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Validation according to ISO/TS 12869:2012 of a molecular method for the isolation and quantification of *Legionella* spp. in water

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ABSTRACT

The aim of the present work was to validate the performances of a new molecular method comprehensive of water sample filtration, DNA extraction and Real-Time PCR for the quantification of *Legionella* spp. in clear water samples, in accordance with the recent ISO Technical Specification 12869:2012. All criteria and requirements were verified considering inclusivity and exclusivity, check of the calibration function, limit of detection and limit of quantification, recovery calculation, robustness and uncertainty of the entire method. The performances were validated as all parameters resulted to be in compliance with values detailed by the above mentioned standard. The described method proved to be specific, sensitive, accurate and it has been fully validated according to ISO/TS 12869:2012. The possibility of using a validated molecular method will improve the reliability of the results making it a promising tool that should be used in addition to cultural analysis. Moreover, these findings make it particularly suitable for a relatively inexpensive screening of water samples, reducing the turnaround time and the workload.

1. Introduction

Legionella is a Gram-negative aerobic bacillus that generally colonizes natural and artificial aquatic environments, especially the hot water of peripheral distribution system [30]. Humans may be infected through inhalation of contaminated aerosolized water droplets [26]. Legionellosis has two clinically distinct forms: Legionnaires' disease, a severe type of infection which includes pneumonia, presenting high lethality in immunocompromised hosts, and Pontiac fever, a milder self-limiting illness [12,20].

Legionnaires' disease remains an important cause of both morbidity and mortality and in 2012, 5852 cases of Legionnaires' disease were reported in the EU Member States, Iceland and Norway. Six countries (France, Italy, Spain, Germany, Netherlands and the United Kingdom) accounted for 84% of all notified cases. Even though sporadic community-acquired cases represent the majority of notified cases, large outbreaks continue to occur, placing temporary pressure on local healthcare services [14].

Currently, more than 50 *Legionella* species are described and 70 serogroups [35] are identified, but *Legionella pneumophila* serogroup 1 is responsible for 39–70% of all *Legionella* nosocomial infections in Europe and for more than 84% of all Legionnaire's disease cases that occur worldwide, [9,32].

This is, primarily, due to the fact that *L. pneumophila* serogroup 1 is the most frequent bacterium of the species circulating in water systems [10]. Various parameters support the presence of *L. pneumophila* in water, such as hardness, temperature (between 25 and 45 °C), corrosion, scale, flow regimes [9,19], ability to produce monospecies biofilms [27] and resistance to disinfectant [18] particularly when chlorine is adopted in *Legionella* risk control.

Environmental surveillance and monitoring of *Legionella* spp. is crucial for evaluating risk and identifying control strategies [11]. U.S. Centers for Disease Control and Prevention (CDC) and the European Health Protection Agency recommend culture analysis to identify *Legionella* in water samples, but this quantitative microbiological culture method reflects measurement error and generally underestimates cell densities by 10–60 % as indicated by results from the CDC Elite proficiency testing program [22] and from McCoy et al. [23], which note that error in estimated counts from *Legionella* culture analysis could arise.

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Furthermore, this approach requires up to ten days and is limited by the physiological state of the cells. For instance, *Legionella* cells that are viable but nonculturable (VBNC) or internalized within amoebae are not detected by conventional culture method [29]. This is further complicated by difficulties in isolating legionellae in samples containing high background levels of other microorganisms. Additionally, some *Legionella* species grow poorly on conventional solid media used for the routine isolation.

As *Legionella* is a pathogen of public health concern, the consequences of reporting false negatives or underreporting the concentration of *Legionella* may be serious [34].

To respond to this need, recently, rapid assays based on quantitative Real-Time PCR (qPCR) have been proposed to quantify the presence of *Legionella* spp. in water [8,13,16,21,24,33,36] underlining the need for reassessment of national regulations or guidelines.

The qPCR methods can be applied to both the routine monitoring of water supply systems and for the follow-up of disinfection treatments and are specific and sensitive [3,13,16]. The development of standardized and validated qPCR methods involving the integration of efficient sample preparation techniques with rapid amplification should significantly improve the detection, prevention and management of *Legionella* infection.

A technical specification [6] has been elaborated on the basis of preexisting standard NF T90-471 [5]. The ISO/TS specifies the method for the detection and quantification of *Legionella* spp. and *L. pneumophila*, defining requirements and performances to ensure the reliability of results of each assay and the equivalence among different Real-Time PCR assays.

The aim of this study was to carry out a validation of a new commercial system for the detection and quantification of *Legionella* spp. in water samples, according to the ISO/TS 12869:2012.

Table 1
List of *Legionella* species used for the study.

Species	Origin	Real-Time PCR result
L. cincinnatiensis	Collection strain	+
L. birminghamensis	Collection strain	+
L. oakridgensis	Collection strain	+
L. micdadei	Collection strain	+
L. bozemanii	Collection strain	+
L. bozemanii	ATCC35545	+
L. anisa	ATCC35292	+
L. cherrii	ATCC35252	+
L. erythra	ATCC35303	+
L. dumoffii	ATCC35850	+
L. feleii	ATCC35849	+
L. gormanii	ATCC33342	+
L. hackeliae	ATCC35250	+
L. jordanis	ATCC 33623	+
L. lansingensis	ATCC 49751	+
L. longbeachae	ATCC33484	+
L. parisiensis	ATCC35299	+
L. sainthelensi	ATCC35248	+
L. tucsonensis	ATCC49180	+
L. wadsworthii	ATCC33877	+
L. gormanii	ATCC33342	+
L. maceachernii	ATCC35300	+
L. pneumophila SG1	ATCC33152	+
L. pneumophila SG9	Collection strain	+
L. pneumophila SG7	ATCC33823	+
L. pneumophila subsp. fraserii SG5	ATCC33216	+
L. pneumophila subsp. pneumophila SG2	ATCC33154	+
L. pneumophila subsp. pneumophila SG3	ATCC33155	+
L. pneumophila subsp. pneumophila SG10	ATCC43283	+
L. pneumophila subsp. pneumophila SG1	ATCC33153	+
L. pneumophila subsp. pneumophila SG6	ATCC33215	+
L. pneumophila subsp. pneumophila SG8	ATCC35096	+
L. pneumophila subsp. pneumophila SG12	ATCC43290	+
L. pneumophila subsp. pneumophila SG14	ATCC43703	+

2. Materials and methods

2.1. Study design

The investigated molecular method entails of a three-step process: filtration of water samples, bacterial DNA isolation and subsequent quantification through Real-Time PCR. The validation of the entire method was conducted on the basis of the protocol, experimental designs and calculation methods described by ISO/TS 12869:2012. The first step of the validation examined the amplification kit and the following parameters were investigated: inclusivity and exclusivity, linearity, limit of detection (LOD), limit of quantification (LOQ) and assessment of the standard curve through a connection with the reference material available from the Legionelles Centre National de Référence (Lyon, France).

The second step was performed considering the whole method (filtration, extraction and amplification) and concerned the evaluation of recovery, robustness and uncertainty measurement.

2.2. Bacterial growth conditions

A total of 50 bacterial strains were supplied by the DSMZ (Braunschweig, Germany) and rehydrated, collected and maintained in culture from the laboratory of the Department of Biomolecular Sciences (University of Urbino, Italy) as recommended by Association Français de Normalisation (AFNOR, Paris, France) NF T90-471 and ISO/TS 12869:2012.

Briefly, all 34 *Legionella* strains used in this study (Table 1) were rehydrated in Nutrient Broth and were cultured on Legionella CYE Agar Base supplemented with L-cysteine HCl (Oxoid Ltd., Hampshire, UK) incubated at 35 ± 2 °C, 3% CO_2 for 3–5 days and examined for the presence of bacterial colonies having characteristics of *Legionella* bacteria.

16 non-*Legionella* bacteria, listed in Table 2, were rehydrated in Nutrient Broth and were cultured according to their appropriate growth agar and temperature (www.dsmz.de/catalogues/catalogues-microorganisms.html).

2.3. Types of water samples

The samples of distilled sterile water, mineral natural water and sulfurous water (all without *Legionella* DNA) were taken in sterile containers and immediately investigated.

Table 2
List of non-*Legionella* species used for the study.

Species	Origin	Real-Time PCR result
Aeromonas hydrophila subsp. hydrophila	ATCC7966	–
Alcaligenes faecalis subsp. faecalis	ATCC8750	–
Bacillus subtilis	ATCC6633	–
Burkholderia cepacia	ATCC17770	–
Clostridium spp.	ATCC25772	–
Enterobacter aerogenes	ATCC13048	–
Escherichia coli	ATCC25927	–
Flavobacterium spp.	ATCC15997	–
Klebsiella oxytoca	ATCC8724	–
Listeria monocytogenes	ATCC9525	–
Proteus vulgaris	Collection strain	–
Pseudomonas aeruginosa	ATCC10145	–
Pseudomonas fluorescens	ATCC13525	–
Pseudomonas putida	ATCC17514	–
Serratia marcescens	ATCC14756	–
Strenotrophomonas maltophila	ATCC19374	–

2.4. Validation of the New Legionella spp. quantitative kit

2.4.1. Inclusivity and exclusivity

The specificity was checked according to the main criteria provided by the ISO/TS 12869:2012 by testing the *New Legionella spp. quantitative kit* (Diatheva, Fano, Italy) on several species of *Legionella* genus (Table 1) and other non target microorganisms likely to be found in the same ecological niches (Table 2).

Genomic bacterial DNAs were extracted from typical colonies using Bacterial genomic DNA isolation kit (Diatheva) according to the manufacturer's instructions. DNA concentrations were determined by spectrophotometry at 260 nm. Genomic units (GU) for *Legionella* species were calculated assuming that the genome of *L. pneumophila* is 4.3 fg (according to AFNOR NF T90-471), while the GUs estimated for non target bacterial species were based on their genome size (www.ncbi.nlm.gov/genomes/lproks.cgi).

Inclusivity and exclusivity tests were performed using the amplification assay on DNA extracts containing 10^2 and 10^5 GU/PCR well respectively.

2.4.2. Study of the calibration function

Five independent series of dilutions starting from 2.5×10^4 to 25 GU/PCR well were prepared on different days and by different operators using *L. pneumophila* ATCC33152 DNA extracted as described in Section 2.4.1 "*Inclusivity and exclusivity*". Each dilution was amplified by the *New Legionella spp. quantitative kit* and the calibration function was calculated by linear regression analysis of threshold cycles (Ct) measured for each amplification vs. the $\log_{10}$ copy number for each standard dilution. The measured quantity for each dilution level was determined in retrospect by using the formula $\log x = [Ct - b]/a$, where a is the slope, b is the y-intercept, and x is the quantity. The mean bias (mb) and the standard deviation (SD) were determined comparing the obtained values to the theoretical quantities for each load level. The accuracy of linearity (E_{lin}) for the entire calibration range was calculated using the formula $E_{lin} = \sqrt{(SD)^2 + (bias)^2}$. Expanded uncertainty of linearity (U_{lin}) was also defined by the formula $U_{lin} = E_{lin} \times t_{k-2}$. Finally, the efficiency (e) of the amplification was assessed starting from the slope value using the formula $e = (10^{-1/a} - 1) \times 100$.

2.4.3. Limit of detection and quantification

The limit of detection (LOD) is defined as the smallest number of GU per assay which gives a qPCR positive result (amplification) in at least 90% of cases. For the LOD determination 14 separate dilutions containing 5 and 10 GU/PCR well were analyzed.

According to the ISO requirements the limit of quantification (LOQ) corresponds to the first level of the calibration range (25 GU) and is the smallest number of GU that can be quantified. Fourteen dilutions series were tested to determine accuracy (E_{LQ}) and uncertainty (U_{LQ}) at the LOQ using the same formulas described in the section 2.4.2 "*Study of the calibration function*".

The theoretical limits of quantification and detection of the whole method (LOQ_{meth}, LOD_{meth}) were calculated by the formula

$$LOQ_{meth} = LOQ \times F/V \text{ and } LOD_{meth} = LOD \times F/V$$

where:

F is a conversion factor of number of GU/PCR well to number of GU/L and V is the filtered volume of sample expressed in liters.

2.4.4. Connection of the primary standard to the reference material

The Standard DNA provided by *New Legionella spp. quantitative kit* (primary standard) was correlated to the French *L. pneumophila* DNA Standard (SMR_LEGDNA_01 standard from Legionelles Centre National de Référence, Lyon, France) provided with a certified value of $10.6 \times 10^6 \pm 1.7 \times 10^6$ GU. Briefly, three independent decimal dilution ranges starting from 2.5×10^4 to 25 GU/PCR well were obtained from both standard DNAs and amplified in the same run.

A calibration function was established by linear regression, using Ct obtained from the amplification of primary standard dilutions. Starting from Ct values for each level of French *Legionella pneumophila* Standard DNA, the decimal logarithm GU values (quantity found per level) were calculated by reverse calibration. The calibration error per level was determined subtracting the theoretical quantity to the quantity found per level according to the ISO technical specification. To verify the equivalence of the slopes (PCR efficiency) the absolute value of the difference between the deviations (calibration error per level) at the highest point and lowest point of the range was calculated as follows $|\log10(25{,}000) - \log10(25)|$.

2.5. Validation of the entire molecular method

2.5.1. Recovery robustness and uncertainty measurement of the whole method

The recovery of *Legionella* was evaluated according to ISO/TS 12869:2012 on artificially contaminated water samples prepared as follows: colonies of *L. pneumophila* ATCC33152, that are less than 72 h old, were inoculated in Tryptone salt (NaCl 8.5 g l^{-1}, Peptone 1 g l^{-1}) to obtain a mother suspension with an optical density (OD_{600nm}) of 0.5 corresponding to 10^9 CFU ml^{-1}. Serial 10-fold dilutions in the same medium were prepared and used for the artificial contamination of water samples (free of nucleic acids of *Legionella*) enabling to obtain the respective quantities of 10^5 and 10^3 CFU. For each level of concentration, at least 10 separate spiked samples were prepared, on different days and by several operators and were processed starting from the filtration, with the entire molecular method. Briefly, the bacteria from the water sample were concentrated by filtration on membrane filters of polyvinylidene fluoride (PVDF) (PALL, Port Washington, USA), with a low adsorption capacity of either protein or DNA, and a porosity of 0.45 μm. The lysis step was carried out by the combined effect of a chaotropic buffer and temperature and DNA purified through silica columns. Columns were washed twice to eliminate molecules that may inhibit subsequent reactions. Finally, DNA was eluted using a low ionic strength-buffer, pH 8.5 and directly used for the amplification by the *New Legionella spp. quantitative kit*. Moreover, *Legionella* DNA from the mother suspension was isolated and amplified to estimate the GU number: three 100 μl-aliquots were subjected to DNA extraction according to the lysis protocol described above without column purification and lysates diluted 1:1000 before the amplification.

The mean value of bacterial concentration (expressed as GU) obtained for mother suspension was used as reference to calculate *A* expressed as decimal logarithm in the formula below, while the GU estimated for each spiked sample was used to calculate *B*. The recovery for each sample was determined using the formula:

$$\log_{10}\eta_x = B - A + D + \log_{10}1000/V_{pe}$$

where:

$\log_{10}\eta x$ is the decimal logarithm of recovery for sample x; *A* is the reference value for the concentration of the mother suspension, expressed as a decimal logarithm of the number of genome units per milliliter; V_{pe} is the volume of the spiking suspension, in microliters, μl; *B* is the value measured from the spiked sample, expressed as a decimal logarithm of the number of genome units per sample; *D* is the decimal logarithm of the dilution factor between the mother suspension and the spiked suspension, considering a value 3 for the 100,000 GU level and 5 for the 1000 GU level.

The robustness of the assay, that is, the effect of the matrix that leads to false negative results, was determined calculating the recovery values as described above by analyzing tap and sulfurous water for a total of 20 samples contaminated with 10^3 and 10^5 CFU l^{-1} as previously described.

The uncertainty measurement ($U_{overall}$) of the whole method was calculated from the average recovery values (η_x) and variance (s^2) for all tested matrices by the formula

$$U_{overall} = 2 \times \sqrt{\eta_x} + s^2.$$

2.5.2. Statistical analysis

All statistical analysis were performed according to the ISO/TS 12869:2012 recommendations.

3. Results

3.1. Validation of the New Legionella spp. quantitative kit

3.1.1. Inclusivity and exclusivity

The specificity of the amplification assay was assessed on a panel of target and non target species. The results showed in Tables 1 and 2 indicated that all the 34 *Legionella* species were positively identified, while none among the 16 non-*Legionella* bacteria produced an amplification signal.

3.1.2. Study of the calibration function of Real-Time PCR assay

Table 3 summarizes the results obtained from the study of calibration function conducted by amplifying five separate serial ten-fold dilutions of *L. pneumophila* ATCC33152 DNA ranging from 25,000 to 25 GU/PCR well. The calibration function was calculated by linear regression of the threshold cycles (Ct) versus the logarithm of the *L. pneumophila* concentrations per reaction and resulted to be $y = -3.48x + 32.34$ corresponding to a validated qPCR efficiency of 93.8%. Accuracy (E_{lin}) and uncertainty (U_{lin}) were also calculated and values are shown in Table 3.

3.1.3. Limit of detection and quantification

LOD and LOQ were investigated using DNA extracted from *L. pneumophila* ATCC33152. The LOD corresponded to 5 GU/PCR well, detected in 100% cases. E_{LQ} and U_{LQ} at the LOQ (25 GU/PCR well) were determined to be 0.14 and 0.3 respectively (see appendices section).

The theoretical limits of quantification and detection of the whole method resulted to be 500 GU l^{-1} and 100 GU l^{-1}, considering an F value of 20, which takes into account all dilution factors deriving from different steps of the method.

Table 3
Verification of the calibration function.

GU/PCR well level	25,000	2500	250	25
Theoretical quantities $\log_{10}$	4.40	3.40	2.40	1.40
Mean Ct ± SD[a]	17.20 ± 0.46	20.43 ± 0.42	23.61 ± 0.34	27.74 ± 0.39
Measured quantities $\log_{10}$	4.35	3.42	2.51	1.32
Mean bias	−0.04	0.03	0.11	−0.08
Standard deviation	0.13	0.12	0.10	0.11
E_{lin}	0.14	0.12	0.14	0.14
U_{lin}	0.44	0.39	0.47	0.44

[a] Ct measurements obtained for 4 different levels of genomic unit quantities obtained from 5 independent amplification assays.

3.1.4. Connection of the primary standard DNA to the reference material

Parameters analyzed in order to evaluate the connection between the Standard DNA provided by *New Legionella spp. quantitative kit* and French *L. pneumophila* Standard DNA are reported in Table 4. The equivalence of the slope was verified and calculated to be 0.09, while the mean calibration error was 0.14, both values were compliant as less than the critical value of 0.15.

3.2. Validation of the entire method

3.2.1. Recovery of Legionella from spiked samples robustness and uncertainty measurement of the whole method

Tests conducted for the calculation of the recovery and robustness of the entire method were performed in order to verify the conformity of the method to the ISO. Distilled sterile water samples and two other matrices (tap and sulfurous water) artificially contaminated with two different concentration levels under intermediate precision conditions (over several days, by several technicians) showed different recovery values, ranging from 38 to 75%. The calculated recovery values for both contamination levels are reported in Table 5.

$U_{overall}$ calculated starting from the average recovery value of −0.29 and from the variance value of 0.04 resulted to be 0.71.

4. Discussion

Nearly 90% of *Legionella* infections in humans are attributable to *L. pneumophila* [25] however, a total of 19 other *Legionella* species such as *Legionella micdadei*, *Legionella dumoffi*, *Legionella bozemanii* and *Legionella longhebeachae* are known to cause disease in humans as they have been isolated from clinical cases [35].

For this reason, National (2000) and European Guidelines (2000) [17] for *Legionella* spp. control and prevention require the risk analysis and microbiological surveillance of all *Legionella* species. These regulations include a culture based detection and enumeration according to the ISO 11731 [4]. Although the microbiological method is considered the gold standard, it is often limited by various issues. For example, there may be considerable losses of legionellae during the concentration, resuspension and pretreatment phases and the growth could be affected by both the presence of other bacterial organisms and VBNC *Legionella* cells.

The availability of rapid, specific and sensitive methods could be of special relevance for water risk management strategy. During the last decade, many molecular assays [28,31] have been proposed as attractive alternative procedures. Most of them, have been developed and subsequently validated for the detection and quantification of *Legionella* carrying out a comparison with culture. The obtained results reported a discrepancy of 50% between culture and molecular method, that could be explained by both an underestimation of culture [34] and differences of molecular methods optimized without following a universal accepted procedure. The first step towards standardization of a qPCR procedure was accomplished by the AFNOR through the publication in 2006 of the NF T90-471 [5]. The experimental standard was subsequently revised and modified in 2010, including the use of a certified reference material [7]. Only at the end of 2012, the ISO/TS 12869 was introduced for validating qPCR methods, and ensuring the equivalence of results obtained by laboratories also when different molecular assays are used.

To the extent of our knowledge, none of the previous published paper report data about validation in accordance with the recent ISO technical specification are available.

The study described here was carried out with the aim of providing a validated method for the detection and enumeration of

Table 4
Connection of DNA standard provided by *New Legionella spp. quantitative kit* and French *L. pneumophila* DNA standard.

	Reference range[a]				Calibration solution[b]			
GU/PCR well level	25	250	2500	25,000	25	250	2500	25,000
Theoretical quantities $\log_{10}$	4.40	3.40	2.40	1.40	4.40	3.40	2.40	1.40
Mean Ct ± SD	28.20 ± 0.03	23.85 ± 0.04	20.94 ± 0.36	17.76 ± 0.18	28.64 ± 0.05	24.84 ± 0.09	21.19 ± 0.08	18.06 ± 0.21
Calibration function		$y = -3.42x + 32.61$						
Quantity found per level					1.16	2.27	3.34	4.26
Calibration error per level					−0.24	−0.13	−0.06	−0.14
Mean calibration error					−0.14			
Equivalence of the slopes					0.09			

[a] Reference range refers to dilution range created from DNA standard provided by New *Legionella* spp. quantitative kit.
[b] Calibration solution refers to dilution range created from French *L. pneumophila* DNA standard.

Table 5
Recovery and robustness of the whole method.

Theoric level of contamination	1000 GU l^{-1}			100,000 GU l^{-1}		
	Sterile water	Tap water	Sulfurous water	Sterile water	Tap water	Sulfurous water
Real level of contamination	1603.73	774.40	900.50	160388.25	77440.00	90055.00
Results for quantification GU l^{-1}	654.00	846.67	580.00	57653.00	32600.00	55060.00
Recovery $\log_{10}$	−0.46	−0.05	−0.19	−0.46	−0.37	−0.21

Legionella spp. according to main parameters defined by the ISO 12869. The method consists of a filtration step of the water sample, followed by bacterial DNA isolation and quantitative Real-Time PCR analysis. The total analysis time was about 3 h, in contrast to the 11 or more days required for the culture method.

Legionella genus is widely distributed in nature and is specifically associated with aquatic environments where many other non target bacteria could be present and interfere with the qPCR. For these reasons, the ISO establishes that the inclusivity of the amplification assay must be verified on a wide list of *Legionella* strains as well as the exclusivity, testing several non target bacteria usually founded in the same ecosystem. The results obtained by testing the amplification assay on requested species yielded for both parameters a satisfactory value of 100%.

The study of the calibration function was performed in order to verify the accuracy and efficiency of the qPCR assay, and the results obtained showed a high level of performance, confirming the suitability of the assay for quantification purposes. The linearity domain was checked on five independent series of dilutions starting from 2.5×10^4 to 25 GU/PCR well of the Standard DNA provided by the kit. E_{lin} parameters calculated for each level of standard curve were less than the critical value of 0.15 required by the ISO 12869, validating the qPCR linearity domain from 25,000 GU to 25 GU/PCR well. The qPCR efficiency resulted to be 93.8%, close to the theoretical optimal that lies between 75 and 125%, proving further validity for quantification.

The overall consistency of the standard curve provided by the molecular assay was confirmed performing additionally tests using a well characterized and certified reference material. Results obtained in the connection study were clearly in compliance with the requirements of the ISO, ensuring the trueness and precision in the quantification of the *Legionella* DNA.

The amplification assay resulted to be very sensitive, in fact the detection limit was estimated to be 5 GU/PCR well, detected in 100% of cases. The limit of quantification was checked at 25 GU/PCR well, in order to verify the accuracy and the uncertainty at this level. The E_{lin} calculated was 0.14 (below the critical value of 0.15) and uncertainty measurement was 0.3, confirming that the quantification limit was repeatable and accurate up to 25 GU/PCR well according to the Student test. The theoretical LOQ_{meth} and LOD_{meth} values were 100 and 500 GU l^{-1} respectively, resulting more sensitive than other reported for some qPCR systems validated according to the AFNOR procedure [15].

With the objective of applying the molecular method for quantification of *Legionella* in water samples the establishment of an optimal recovery is extremely important to ensure not only high sensitivity but also the consistency of results. At the same time, the quantification of the *Legionella* DNA must not be affected by the type and nature of water samples that are analyzed by laboratories.

For these reasons, the recovery and robustness of molecular assay were evaluated with distilled sterile water, sulfurous and tap waters, artificially contaminated with 1000 and 100,000 GU l^{-1} of *L. pneumophila*. The average recovery rates obtained for all tested matrices were greater than the minimum yield requested by the ISO (>25%), ranging from 38 to 75%, with an uncertainty of 0.71. The recovery evaluation has allowed to demonstrate the reliability of results, ensuring that during the filtration and DNA extraction processes, there is only a minimum loss of bacterial cells. Moreover the presence of an internal amplification control permitted to exclude any possible inhibition effects, differently to culture process where there is no similar control.

The performance on real samples is unknown, and the evaluation is exclusively a technical evaluation.

Despite the fact that ISO/TS 12869:2012 did not take into account the possibility to distinguish live from dead cells, we know that this could a limiting factor for the application of Real-Time PCR. For this reason, further improvement of the method, concerning the introduction of a DNA-intercalating agent, such as ethidium monoazide, able to selectively amplify only the DNA of living cells [15,34], is under investigation.

5. Conclusions

In conclusion, we would like to underline that the investigated method has been validated showing appropriate performances characteristics complying with the international standard TS 12869 and, although culture represents the reference method, as suggested by other Authors qPCR should be used in addition to culture rather than an alternative [21,34]. For example, as a screening tool offering a lot of advantages in terms of specificity, sensitivity and a considerable reduction of analysis time that could be convenient when timely corrective actions are required.

Appendices

Table 6
Verification of detection limit.

GU/PCR well level	25
Theoretical quantities $\log_{10}$	1.40
Mean Ct ± SD	27.71 ± 0.42
Regression curve	y = −3.48x + 32.34
Measured quantities $\log_{10}$	1.33
Mean bias	−0.07
Standard deviation	0.12
E_{LQ}	0.14
U_{LQ}	0.30

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