Received: 14 February 2021 | Revised: 4 April 2021 | Accepted: 9 April 2021

DOI: 10.1111/jam.15103

ORIGINAL ARTICLE

Journal of Applied Microbiology

Comparison of the culture method with multiplex PCR for the confirmation of *Legionella* spp. and *Legionella pneumophila*

D. Eble[1] | V. Gehrig[2] | P. Schubert-Ullrich[1] | R. Köppel[2] | H.P. Füchslin[2]

[1]Industrielle Werke Basel, Basel, Switzerland

[2]Kantonales Labor Zürich, Zürich, Switzerland

Correspondence
Hans Peter Füchslin, Kantonales Labor Zürich, Fehrenstrasse 15, Zürich 8032, Switzerland.
E-mail: hanspeter.fuechslin@kl.zh.ch

Abstract

Aims: The detection and enumeration of *Legionella* spp. in water samples are typically performed via a cultural technique standardized in ISO 11731. This method is time-consuming (up to 15 days), and the specificity of the confirmation step is questionable. This study proposes the use of multiplex polymerase chain reaction (PCR) to confirm presumptive *Legionella* colonies directly from the culture plate; this shortens the response time by 2–5 days while still reporting results in colony forming units (CFU).

Methods and Results: Two laboratories analysed a total of 290 colonies to compare the confirmation step of *Legionella* spp. and *Legionella pneumophila* in accordance with ISO 11731 by culture growth and agglutination *vs* multiplex PCR. Discordant results were resolved by the swiss national reference laboratory. The data were evaluated following ISO 16140 and showed that the PCR-technique had higher specificity.

Conclusions: The confirmation of *Legionella* spp., *L. pneumophila* and *L. pneumophila* serogroup 1 by multiplex PCR allows detection of positive colonies more rapidly and with higher specificity.

Significance and Impact of the Study: The study highlights a possibility to shorten the response time significantly during the enumeration of *Legionella* spp. and achieving a higher specificity while adhering to the legally recognized reporting in CFU.

KEYWORDS

confirmation, culture, identification, *Legionella pneumophila*, *Legionella* spp., multiplex PCR, serogroups, water

INTRODUCTION

Legionella spp. are gram-negative bacteria that can cause legionellosis in humans after being inhaled together with aerosols containing them. The symptoms of legionellosis can vary from a flu-like Pontiac fever to Legionnaires' disease—an atypical and acute pneumonia (Stojek and Dutkiewicz 2006) Therefore, *Legionella* spp. represent a significant health risk, especially for the elderly and people with a weakened immune system or underlying morbidities (Collins *et al*. 2015).

Legionella spp. tend to colonize artificial water systems with temperatures between 20 and 45°C and can reach high concentrations. Systems with the potential for aerosol formation, such as hot and cold water systems, cooling towers, evaporation condensers and whirlpools, are considered to be particularly risky (Bartram 2007). In response to these risks, national health authorities and drinking water legislations in

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.
© 2021 The Authors. *Journal of Applied Microbiology* published by John Wiley & Sons Ltd on behalf of Society for Applied Microbiology

Europe recommend regular testing for *Legionella* spp. and prescribe legal maximum values (e.g., Bundesministerium der Justiz und für Verbraucherschutz, 2016; Eidgenössische Departement des Innern, 2016). Moreover, the operators of public bath and shower facilities are obliged to perform checks, leading to an increased number of samples for analytical testing laboratories.

For the testing of environmental samples, the culture method (ISO 11731:2017) represents the standard technique for the detection and quantification of viable *Legionella* spp. in Europe (Collins *et al*. 2015). Results can take up to 15 days (ISO 11731:2017) after sampling, making the culture method a relatively slow technique. This limitation arises from the growth behaviour of *Legionella*, whose growth can be quickly inhibited by the presence of other microorganisms (Leoni and Legnani, 2001). Further limitations include poor reproducibility of the culture method (Boulanger and Edelstein 1995; Yáñez *et al*. 2005; in't Veld, 2017) as well as its inability to detect viable but non-culturable cells (VBNC) that occur under specific environmental conditions (Shih and Lin, 2006). As an alternative to the culture method, the development of molecular methods, such as quantitative real-time polymerase chain reaction (real-time PCR), has been described by various authors (Wilson *et al*. 2003; Yáñez *et al*. 2005; Yang *et al*. 2010). At present, different studies have compared the detection and quantification of *Legionella* in different water samples by the culture method and real-time PCR (Villari *et al*. 1998; Bonetta *et al*. 2010; Collins *et al*. 2015; Farhat *et al*. 2018). They were able to show that real-time PCR can be used as a supplementary enumeration technique to screen for negative samples, thus, enabling results the same day. However, results in colony forming units (CFU) and genome units (GU) only show a weak correlation and therefore, the values cannot be directly converted (Collins *et al*. 2015). National legislations define the legal maximum value for *Legionella* in CFU. Therefore, PCR-positive samples still need to be tested using the culture method to prove conformity with legislation.

When applying the culture method, it is almost impossible to distinguish different *Legionella* spp. based on the appearance of the colonies on agar media. Presumptive colonies should consequently be confirmed, according to ISO 11731:2017, by subculture on BCYE Agar with L-cysteine (buffered charcoal yeast extract with α-ketoglutarate) and BCYE-Cys-Agar (BCYE Agar without L-cysteine). Blood agar, nutrient agar or tryptone soy agar can be used instead of BCYE-Cys agar. This subculture step requires two to five additional days. Further confirmation of *Legionella* and identification of the species and serogroup can be done using commercially available latex agglutination kits, which allow for rapid identification of *Legionella pneumophila* and *other clinical relevant strains* with high specificity (Reyrolle *et al*. 2004; Helbig *et al*. 2006). One further advantage is that the method requires no expensive analytical equipment. However, the confirmation procedure based on subculture is laborious. Our experience shows that the agglutination test can lead to uncertain results and misinterpretations in cases where the reaction is delayed. The following alternative methods are routinely applied in laboratories to confirm presumptive colonies:

Polymerase chain reaction

PCR methods are an interesting alternative for the confirmation of presumptive *Legionella* colonies. In recent years, several PCR-based methods aimed at detecting and quantifying of *Legionella* in water have been described. Most published PCR systems typically target the 5S and 16S rRNA genes for identification of the genus *Legionella* and the macrophage infectivity potentiator (*mip*) gene for the specific detection of *L. pneumophila* strains (Villari *et al*. 1998; Bonetta *et al*. 2010; Collins *et al*. 2015; Farhat *et al*. 2018). The ribosomal 23S–5S spacer region's DNA-sequence information was used to develop a genus specific detection and identification system for *Legionella* spp., using PCR (Herpers *et al*. 2003). PCR methods can provide information on the serogroup as well as additional information. For example, by sequencing the PCR amplicon with subsequent sequence analysis, e.g., in a BLAST algorithm search, the strain under investigation can be identified at the species or even strain level.

Matrix-assisted laser desorption/ionization time-of-flight

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) has recently emerged as a rapid bacterial spp. identification method. The disadvantages are the high cost associated with instrument acquisition. The method is therefore only suitable for high sample throughput. It has been shown that most bacterial spp. exhibit specific protein profiles (Hsieh *et al*. 2008). The method was further developed for the identification of *Legionella* spp. and *L. pneumophila*. The technique allows to perform a full analysis from a single colony in only a few minutes, thus, providing an inexpensive (after the initial investment costs) and rapid screening of a large number of colonies within a short time (Gaia *et al*. 2011). Generally, MALDI-TOF is simpler to use than PCR, and results can be obtained faster. MALDI-TOF cannot identify *Legionella* strains at the serogroup level but is a reliable tool for the rapid identification of *Legionella* strains at the species level (Moliner *et al*. 2010). However, colonies which have grown together can no longer be directly identified by MALDI-TOF without subculturing.

Other methods

A range of other methods can also be used for the identification of presumptive colonies, such as fluorescent in situ hybridization (Baudart *et al*. 2015), Raman spectroscopy (Li *et al*. 2015), pulsed field gel electrophoresis, multilocus sequence typing and whole genome sequencing (Zourob 2008). These methods are typically labour-intensive and require dedicated instrumentation and expertise. To our knowledge, these techniques are, therefore, not routinely applied to the identification of presumptive *Legionella* colonies. Technical improvements could change this in the future.

The confirmation step based on the non-growth of presumptive *Legionella* spp. on only one culture medium, as described in ISO 11731:2017, is prone to errors. Alternative confirmation methods, such as multiplex PCR, should be considered for routine application to achieve a higher specificity and reduce the time expenditure by 2–5 days. This study aims to evaluate multiplex PCR for colony confirmation directly from the culture plate. Two different PCR systems—a commercial one (Biotecon Diagnostics, Potsdam, Germany) and an in-house method—were applied by the two laboratories in this comparison study.

MATERIALS AND METHODS

Laboratories

Industrielle Werke Basel is the water supplier for the region of Basel, Switzerland. Its quality control laboratory is ISO 17025 (STS 0211) accredited and henceforth referred to as laboratory 1.

The Cantonal Laboratory of Zurich (Kantonales Labor Zurich) is responsible for enforcing the Federal Act on Food and Utility Articles in the Canton Zurich, Switzerland. It is ISO 17025 (STS 0172) accredited and will in the following be referred to as laboratory 2.

Samples, sample exchange and storage conditions

Between July 2019 and April 2020, 90 samples of presumptive *Legionella* colonies were collected in laboratory 1 and 200 samples in laboratory 2. All samples were analysed according to the confirmation procedure of the culture method and by PCR confirmation in the laboratory of origin. Furthermore, all samples from laboratory 1 and 50 samples from laboratory 2 were sent to the other laboratory for confirmation with PCR. Hence, a total of 140 samples were additionally subjected to an interlaboratory comparison for both PCR systems.

For confirmation with PCR, the presumptive colonies were stored up to 6 months to analyse several samples in one PCR run and pass on a larger number of samples between the laboratories. Both laboratories agreed on the following storage conditions: the presumptive colonies must be labelled clearly, and the culture plates must be stored upside-down at 2–8°C. Additionally, presumptive colonies can be transferred into 0·5 Tris-EDTA (TE) buffered water and stored at 2–8°C for a maximum of 14 days. The storage conditions ensured that both laboratories could perform their whole PCR protocol, starting with the lysis step.

Confirmation via culture method

The isolation of *Legionella* spp. from the water samples was performed according to ISO 11731:2017. For filtration, 0·45-µm nitrocellulose membrane filters were used. The specific procedure was chosen according to the water type and the resulting concentration of accompanying flora. For water with low accompanying flora, direct plating and membrane filtration without washing were used. For water with high accompanying flora direct plating, membrane filtration without washing and if necessary, dilutions were used. Both laboratories used BCYE Agar and BCYE+AB (BCYE Agar with antibiotics) or GVPC Agar (Glycine-Vancomycin-Polymyxin-B-Cycloheximide-Agar). Independent of the first step of the procedure, the inoculated plates were incubated for 7–10 days at 37 ± 1°C. All colonies with a characteristic morphology according to ISO 11731:2017 were typed as presumptive colonies. One to three colonies of each type were randomly chosen and subcultured on BCYE Agar and BCYE-Cys-Agar or Columbia Blood Agar. Colonies that grew only on BCYE Agar were counted as *Legionella* spp. For further identification of *L. pneumophila* serogroup 1 and *L. pneumophila* serogroup 2–14, a latex agglutination test (Thermo Fisher Diagnostic Switzerland, DR800 M) was conducted.

Commercial PCR kit (laboratory 1)

Laboratory 1 used the microproof® Legionella Quantification LyoKit (Biotecon Diagnostics GmbH, R 602 45) combined with the foodproof® StarPrep Three (Biotecon Diagnostics GmbH, S 400 18) for DNA extraction.

A small amount of the presumptive colony was taken with a sterile dee and resuspended in either 50- or 100-µl lysis buffer. *Pseudomonas aeruginosa* (ATCC 27853) was used as negative control. *Legionella pneumophila* serogroup 1

(ATCC 33152) was used as positive control. The samples and controls were lysed at 98 ± 2°C for 5 min, briefly vortexed and centrifuged at 13 000 ***g*** for 2 min.

For each sample and control, 5-µl DNA-extract was diluted with 20-μl 0·5 TE buffer and added to the lyophilized master mix. The PCR strips were centrifuged for 0.5 min at 200 x ***g*** or 5 s at 2000 x ***g***. PCR was performed with a CFX96 (Bio-Rad, Potsdam, Germany; 1855195).

Legionella spp. was detected in the HEX channel via a specific region in the rDNA operon sequence, of which three copies are present in the *Legionella* genome. *Legionella pneumophila* was detected in the FAM channel via the *mip* gene and strains belonging to serogroup 1 by amplifying the *wzm* gene in the ROX channel. All three genes have been shown to allow specific detection of the respective targets (Mérault *et al*. 2011). The kit provides synthetic DNA as part of the lyophilized master mix that works as an internal control. It therefore allows monitoring of successful DNA amplification in each reaction tube by using a CY5-probe. The kit was validated by the manufacturer in accordance with ISO/TS 12869: 2012. All parameters met the criteria set by the aforementioned norm. The validation data are available from the manufacturer.

Real-time PCR protocol was as follows: pre-incubation for 4 min at 37°C and incubation for 5 min at 95°C followed by 50 cycles of denaturation for 5 s at 90°C, annealing for 60 s at 60°C and extension for 60 s at 72°C. Data were collected after each annealing step. CFX Manager Software Industrial Diagnostic Edition ver. 3.0 was set to automatic baseline setting with activated baseline drift correction. For each sample, the result was given as C_t value.

As a bacterial colony typically contains billions of cells, amplification is achieved already after a few cycles. Therefore, samples with a C_t value ≤ 25 were rated as positive. Samples with a C_t value > 25 were regarded as potentially cross-contaminated and consequently rated as negative. For a successful PCR run, the positive control had to be rated positive, whereas the negative control had to be rated negative for all three parameters/channels. Furthermore, all samples rated as negative, needed positive internal controls to exclude PCR inhibition, to be counted as a validated result. PCR-inhibition was observed in only one sample. In this case, a successful retest was performed, otherwise a 1 : 5 dilution (v/v) of the DNA extract with PCR water would have been applied (as recommended by the kit manufacturer) to prevent PCR inhibition (e.g., due to DNA-overload or presence of inhibiting substances).

In-house PCR (laboratory 2)

Presumptive colonies (as described above) for *Legionella* spp. were picked and resuspended in 100-µl DNA-free pure water. For the DNA extraction, the tubes containing the samples were heat-treated at 95°C for 15 min.

Real-time PCR was performed for the qualitative analysis of *Legionella* spp. and *L. pneumophila* with the Rotor Gene Q6000 (Qiagen Switzerland, 9001720). Five microliter of the DNA-sample were added to a PCR master mix of total 20 µl, which consisted of 6·9-μl 0·5 TE buffer, specific primers 3 × 0·1 µl, 20 µmol l^{-1} (LegP F/R, Legio F/R, TEcol1553), specific Probes 3 × 0·1 µl, 50 µmol l^{-1} (Legio Fam, LegPROX, Tecol573Hex) and 12·5-μl Sensifast (Bioline, London, UK, BIO-86020). The following primers and probes were used for the detection: Detection of *Legionella* spp. using the 23S-5S spacer region by primer Legio F (5′-CTA ATT GGC TGA TTG TCT TGA C-3′), primer Legio R (5′-GGC GAT GAC CTA CTT TCG-3′) and probe LegioFam (5′-CGA ACT CAG AAG TGA AAC-3′) (Herpers *et al*. 2003). Detection of *L. pneumophila* using the *mip* gene by primer LegP F (5′-AAA GGC ATG CAA GAC GCT ATG-3′, primer LegP R (5′-TGT TAA GAA CGT CTT TCA TTT GCT G-3′) and probe LegPRox (5′-TGG CGC TCA ATT GGC TTT AAC CGA-3′) (Welti *et al*. 2003). Detection of *Escherichia coli* using the elongation factor Tu (*tuf*) by primer TEcol1553 (5′-TGG GAA GCG AAA ATC CTG-3′), primer TEcol1754 (5′-CAG TAC AGG TAG ACT TCT G-3′) and probe TEcol573Hex (5′-AACTGGCTGGCTTCCTGG-3′) (Maheux *et al*. 2011). Thus, the FAM channel detects *Legionella* spp. and the ROX channel *L. pneumophila*. The internal control *E. coli* is detected in the HEX channel. DNA of *E. coli* can be added to the isolated DNA and can serve as an inhibition control. The in-house PCR was validated by laboratory 2 according to Swiss national standard (Schweizerische Akkreditierungsstelle 2017).

Every real-time PCR run contained a standard range of *L. pneumophila* DNA to optionally calculate a standard curve, a positive control (*L. pneumophila* ATCC 33152) and a negative control sample (0·5 TE buffer).

The two step cycling program for the PCR consisted of initial denaturation for 5 min at 95°C followed by 45 cycles of 5 s at 95°C for denaturation and 15 s at 55°C for annealing and elongation.

The PCR results were analysed with the Rotor-Gene Q Series Software 2.0.2 according to the Operators Instructions (Rotor-Gene® Q User Manual, ver. 2.0, 2009). The threshold was set automatically with 'Auto-Find Threshold' to get the C_t value of each sample. For this analysis process, the 'Dynamic Tube Normalization' option was activated as well as 'Slope Correct', 'Linear Scale' and 'Outlier Removal' if required. Samples with a *Legionella* spp. C_t value ≤ 28 and a *L. pneumophila* C_t value ≤ 25 were rated as positive. Samples with higher C_t values were rated as negative.

This in-house developed PCR-method does not deliver information on serogroups.

Review by the reference laboratory

Samples with divergent results regarding the culture method and PCR were analysed by the Swiss reference laboratory (Centro Nazionale di Referenza per Legionella in Bellinzona) for verification. The corresponding colonies from the stored plates were first recultivated on BCYE Agar and then analysed using MALDI-TOF. The reference laboratory uses the Vitek MS system from bioMérieux with a validated SARAMIS database and in-house spectra. *Legionella* spp. and *L. pneumophila* positive colonies, respectively, were further examined using agglutination tests.

Statistical methods

The four-field test described in ISO 16140:2003 was used to compare the qualitative methods assessing the following performance indicators: specificity, sensitivity and accuracy. The Kappa concordance index (ISO 16140:2003; Schweizerische Akkreditierungsstelle 2017) was chosen for statistical analysis, which shows the degree of agreement between the two test methods.

RESULTS

Comparison of culture method and commercial PCR kit

Laboratory 1 analysed 90 samples. The results can be seen in Table 1.

For *Legionella* spp., the method comparison (PCR *vs* culture) showed an accuracy of 96%, a specificity of 97%, a sensitivity of 94% and a statistical agreement between the two methods of 0·90, as assessed by the Kappa concordance index. For *L. pneumophila*, the method comparison showed an accuracy of 93%, a specificity of 92%, a sensitivity of 100% and a statistical agreement between the two methods of 0·79. For *L. pneumophila* serogroup 1, the method comparison showed an accuracy and specificity of 99%, a sensitivity of 100% and a statistical agreement between the two methods of 0·90.

Nine of the 90 samples showed a difference between the result of the culture method and PCR. Five of the nine samples were successfully recultivated (plating on BCYE, up to 5 days at 37°C) for analysis at the Swiss reference laboratory using MALDI-TOF analysis and agglutination tests. Table 2 shows an overview of the samples with divergent PCR and culture results together with the results of the reference laboratory. For all recultivated samples, the Swiss reference laboratory confirmed the PCR result. One sample initially showed a discrepancy between culture and PCR. After re-cultivating and re-testing the sample, all methods agreed. For the statistical evaluation, the diverging first result has been used in the method comparisons.

Comparison of culture method and in-house PCR

Laboratory 2 analysed a total of 200 samples. The results of method comparison (PCR *vs* culture) for *Legionella* spp. and *L. pneumophila* are presented in Table 3. For *Legionella* spp., an accuracy of 96%, a specificity of 100%, a sensitivity of 94% and a statistical agreement of 0·91 were obtained. For *L. pneumophila*, an accuracy of 97%, a specificity of 94%, a sensitivity of 99% and a statistical agreement between test methods of 0·94 were obtained. Thirteen of the 200 samples showed a difference between the culture method and the PCR result. All samples were successfully recultivated for analysis in the Swiss reference laboratory. Table 4 combines an overview of the samples with divergent PCR and culture results with the reference laboratory results using MALDI-TOF analysis and agglutination tests. For all but one sample, the Swiss reference laboratory confirmed the PCR result. For the one divergent sample, the culture method analysed *Legionella* spp., the PCR method analysed *L. pneumophila* and the reference laboratory reported the colony identity as *Mycobacterium* spp.

TABLE 1 Comparison of culture method and commercial PCR kit (laboratory 1)

Total = 90	***Legionella* spp. (sample IDs)**[‡]	***Legionella pneumophila* (sample IDs)**	***Legionella pneumophila* serogroup 1 (sample IDs)**
Culture[*] + and PCR[†] +	31	15	5
Culture − and PCR −	55	69	85
Culture + and PCR −	2 (ID 061, 025)	0 (no samples)	0 (no samples)
Culture − and PCR +	2 (ID 004, 034)	6 (ID 003, 012, 013, 032, 033, 034)	1 (ID 003)

[*]Culture describes confirmation on specific agar. + and − describe the final confirmation result.

[†]PCR describes confirmation using PCR. + and − describe the final confirmation result.

[‡]For samples with divergent PCR and culture method result, sample IDs are given (for more information, see Table 2).

TABLE 2 Comparison of samples with divergent results using the commercial PCR kit and culture method

Sample ID	Culture method (ISO 11731:2017)	Commercial PCR kit (laboratory 1)	MALDI-TOF and agglutination test (Swiss reference laboratory)
003	*Legionella* spp.	*Legionella pneumophila* serogroup 1[*] (*Legionella* spp.)[†]	*Legionella anisa*
004	No *Legionella* spp.	*Legionella* spp.	Not analyzed
012	*Legionella* spp.	*Legionella pneumophila* serogroup 2–14	*Legionella pneumophila* serogroup 2–14
013	*Legionella* spp.	*Legionella pneumophila* serogroup 2–14	*Legionella pneumophila* serogroup 2–14
025	*Legionella* spp.	No *Legionella* spp.	*Bradyrhizobium* spp.
032	*Legionella* spp.	*Legionella pneumophila* serogroup 2–14	*Legionella pneumophila* serogroup 2–14
033	*Legionella* spp.	*Legionella pneumophila* serogroup 2–14	Not analyzed
034	No *Legionella* spp.	*Legionella pneumophila* serogroup 2–14	Not analyzed
061	*Legionella* spp.	No *Legionella* spp.	Not analyzed

[*]Original result.

[†]Result after recultivation (just for information purpose, was not included in the statistical evaluation).

TABLE 3 Comparison of culture method and the in-house PCR

Total = 200	*Legionella* spp. (sample ID)[‡]	*Legionella pneumophila* (sample ID)
Culture[*] + and PCR[†] +	126	117
Culture − and PCR −	66	77
Culture + and PCR −	8 (ID 141, 142, 143, 144, 174, 175, 264, 290)	1 (ID 290)
Culture − and PCR +	0 (no samples)	5 (ID 168,169,170,172,173)

[*]Culture describes confirmation on specific agar. + and − describe the final confirmation result.

[†]PCR describes confirmation using PCR. + and − describe the final confirmation result.

[‡]For samples with divergent PCR and culture method result, sample IDs are given (for more information, see Table 4).

TABLE 4 Comparison of samples with divergent in-house PCR and culture results

Sample ID	Culture method (ISO 11731:2017)	In-house PCR (laboratory 2)	MALDI-TOF and agglutination test (Swiss reference laboratory)
141	*Legionella* spp.	No *Legionella* spp.	Acinetobacter
142	*Legionella* spp.	No *Legionella* spp.	Acinetobacter
143	*Legionella* spp.	No *Legionella* spp.	Acinetobacter
144	*Legionella* spp.	No *Legionella* spp.	Acinetobacter
168	*Legionella* spp.	*Legionella pneumophila*	*Legionella pneumophila* serogroup 2–14
169	*Legionella* spp.	*Legionella pneumophila*	*Legionella pneumophila* serogroup 2–14
170	*Legionella* spp.	*Legionella pneumophila*	*Legionella pneumophila* serogroup 2–14
172	*Legionella* spp.	*Legionella pneumophila*	*Legionella pneumophila* serogroup 2–14
173	*Legionella* spp.	*Legionella pneumophila*	*Mycobacterium* spp.
174	*Legionella* spp.	No *Legionella* spp.	*Bacillus* spp.
175	*Legionella* spp.	No *Legionella* spp.	*Bacillus* spp.
264	*Legionella pneumophila*	No *Legionella* spp.	*Bacillus* spp.
290	*Legionella* spp.	No *Legionella* spp.	*Bacillus* spp.

Comparison of commercial PCR and in-house PCR (interlaboratory comparison)

In total, 140 samples (90 samples of laboratory 1 and 50 samples of laboratory 2) were analysed with PCR by both laboratories. Two samples have been excluded due to inconclusive labelling. The results of the interlaboratory comparison can be seen in Table 5.

In the statistical evaluation, the results of the commercial PCR kit have been stated as reference method. For *Legionella* spp., the interlaboratory comparison showed an accuracy of 97%, a specificity of 98% and a sensitivity of 96%. For *L. pneumophila*, the method comparison showed an accuracy of 97%, a specificity of 99% and a sensitivity of 95%. With a statistical agreement of 0·94 for *Legionella* spp. and *L. pneumophila*, a significant correlation was found.

Seven of the 138 samples showed divergent results between the commercial PCR kit and the in-house PCR (see Table 6). As described above, one sample of laboratory 1 showed a discrepancy between the PCR result of laboratory 1 before and after recultivation for the analysis in the Swiss national laboratory. For the statistical evaluation, the diverging first result has been used in the method comparisons.

DISCUSSION

ISO 11731:2017 (Appendix G) indicates the possibility to use the PCR method to identify *Legionella* colonies. In previous studies, the PCR method has also been successfully applied to confirm presumptive colonies (Saint 1998; Giglio *et al*. 2005; Tabatabaei *et al*. 2016). In contrast to these studies, we used a multiplex PCR that enables us to check presumptive colonies for *Legionella* spp. and identify *L. pneumophila* simultaneously with the same measurement experiment. Additionally, the commercial PCR kit delivers information on *L. pneumophila* serogroup 1.

The PCR-confirmation of presumptive colonies directly from the cultivation plate has various advantages compared to the standard procedure involving subculturing and subsequent agglutination test:

(i) The specificity is significantly higher. The subculture yields a substantially higher proportion of false-positive results than the PCR-method. Of the 18 colonies verified by the Swiss reference laboratory, 10 of the culture method results were false-positive regarding *Legionella* spp. Furthermore, the agglutination method has failed to identify *L. pneumophila* positive samples in seven cases, where PCR showed positive results.

(ii) The confirmation by PCR needs less analysis time and final results can be obtained 2–5 days faster. Subculturing on blood-agar or BCYE-Cys-Agar takes additional 2–5 days for incubation. In contrast, PCR identification of presumptive colonies directly from the primary plate delivers results within 2–4 h, including both DNA extraction and PCR run time. Different kit manufacturers for multiplex PCR recently offer even shorter DNA extraction procedures and PCR run times, especially optimized for identification purposes, only. However, for quantification of *Legionella* according to ISO 11731:2017, the primary plate must be incubated for at least 7–10 days to give all possible *Legionella* spp. enough time to grow.

(iii) The two-step procedure of subculturing and subsequently agglutination test can be reduced to one step using PCR confirmation. The PCR detection has the potential for automatization and to reduce the time for procedure. In the future, multiplex PCR can be further expanded to other Legionella strains and serogroups. For example, Biotecon Diagnostics GmbH already offers a commercial kit for the determination of *Legionella* spp., *L. pneumophila* and *L. pneumophila* serogroup 1. Additionally, the infection potential of an isolate can be determined by multiplex PCR for pathogenesis markers (Huang *et al*. 2006).

Our comparison study shows that taking presumptive colonies directly from the culture plate and confirming them by multiplex PCR has a faster response time and is more specific than the standard confirmation method by subcultivation according to ISO 11731:2017. Thereby, it is also possible to directly confirm the presence and absence of *L. pneumophila* serogroup 1 already after 7–10 days while still reporting the result in CFU as most legislations still demand.

TABLE 5 Interlaboratory comparison of commercial PCR kit and in-house PCR

Total = 138	***Legionella* spp. (sample ID)**[†]	***Legionella pneumophila* (sample ID)**
Commercial PCR[*] + and in-house PCR +	71	57
Commercial PCR − and in-house PCR −	63	77
Commercial PCR + and in-house PCR −	3 (ID 014,058,067)	3 (ID 003,014,083)
Commercial PCR − and in-house PCR +	1 (ID 057)	1 (ID 090)

[*]PCR describes confirmation using PCR. + and − describe the final confirmation result.

[†]For samples with divergent PCR result between the laboratories, sample IDs are given (for more information, see Table 6)

TABLE 6 Samples with divergent PCR results in the interlaboratory comparison

Sample ID	Commercial PCR kit (laboratory 1)	In-house PCR (laboratory 2)	Culture method (ISO 11731:2017)
003	*Legionella pneumophila* serogroup 1[*] (*Legionella* spp.)[†]	*Legionella* spp.	*Legionella* spp.[‡]
014	*Legionella pneumophila* serogroup 2-14	No *Legionella* spp.	*Legionella pneumophila* serogroup 2-14
057	No *Legionella* spp.	*Legionella* spp.	No *Legionella* spp.
058	*Legionella* spp.	No *Legionella* spp.	*Legionella* spp.
067	*Legionella* spp.	No *Legionella* spp.	*Legionella* spp.
083	*Legionella pneumophila* serogroup 1	*Legionella* spp.	*Legionella pneumophila* serogroup 1
090	*Legionella* spp.	*Legionella pneumophila*	*Legionella* spp.

[*]Original result.

[†]Result after recultivation (just for information purpose, was not included in the statistical evaluation).

[‡]MALDI-TOF result was *Legionella anisa* (available due to different results during the comparison of culture method and the commercial PCR kit).

The method comparison of PCR-identification *vs* the subculture identification described in ISO 11731:2017 was performed according to ISO 16140:2003. Molecular detection of *Legionella* by PCR met all criteria for the qualitative validation of an alternative method. According to our results, the confirmation step of ISO 11731:2017 can be safely substituted by PCR.

In the future, we expect further applications of PCR methods emerging in routine analysis of *Legionella*. It needs to be examined whether a PCR threshold value in genomic units per litre (GU l^{-1}) can be defined for which the current legal limit for *Legionella* spp. (1000 CFU per litre) can be reliably met. Such thresholds were already proposed by several studies (Lee *et al*. 2011; Collins *et al*. 2017). An alternative could be the establishment of real-time PCR as a cost-effective and fast screening method for routine inspections, e.g., via a first PCR screening and subsequent cultivation only for positive samples or in case a specific threshold value of GU l^{-1} is exceeded. Similar procedures are already accepted or mentioned as possible in some countries such as Canada (Public Works and Government Services Canada 2016) and Italy (Ministero della Salute 2015).

Several PCR-methods have been developed that favour DNA detection and quantification of viable (meaning intact) *Legionella* cells (Nocker *et al*. 2006; Chen and Chang 2010). These qPCRs are directly applied without precultivation. They are affected by different factors such as density of the cells and methods applied (Taylor *et al*. 2014; Braun *et al*. 2019). Another approach to differentiate between viable and non-viable *Legionella* cells is to detect precursor RNA produced only by viable cells on contact with fresh nutrients during cultivation (Boss *et al*. 2018). Although real-time PCR seems promising, additional studies are needed to develop a stable viability qPCR-method for different environmental samples. In the future, the combined application of conventional culture methods and new culture-independent molecular biological methods will be purposeful and helpful to detect and assess microbial contamination in drinking water installations.

ACKNOWLEDGEMENTS

We kindly acknowledge V. Gaia, National Reference Center for Legionella, Bellinzona, Switzerland, for MALDI-TOF MS analysis.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS CONTRIBUTION

Dana Eble and Vanessa Gehrig contributed data or analysis tools, performed the analysis and wrote the paper. Patricia Schubert-Ullrich conceived and designed the analysis and wrote the paper. René Köppel conceived and designed the analysis, contributed data or analysis tools, performed the analysis and wrote the paper. Hans Peter Füchslin conceived and designed the analysis, collected the data, contributed data or analysis tools, performed the analysis and wrote the paper.

ORCID

H.P. Füchslin https://orcid.org/0000-0002-4424-8556

REFERENCES

Bartram, J. (2007) *Legionella and the Prevention of Legionellosis*. Geneva: World Health Organization (ISBN: 9 241562 978).

Baudart, J., Guillaume, C., Mercier, A., Lebaron, P. and Binet, M. (2015) Rapid quantification of viable Legionella in nuclear cooling tower waters using filter cultivation, fluorescent in situ hybridization and solid-phase cytometry. *J Appl Microbiol* 118, 1238–1249.

Bonetta, S., Bonetta, S., Ferretti, E., Balocco, F. and Carraro, E. (2010) Evaluation of *Legionella pneumophila* contamination in Italian

hotel water systems by quantitative real-time PCR and culture methods. *J Appl Microbiol* 108, 1576–1583.

Boss, R., Baumgartner, A., Kroos, S., Blattner, M., Fretz, R. and Moor, D. (2018) Rapid detection of viable *Legionella pneumophila* in tap water by a qPCR and RT-PCR-based method. *J Appl Microbiol* 125, 1216–1225.

Boulanger, C.A. and Edelstein, P.H. (1995) Precision and accuracy of recovery of *Legionella pneumophila* from seeded tap water by filtration and centrifugation. *Appl Environ Microbiol* 61, 1805–1809.

Braun, R.S., Mendis, N., Li, L. and Faucher, S.P. (2019) Quantification of viable but non-culturable cells of *Legionella pneumophila*. *Methods Mol Biol* 1921, 45–53.

Bundesministerium der Justiz und für Verbraucherschutz (2016) Verordnung über die Qualität von Wasser für den menschlichen Gebrauch (Trinkwasserverordnung - TrinkwV): BGBl. I S. 459.

Chen, N.-T. and Chang, C.-W. (2010) Rapid quantification of viable legionellae in water and biofilm using ethidium monoazide coupled with real-time quantitative PCR. *J Appl Microbiol* 109, 623–634.

Collins, S., Jorgensen, F., Willis, C. and Walker, J. (2015) Real-time PCR to supplement gold-standard culture-based detection of Legionella in environmental samples. *J Appl Microbiol* 119, 1158–1169.

Collins, S., Stevenson, D., Walker, J. and Bennett, A. (2017) Evaluation of Legionella real-time PCR against traditional culture for routine and public health testing of water samples. *J Appl Microbiol* 122, 1692–1703.

Eidgenössisches Departement des Innern (EDI) (2016) Verordnung des EDI über Trinkwasser sowie Wasser in öffentlich zugänglichen Bädern und Duschanlagen (TBDV). SR 817.022.11.

Farhat, M., Shaheed, R.A., Al-Ali, H.H., Al-Ghamdi, A.S., Al-Hamaqi, G.M., Maan, H.S., Al-Mahfoodh, Z.A. and Al-Seba, H.Z. (2018) Legionella confirmation in cooling tower water. Comparison of culture, real-time PCR and next generation sequencing. *Saudi Med J* 39, 137–141.

Gaia, V., Casati, S. and Tonolla, M. (2011) Rapid identification of *Legionella* spp. by MALDI-TOF MS based protein mass fingerprinting. *Syst Appl Microbiol* 34, 40–44.

Giglio, S., Monis, P.T. and Saint, C.P. (2005) Legionella confirmation using real-time PCR and SYTO9 is an alternative to current methodology. *Appl Environ Microbiol* 71, 8944–8948.

Helbig, J.H., Lück, P.C., Kunz, B. and Bubert, A. (2006) Evaluation of the Duopath Legionella lateral flow assay for identification of *Legionella pneumophila* and Legionella species culture isolates. *Appl Environ Microbiol* 72, 4489–4491.

Herpers, B.L., de Jongh, B.M., van der Zwaluw, K. and van Hannen, E.J. (2003) Real-time PCR assay targets the 23S–5S spacer for direct detection and differentiation of *Legionella* spp. and *Legionella pneumophila*. *J Clin Microbiol* 41, 4815–4816.

Hsieh, S.-Y., Tseng, C.-L., Lee, Y.-S., Kuo, A.-J., Sun, C.-F., Lin, Y.-H. and Chen, J.-K. (2008) Highly efficient classification and identification of human pathogenic bacteria by MALDI-TOF MS. *Mol Cell Proteomics* 7, 448–456.

Huang, B., Yuan, Z., Heron, B.A., Gray, B.R., Eglezos, S., Bates, J.R. and Savill, J. (2006) Distribution of 19 major virulence genes in *Legionella pneumophila* serogroup 1 isolates from patients and water in Queensland, Australia. *J Med Microbiol* 55, 993–997.

ISO (2003) *Microbiology of Food and Animal Feeding Stuffs — Protocol for the Validation of Alternative Methods*. ISO 16140:2003, Geneva: International Organisation for Standardisation.

ISO (2017) *Water Quality – Detection and Enumeration of Legionella*. ISO 11731:2017. Geneva: International Organisation for Standardisation.

ISO (2019) *Water Quality — Detection and Quantification of* Legionella *spp. and/or* Legionella pneumophila *by Concentration and Genic Amplification by Quantitative Polymerase Chain Reaction (qPCR)*. Geneva: International Organisation for Standardisation.

Lee, J.V., Lai, S., Exner, M., Lenz, J., Gaia, V., Casati, S., Hartemann, P., Lück, C. *et al*. (2011) An international trial of quantitative PCR for monitoring Legionella in artificial water systems. *J Appl Microbiol* 110, 1032–1044.

Leoni, E. and Legnani, P.P. (2001) Comparison of selective procedures for isolation and enumeration of Legionella species from hot water systems. *J Appl Microbiol* 90, 27–33.

Li, J., Qin, T., Jia, X.X., Deng, A.H., Zhang, X., Fan, W.H., Huo, S.D., Wen, T.Y. *et al*. (2015) Rapid identification of legionella pathogenicity by surface-enhanced Raman spectroscopy. *Biomed Environ Sci* 28, 437–444.

Maheux, A.F., Bissonnette, L., Boissinot, M., Bernier, J.-L.-T., Huppé, V., Picard, F.J., Bérubé, É. and Bergeron, M.G. (2011) Rapid concentration and molecular enrichment approach for sensitive detection of *Escherichia coli* and *Shigella* species in potable water samples. *Appl Environ Microbiol* 77, 6199–6207.

Mérault, N., Rusniok, C., Jarraud, S., Gomez-Valero, L., Cazalet, C., Marin, M., Brachet, E., Aegerter, P. *et al*. (2011) Specific real-time PCR for simultaneous detection and identification of *Legionella pneumophila* serogroup 1 in water and clinical samples. *Appl Environ Microbiol* 77, 1708–1717.

Ministero della Salute (2015) Linee guida per la prevenzione ed il controllo della legionellosi: Conf. Rapporti SR 2005.

Moliner, C., Ginevra, C., Jarraud, S., Flaudrops, C., Bedotto, M., Couderc, C., Etienne, J. and Fournier, P.-E. (2010) Rapid identification of *Legionella* species by mass spectrometry. *J Med Microbiol* 59, 273–284.

Nocker, A., Cheung, C.-Y. and Camper, A.K. (2006) Comparison of propidium monoazide with ethidium monoazide for differentiation of live vs. dead bacteria by selective removal of DNA from dead cells. *J Microbiol Methods* 67, 310–320.

Public Works and Government Services Canada (2016) *Control of Legionella in Mechanical Systems - Standard for Building Owners, Design Professionals, and Maintenance Personnel*. MD 15161-2013. Quebec: Public Works and Government Services Canada.

Reyrolle, M., Ratat, C., Leportier, M., Jarraud, S., Freney, J. and Etienne, J. (2004) Rapid identification of *Legionella pneumophila* serogroups by latex agglutination. *Eur J Clin Microbiol Infect Dis* 23, 864–866.

Saint, C.P. (1998) A colony based confirmation assay for Legionella and *Legionella pneumophila* employing the EnviroAmp Legionella system and seroagglutination. *Lett Appl Microbiol* 26, 377–381.

Schweizerische Akkreditierungsstelle (2017) Leitfaden zur Validierung mikrobiologischer Prüfverfahren und zur Abschätzung der Messunsicherheit im Bereich Lebensmittel- und Umweltmikrobiologie: Dokument Nr. 328.dw.

Shih, H.-Y. and Lin, Y.E. (2006) Caution on interpretation of legionella results obtained using real-time PCR for environmental water samples. *Appl Environ Microbiol* 72, 6859.

Stojek, N. and Dutkiewicz, J. (2006) Legionella and other gram-negative bacteria in potable water from various rural and urban sources. *Ann Agric Environ Med* 13, 323–335.

Tabatabaei, M., Hemati, Z., Moezzi, M. and Azimzadeh, N. (2016) Isolation and identification of *Legionella* spp. from different aquatic sources in south-west of Iran by molecular & culture methods. *Mol Biol Res Commun* 5, 215–223.

Taylor, M.J., Bentham, R.H. and Ross, K.E. (2014) Limitations of using propidium monoazide with qPCR to discriminate between live and dead Legionella in biofilm samples. *Microbiol Insights* 7, 15–24.

in't Veld, S. (2017) Development of ISO 11731:2017, Legionella workshop organised by Xebios Diagnostics GmbH, Düsseldorf.

Villari, P., Motti, E., Farullo, C. and Torre, I. (1998) Comparison of conventional culture and PCR methods for the detection of *Legionella pneumophila* in water. *Lett Appl Microbiol* 27, 106–110.

Welti, M., Jaton, K., Altwegg, M., Sahli, R., Wenger, A. and Bille, J. (2003) Development of a multiplex real-time quantitative PCR assay to detect *Chlamydia pneumoniae*, *Legionella pneumophila* and *Mycoplasma pneumoniae* in respiratory tract secretions. *Diagn Microbiol Infect Dis* 45, 85–95.

Wilson, D.A., Yen-Lieberman, B., Reischl, U., Gordon, S.M. and Procop, G.W. (2003) Detection of *Legionella pneumophila* by real-time PCR for the mip gene. *J Clin Microbiol* 41, 3327–3330.

Yáñez, M.A., Carrasco-Serrano, C., Barberá, V.M. and Catalán, V. (2005) Quantitative detection of *Legionella pneumophila* in water samples by immunomagnetic purification and real-time PCR amplification of the dotA gene. *Appl Environ Microbiol* 71, 3433–3441.

Yang, G., Benson, R., Pelish, T., Brown, E., Winchell, J.M. and Fields, B. (2010) Dual detection of *Legionella pneumophila* and Legionella species by real-time PCR targeting the 23S–5S rRNA gene spacer region. *Clin Microbiol Infect* 16, 255–261.

Zourob, M.M. (ed.) (2008) *Principles of Bacterial Detection: Biosensors, Recognition Receptors and Microsystems*. New York, NY: Springer (ISBN 978-0-387-75113-9).

How to cite this article: Eble D, Gehrig V, Schubert-Ullrich P, Köppel R, and Füchslin H. (2021) Comparison of the culture method with multiplex PCR for the confirmation of Legionella spp. and Legionella pneumophila. *J Appl Microbiol*. 131, 2600–2609. https://doi.org/10.1111/jam.15103