	840	1,800	68.2
	105	120	53.3
	870	1,200	58.0
	80	70	46.7
	8	12	58.8
Potable water	294	230	43.9
	1,260	1,600	55.9
	1,260	1,600	55.9
	126	160	55.9
	126	160	55.9
	4	12	74.1
	2	2	48.8
	105	120	53.3
	8	12	58.8
	80	70	46.7
	46	28	37.7
	40×10^7	40×10^7	50.0
	40×10^6	40×10^6	50.0
	1,050	280	21.0
	105	28	21.0
	11	2	16.1
Cooling tower water	462.2	3,778.8	89.1
	22,501.6	6,734.7	23.0
	951.7	452.9	32.2
	184.2	15.6	7.8
	17,3975.9	14,626.3	7.7
	418.7	1,254.7	75.0
	5,069.8	1,053.4	17.2

Design, optimization, and specificity of primers and probes. The primers and probes were designed based on a conserved region of the *L. pneumophila dotA* gene. The sequences were compared to all the nucleotide databases of GenBank (http://www.ncbi.nlm.nih.gov) to ensure their specificity, and the sequences of the *L. pneumophila dotA* gene were unique. The selected regions were analyzed for the requirements imposed by real-time quantitative PCR using Primer Express software (version 2.0), with the resulting primer set (Table 3) producing a product of 80 bp. Nevertheless, a conventional TaqMan probe did not fulfill the requirements imposed by the software, so an MGB probe was designed to maintain the same nucleotide sequence but have a higher melting temperature. In our case, the TaqMan probe had a T_m of 46°C, whereas the MGB probe had a T_m of 66.4°C.

The specificity of the primers and the MGB probe was verified experimentally by using all the species listed in Table 1. The primers set and the probe amplified *L. pneumophila* strains but not any other bacteria or *Legionella* spp. tested in this work.

The concentration of primers was optimized by using 6 pg of DNA from *L. pneumophila* (NCTC 11192) as a template and performing 5′-nuclease assay reactions with different concentrations of forward and reverse primers. This allowed determination of the primer concentrations that gave the lowest C_t values and the highest fluorescence intensity for a normalized reported value. The primer concentrations tested ranged from 300 to 900 nM, while the rest of the parameters were kept invariant, including the annealing temperature and the MGB probe concentration (250 nM, excess following the manufacturer's recommendations). The C_t values we obtained were approximately the same with all combinations, so we used the lowest concentration (300 nM) of each primer.

The optimal concentration of the internal positive control was determined to minimize its competition with the *L. pneumophila* target. Serial 10-fold dilutions of the internal positive

TABLE 3. Oligonucleotides used for real-time amplification of *L. pneumophila* and the internal positive control

Name	Positions on gene[a]	Sequence (5′ → 3′)	Accession no.
*dotA*F	986–1004	ATTGTCTCGCGCGATTGC	AY720956
*dotA*R	1066–1043	CCGGATCATTATTAACCATCACC	AY720956
dotA probe	1006–1027	ATACAGCAAATGTATGTGACTT	AY720956
gyrB probe	590–614	AACAAGACCCCGATCCACCCGAAG	AY101778

[a] Primer and probe nucleotide positions are given according to the complete sequence of the *dotA* gene of *L. pneumophila* ATCC 33152 (AF095231). In the case of the internal control (*gyrB* gene of *A. hydrophila* CECT 839), the probe positions are given using the *Escherichia coli* numbering.

control amplicon were combined with different concentrations of *L. pneumophila* DNA and amplified with the primers *dotA*F and *dotA*R. The concentration containing 39 copies of the internal positive control was selected to be used in each run since no competition was observed with the *Legionella* target (Table 6).

Determination of the standard curve. To develop an external standard curve for quantitative PCR, 10-fold serial dilutions of known *L. pneumophila* DNA concentrations were prepared. Three different analysts performed 21 different amplification experiments in triplicate, for a total of 507 samples. Linear regression analysis, plotting the obtained C_t values versus the logarithm of the *dotA* gene copy number, gave a straight-line plot and a correlation coefficient (r) of -0.973 ($R^2 = 0.947$). The equation resulting from the regression curve obtained was $C_t = -3.03 \log_{10}$ [copy number] + 39.61, and it was linear over at least a 9-$\log_{10}$ range of DNA concentrations, and the reaction had an efficiency of 2.13 (Fig. 1).

The results obtained from the evaluation of the within-run (intra-assay) and between-run (interassay) variations in the multiple assays are shown in Table 4. As determined from the values, the coefficient of variation for the C_t was 1.85% in the intra-assay study (repetitivity) and 3.09% in the interassay study (reproducibility). In contrast, when the intra- and interassay variations were calculated for the number of copies of target DNA, the values were 39.61% and 65.06%, respectively, which are higher than the C_t values. Moreover, differences were found in the coefficient of variation values for both intra- and interassay between samples containing high and low copy numbers: as the number of copies decreased, the coefficient of variation values increased (Table 4 and Fig. 1).

Concerning the limit of detection of the PCR method, *L. pneumophila* was detected in 100% of samples containing as few as 6.9 copies of target DNA, in 85% of samples containing 2.6 copies, in 71% of samples containing 1.6 copies, and in 60% of samples containing 1.4 copies. Due to the low reproducibility at these extremely low concentrations, the limit of quantification was calculated from the standard deviation of C_t values at the limit of detection. For this reason, when the average C_t value at this concentration was increased by three times the standard deviation, the new C_t value was 36.16. Thus, in accordance with the standard curve, the number of copies calculated was 13.8, indicating that reliable quantification was possible above this limit.

Relative accuracy of quantification. A series of experiments were conducted with *L. pneumophila* cultures to determine the correlation between the results obtained by real-time quantitative PCR assays and those obtained by the culture isolation technique. The C_t values obtained from the analysis of 10-fold serial dilutions of *L. pneumophila* cultures were extrapolated to the corresponding external standard curve, previously calculated experimentally. The resulting theoretical number of cells was compared to the CFU obtained from culture isolation (Table 5). A strong positive correlation was found for both methods for the total sample group ($R^2 = 0.94$; $r = 0.97$) (Fig. 2). The slope of the corresponding curve was 1.05.

Quantitative detection of *L. pneumophila* in water samples. The purification of *L. pneumophila* by immunomagnetic separation coupled with the detection by real-time PCR was used to analyze 25 potable water samples and 35 cooling tower water samples. To determine the number of *L. pneumophila* cells presents in each sample, the MGB probe fluorescence was

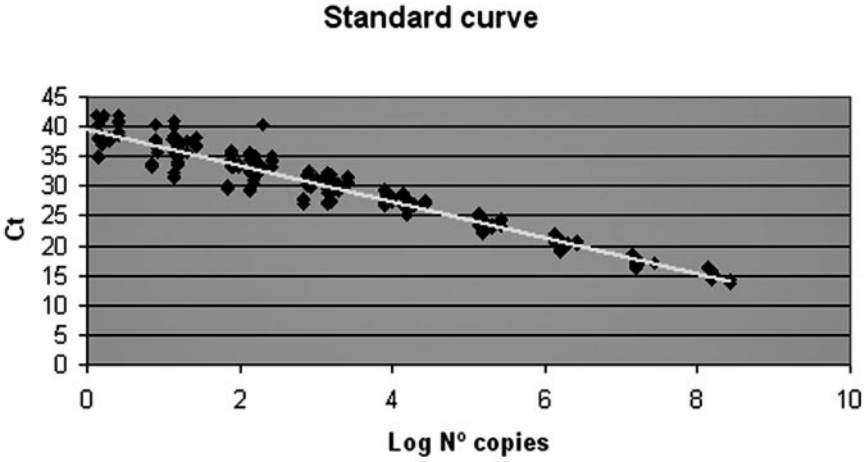

FIG. 1. External standard curve of *L. pneumophila dotA* gene.

TABLE 4. Reproducibility of *dotA* real-time PCR

No. of copies	Coefficient of variation (%)			
	Intra-assay[a]		Interassay[b]	
	C_t	No. of copies	C_t	No. of copies
1,894,405.6	0.47	5.97	0.61	8.0
189,440.6	1.14	17.85	2.96	45.58
18,944.1	0.75	13.12	3.65	60.84
1,894.4	1.59	32.65	3.24	60.31
189.4	2.91	67.37	3.57	91.29
18.9	1.93	50.87	3.42	87.06

[a] Dilution series of DNA of *L. pneumophila* were analyzed in triplicate by two analysts in four different runs. The coefficients of variation (CVs) represent the quadratic mean CVs of the personal means. The personal mean CV was calculated from the 12 triplicates analyzed by one analyst.
[b] The CVs represent the mean of the CVs obtained in samples with the same number of copies by two analysts in four separate runs (four triplicates).

processed, and the C_t values were compared to the external standard curve. For the routine quantification of unknown samples, two known concentrations of *L. pneumophila*, containing 16 and 160 copies of target DNA, were included in every run to control the robustness of the external standard curve (Table 6). The same samples were analyzed in parallel by culture isolation. For potable water, 13 samples yielded positive results by both methods, whereas two were positive only by PCR. In the case of cooling tower water, 22 samples were positive by both methods and 6 samples only by PCR.

In all samples found positive by both culture isolation and real-time PCR, the number of cells obtained by real-time PCR was consistently higher than the CFU obtained by culture counts (Table 5). The regression analysis showed that the average number of cells calculated from the PCR analysis was 20-fold higher than the culture value.

DISCUSSION

Nucleic acid amplification by PCR and more recently real-time PCR can successfully detect legionellae in environmental samples, avoiding the inherent problems of conventional culture isolation (4, 15, 36, 45). PCR techniques have targeted the *mip* gene (4, 15), 5S ribosomal DNA (15), and 16S ribosomal DNA (17, 36, 45). More recently, methods were developed for the simultaneous detection of *L. pneumophila* and *Legionella* spp. based on the 23S-5S internal spacer (17). In this work we have described a new assay based on the specific amplification by real-time PCR of the *dotA* gene, chosen due to its relationship to the virulence of *L. pneumophila*. The specificity of the primers and the MGB probe was confirmed by performing multiple searches of nucleotide databases and by screening 50 different strains of *L. pneumophila*, 12 other *Legionella* species, and six different bacteria common in water.

In this work we have proposed an external standard curve for the quantification of *L. pneumophila* DNA in different real-time PCR runs. By using two control DNA sequences in each run, we can verify the robustness of the standard curve. This external standard curve provides laboratories an easier, faster, and cheaper method for the precise and reproducible quantification of DNA by real-time PCR.

The regression coefficient of the standard curve indicated a

TABLE 5. Accuracy of real-time PCR assay for *L. pneumophila* in dilutions of *L. pneumophila* cultures and water samples

Sample	Theoretical no. of cells	CFU
Dilutions[a]	290,009.33	260,000
	20,571.56	80,000
	890.19	460
	144.63	170
	7.41	12
	7,149.40	5,700
	1,126.81	730
	91.65	125
	19,065.39	20,100
	2,070.24	2,100
	223.10	170
	15.35	70
	37,224.95	21,200
	1,253.37	1,200
	33.34	150
	4,634.98	3,800
	1,404.79	340
	71.86	25
	1.35	5
	21,046.20	22,300
	3,169.13	1,340
	216.41	120
	20.65	12
	2,233.79	2,500
	442.27	290
	18,802.82	2,670
	1,549.30	220
	2,183.10	310
	232.39	33
	35.21	5
Water samples[b]	17,029	1,800
	13,555	120
	54,088	1,200
	2,057	70
	826	12
	6,735	200
	453	20
	16	2
	13,555	120
	826	12
	2,057	70
	62	6
	274	2
	4,268	28
	21,230	280
	4,268	28
	274	2
	7,267	230
	62	6
	9,775	1,600
	1,364	1,600
	1,061	160
	227	160
	146	12
	43	2
	1,761	40
	106	4
	9,731	70
	67	16
	11,568,079	236,000
	60,898	8,148
	1,256	580
	3,620,056	24,638
	31,442	10,670
	3,819	1,261

[a] C_t values determined from the analysis of different dilutions of *L. pneumophila* cultures were extrapolated to the external standard regression curve to calculate the theoretical number of cells per reaction.
[b] C_t values determined from the analysis of different water samples were extrapolated to the external standard regression curve to calculate the theoretical number of cells per reaction.

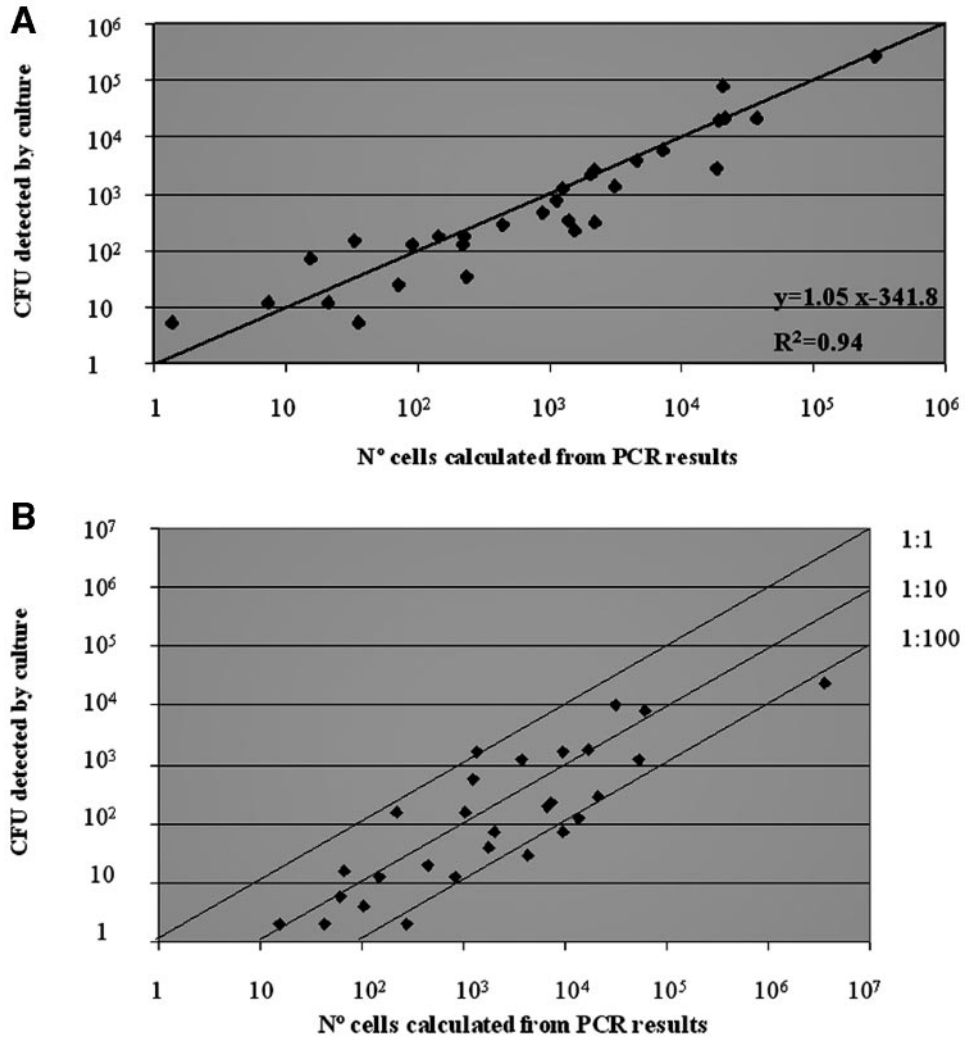

FIG. 2. Correlation between real-time PCR and culture for *L. pneumophila*. (A) Dilutions of *L. pneumophila* cultures. (B) Water samples.

good correlation between the number of copies of target DNA and the amount of amplified product (represented by the C_t value). The obtained value for the slope of the standard curve (−3.03) indicated that the amplification efficiency was very near the optimal slope value of −3.32. Moreover, the R^2 value was 0.947, indicating that the PCR system was highly linear. Therefore, the high correlation and linearity of the standard curve indicated that the assay was suitable for quantitative measurements.

TABLE 6. Optimization of internal positive control (IPC) concentration

No. of copies of *L. pneumophila dotA*	C_t with IPC and *dotA* probes at the indicated no. of IPC copies:					
	390		39		0	
	IPC	*dotA*	IPC	*dotA*	IPC	*dotA*
1,000	35.41	30.83	37.55	30.87		30.68
100	36.22	34.22	36.17	32.79		32.67
10	34.05	36.69	35.59	36.38		35.11
0	34.24		34.48			

The detection limit of our assay was approximately 6.85 copies of target DNA (29.9 fg), slightly more than the 4.3-fg *L. pneumophila* genome (5), and is comparable with data obtained by other authors (16, 31, 36, 37, 41, 46). Recently, it has been described that minor groove binding probes, such as we have designed, increase the sensitivity of bacterial detection 10- to 100-fold (33).

On the other hand, the standard curve is accurate enough to be employed for different runs since it is generated from a wide range of concentrations that were analyzed in triplicate and repeated several times. Moreover, the robustness of the standard curve is controlled in each run by the amplification of two different *L. pneumophila* DNA standards. The method for creating the calibration curve has recently been introduced into the Light Cycler software (24). Thus, the previous publication, combined with our current results, demonstrates that it is not necessary to prepare a standard curve with each run. This new methodology provides a much easier and faster method for

precise and reproducible quantification of both DNA and target cell numbers present in a given sample.

The main problem for real-time PCR of samples containing low DNA concentrations is that the C_t values are greatly scattered, even for triplicates of the same concentration. The reproducibility of our PCR method was evaluated using the coefficient of variation values of several intra- and interassay studies. The coefficient of variation was calculated for C_t as well as for calculated copy numbers. However, because of the exponential nature of PCR amplification, the concentration and C_t have a log-linear relationship, so the expected coefficients of variation for C_t are lower than those for the concentration. Additionally, the imprecision of PCR assays is unavoidably larger than that observed in classical clinical chemistry or immunological assays. The results we obtained in this work are comparable to those obtained by other authors (16, 36, 41).

When *Legionella* cultures were used, we found that the correlation between our PCR method and the standard culture isolation method agreed with previous findings by Rodriguez-Lázaro et al. (37). By contrast, when unknown water samples were analyzed, we found that PCR results yielded higher values in all cases, in agreement with other reports (18, 45). All of these results appear plausible since amplification of exponential cultures of legionellae occurs with the majority of cells in a viable cultivable state. Thus, PCR and culture values should coincide. By contrast, when environmental samples are analyzed by PCR, all DNA is amplified, including DNA from dead bacteria and from viable but noncultivable bacteria. Moreover, in the case of legionellae, recovery rates of culture are usually less than 100% due to specific requirements of growth, overgrowth by other bacteria, and legionella loss and damage during sample preparation. For these reasons the number of cells calculated by PCR is always higher than the number of CFU.

The abundant presence of PCR inhibitors, such as heavy metals and organic matter, has been demonstrated in environmental samples. Different methods of DNA purification have been developed (1, 19, 23, 43), but despite their advantages, inhibition of PCRs is frequent in some problematic samples. For this reason the use of an internal positive control in PCRs is very important to monitor the efficiency of the reaction and to evaluate the possible presence of false-negative results. In this work, a real-time PCR method has been developed that uses the same primer set for simultaneous amplification of the *L. pneumophila dotA* gene and an internal positive control. This method has the advantage of rapid detection of both the target gene and possible PCR inhibitors while maintaining the sensitivity of the PCR assay. We have also reported the usefulness of an immunomagnetic separation method to isolate *L. pneumophila* from heterogeneous bacterial suspensions in water, avoiding PCR inhibitors. In this way the strategy was to purify intact cells rather than DNA. Such immunomagnetic separation techniques have been required to facilitate the rapid DNA detection of some bacteria, viruses, and parasites (26, 30, 32, 40).

In this work we used polyclonal antibodies specific for a number of *L. pneumophila* surface antigens to coat magnetic beads. This avoided the problem of the high level of specificity that can sometimes occur with monoclonal antibodies, and it also increased the likelihood of isolating the desired organism.

The efficiency of recovering *L. pneumophila* cells was similar to that obtained by various authors, ranging from 47% to 87% for microorganisms as different as *Mycobacterium avium*, *Campylobacter jejuni*, and *Cryptococcus neoformans* (20, 26, 44). The efficiency we obtained was for water samples spiked with known concentrations of *L. pneumophila* and without other enrichment techniques commonly performed for other pathogens (11). Some bacteria always remained in the supernatant because the immunobeads could not bind all cells even at the higher dilutions. Water from cooling towers and highly contaminated samples exhibited still smaller recoveries. The efficiency of the method was likely reduced by the presence of debris and other microorganisms that severely compromise the method by acting as nontarget objects (13, 27). In some samples the beads were hardly attracted to the magnetic particle concentrator because of physical impediments. Sample turbidity, for example, is known to reduce the sensitivity of the method (27). Also, subsequent PCRs were inhibited in some of these samples, as shown by the addition of internal control DNA to the PCR mixtures. Although immunomagnetic separation can separate microorganisms of interest from polymerase-inhibitory factors (26) present in environmentally contaminated samples, this did not occur in all cases (27), possibly due to the environmental origin of the samples.

In conclusion, the real-time PCR system that was evaluated and validated in this study, combined with immunomagnetic separation, provides many benefits (speed, specificity, accuracy, sensitivity, stability, and cost-effectiveness) for the quantitative detection of *L. pneumophila* in potable water and other relatively clean environmental water samples. The use of immunomagnetic purification may be limited in extremely contaminated samples, where the recovery efficiencies are highly variable due mainly to the principle of the method and the special characteristics of the sample. For these reasons, more efficient purification methods should be investigated and current purification methods should be improved. The integration of rapid and efficient sample preparation methods with rapid amplification and detection technologies, such as those described here, should improve the management and prevention of *Legionella* outbreaks.

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