Comparative evaluation of three modern PCR methods for quantitative and qualitative analysis of *Legionella* spp. for routine monitoring of premise water system samples.

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Abstract

Aims: To evaluate the capability of modern, PCR-based technologies, to detect and/or enumerate and/or characterize *Legionella* spp. and to determine the role these technologies should play in evaluation of routine monitoring for *Legionella* spp. in premise water systems.

Methods and Results: A total of 120 artificially contaminated water samples were prepared in sterile tap water. Artificial contamination consisted of spiking with a variety of both live and dead *Legionella* spp., including *L. pneumophila* serogroup 1, at various concentrations from <1 CFU/mL up to 3 Log CFU/mL. Inoculated waters were prepared and equally divided for analysis via three modern PCR methods (anonymously referred to as Methods A, B, and C), and the cultural method as outlined in ISO 11731 (and CDC recommendations). All PCR methods met or exceeded their stated limits of detection (LOD) claims, and accurately and consistently distinguished live from dead cells (where applicable). Additionally, all PCR methods showed equivalent or better qualitative *Legionella* spp. detection in less than 24 hours when compared to 7-14 days with the traditional cultural method. The PCR methods were also more consistent in their reporting than the cultural method. Furthermore, Methods B and C showed accurate quantitation across the inoculation range. Method C's multilocus amplification provided additional qualitative characterization which allowed it to accurately distinguish and quantitate *Legionella* spp., *L. pneumophila* serogroup 1 (LP SG1), and *L. pneumophila* serogroup 2-15 (LP SG2-15).

Conclusions: Quantitative, real-time PCR with multilocus amplification, is an efficient means to positively detect, quantify, and characterize, viable *Legionella* spp. with a higher degree of accuracy, specificity, and sensitivity than traditional, "gold-standard", cultural methods.

Significance and Impact: Modern, rapid, PCR-based technologies could prove to be more accurate and reliable alternatives for detection of *Legionella* spp., and in time, replace culture as the "gold-standard" for routine *Legionella* spp. monitoring in premise water systems.

Introduction

It has long been understood that *Legionella* spp. are the causative agent of Legionnaires' Disease, which has a mortality rate of approximately 10% or more according to the Centers for Disease Control and Prevention (CDC). Nearly 10,000 cases of Legionnaires' Disease were reported in 2018. However, because it is believed that Legionnaires' Disease is underdiagnosed and underreported, the true case rate is estimated to be 1.8-2.7 times higher (Collier, et al., 2021).

Legionella spp. are commonly present to varying degrees in natural aquatic environments but can quickly multiply to dangerous concentrations in premise water systems (Albert-Weissenberger, Cazalet, & Buchrieser, 2007). As such, maintaining clean water through a rigorous Water Management Program (WMP), including routine testing for *Legionella* spp., is critical to controlling these waterborne pathogens and avoiding public health crises.

WMPs typically specify location and frequency of monitoring by testing for the presence of *Legionella* following a cultural method. Unfortunately, traditional *Legionella* cultural methods may

require up to 14 days or longer to obtain valid results which delays incidence response (Collins, Stevenson, Walker, & Bennett, 2017).

In addition, the cultural methods have several limitations including poor sensitivity and efficiency. Boulanger and colleagues determined the *in vitro* recovery efficiency for artificially contaminated waters to be only 53% on average. They determined that this efficiency dropped further, to 38% on average, with acidic treatment. While these cultural methods have been considered the "gold standard" for decades, Boulanger *et al* concluded based on their results and similar results obtained by others (Voss and colleagues, Szewyk and colleagues), that cultural quantification of *Legionella* spp. are erratic and unreliable (Boulanger & Edlestein, 1995).

Another limitation with cultural methods is that they rely, by definition, on cells not only being viable, but culturable. In premise water systems, organisms are typically alive, but in a stressed state. As such, only a portion of the organisms present may be culturable even under ideal circumstances. Furthermore, attempts to culture these organisms may be suppressed anywhere from 5-99% due to the selective nature of the medium used, and isolation techniques applied in the traditional culture method (Roberts, August, & Nelson, 1987). Still others are in a state of metabolic dormancy referred to as Viable But Non-Culturable (VBNC). In this state, cells can regain metabolic activity through a process known as "resuscitation" and therefore do pose a risk to human health (Fleischmann, Robben, Alter, Rossmanith, & Mester, 2021).

In an effort to solve this problem, laboratories have attempted to turn to molecular methodologies, namely Polymerase Chain Reaction (PCR) in lieu of cultural methods. Historically, however, PCR methods had several issues. First, because they are an assay for genetic material, they did not initially distinguish between genetic material from live versus dead cells. Additionally, they were not initially quantitative, and qualitative capabilities were narrow in scope. For example, they might assay for *Legionella pneumophila* very specifically, but would not detect other members of the genus. This problem has only increased in relevance in recent years as more cases of legionellosis are attributed to *Legionella* spp. other than *L. pneumophila* (Muder & Yu, 2002).

In this study, three separate, modern PCR methods (anonymously referred to throughout this paper as Methods A, B, and C) that claim to solve many or all of the problems listed above, were evaluated against the CDC/ISO recommended cultural method. Each of the rapid technologies had specific capabilities as outlined in Table 1.

Table 1 – Summary of technologies evaluated.

Method	Brief Technical Description	Quantitative Capabilities[1]	Qualitative Capabilities
A	Nested PCR and "viability" solution with lateral flow cartridge read	N/A	• *Legionella* spp. • Live/Dead Differential
B	qPCR with optional EMA[2] differential	• Quantifies *Legionella* spp. o LOQ = 10 GU/reaction	• *Legionella* spp. • Live/Dead Differential (with optional EMA)
C	qPCR with multilocus amplification and "viability" solution	• Quantifies *Legionella* spp. o LOQ = 3 GU/reaction • Quantifies LP SG1 o LOQ = 5 GU/reaction • Quantifies LP SG 2-15 o LOQ = 5 GU/reaction	• *Legionella* spp. • Characterizes LP SG1 • Characterizes LP SG 2-15 • Live/Dead Differential

[1]*Quantification is via genomic units (GU)*

[2]*EMA=ethidium monoazide is a DNA-intercalating agent that penetrates membrane-damaged cells and covalently bonds to DNA thus allowing only amplification of DNA from membrane-intact cells* (Chen & Chang, 2010)

When choosing rapid technologies for this evaluation, several factors were considered.

First, it was noted that detecting *Legionella* spp. as opposed to only *L. pneumophila* (LP) was critical. Although LP has historically been considered the most clinically significant of *Legionella* spp., it is not necessarily the most common in premise water systems, with *L. anisa*, having been shown to be one of the most recovered *Legionella* spp. from environmental samples (Doleans, et al., 2004). Not only is the presence of any *Legionella* spp. indicative of the ability of *L. pneumophila* to survive and proliferate, but many other *Legionella* spp. including *L. micdadei*, *L. bozemanii*, and *L. dumoffii*, have been shown to be significant etiologic agents (Muder & Yu, 2002). Indeed, this is echoed in the CDC "*Legionella* Toolkit" which states "**There is no 'safe' level or type of *Legionella*** [*sic*]". (United States Centers for Disease Control and Prevention, n.d.). Therefore, it was determined that the ideal PCR method would be able to detect all *Legionella* spp., with the capability to additionally characterize the detection as *L. pneumophila*.

Second, the limit of detection (LOD) of each method was considered. The current CDC "*Legionella* Toolkit" indicates that acceptable results in routine *Legionella* spp. monitoring is <1 CFU/mL for potable water samples, and <10 CFU/mL for non-potable water samples such as cooling towers and decorative water features. CDC suggests that exceeding these limits once indicates that intervention is needed, that observing a 1-2 Log increase demonstrates that *Legionella* spp. growth in the system is poorly controlled, and that >2 Log increase demonstrates that *Legionella* spp. growth in the system is uncontrolled (United States Centers for Disease Control and Prevention, n.d.). Given these suggested limits, it was imperative that rapid methods chosen have an LOD of <1 CFU/mL.

Third, quantitation capabilities were assessed. Historical PCR methods were purely qualitative. That is, they reported "presence" or "absence" of the target organism(s). The CDC expresses the importance of quantitating and trending *Legionella* spp. over time to monitor the health of a water system as part of a holistic WMP (United States Centers for Disease Control and Prevention, n.d.). Consequently, those technologies purporting quantitative capabilities would be considered preferable if the quantitative capabilities could be verified to be precise, accurate, and consistent.

Finally, the ability of the technologies to distinguish "live" from "dead" cells was deemed paramount. Historical PCR methods were focused only on amplifying target genetic material regardless of whether that genetic material is from a live or dead cell. Modern PCR methods employ several techniques to allow amplification of the genetic target from only live cells. This is critical for two reasons. First, it eliminates the need for cultural confirmation of results, which would have been advisable with historical methods to ensure the signal detection came from living organisms. Second, and perhaps most importantly, it provides a much more accurate picture of the true bioburden of the sample. This is because only a small percentage of the live cells present in any sample are culturable with existing methods. The ability of modern PCR methods to detect signal from a much larger population of the bioburden in a system (both culturable and living VBNC cells), was the major focus of this study.

Materials and Methods

Organism Preparation and Artificial Contamination

A total of 120 artificially contaminated water samples were prepared in sterile tap water. Artificial contamination consisted of spiking with a variety of *Legionella* strains at three target concentrations: a low level (<1 CFU/mL), a medium level (1 – 99 CFU/mL) and a high contamination level (2-3 Log CFU/mL).

In addition to utilizing live cultures, several sample sets were spiked with heat-killed ("dead") *Legionella* at known concentrations to determine the reliability of each methods' viability determination capabilities. To challenge the PCR assays realistically, a selection of interfering organisms commonly isolated from environmental water samples was also included. See Tables 2 and 3 below for a summary of the organisms used for artificial contamination and the levels selected for each test sample series.

Table 2 - Artificial Contamination Organisms and Theoretical Target Levels

Sample Series	Target Organisms	Level	Interfering Organisms	Level
1 – 10	*L. pneumophila* SG1 ATCC[a] 33152	Low	Refer to Table 3	Medium
11 – 20	*L. pneumophila* SG2-15 QL0515290-8[b]	Medium		High
21 – 30	*L. pneumophila* SG2-15 QL346945-6	Low		High
31 – 40	*L. pneumophila* SG1 QL056619 (heat-killed)	Medium		Medium
41 – 50	*L. feeleii* QL021116-1	Medium		Medium
51 – 60	*L. oakridgensis* QL14522-2A	Low		Medium
61 – 70	*L. dumoffii* ATCC 33279 (heat killed)	High		Medium
71 – 80	*L. dumoffii* QL0584231-6	High		Medium
81 – 90	*L. micdadei* QL14522-1A	High		Medium
91 – 100	*L. pneumophila* SG2-15 QL346945-6	Low		Medium
	L. oakridgensis QL14522-2A	High		
101 – 110	*L. micdadei* QL14522-1A	High		Medium
111 – 120	*L. dumoffii* QL0584231-6	High		Medium

[a] American Type Culture Collection, Manassas, Virginia
[b] Q Laboratories Culture Collection, Cincinnati, Ohio

Table 3 – Interfering Organisms

	Organism	Source	Origin
Mixed Culture	*Acidovorax temperans*	QL21050-2	Potable Water
	Klebsiella pneumoniae	ATCC 13883	Potable Water
	Kluyvera intermedia	ATCC 33110	Surface Water
	Sphingomonas koreensis	QL21050-1	Potable Water
	Serratia marcescens	QL11007-1	Potable Water

Selected organisms were retrieved from Q Laboratories' freezer stock cultures maintained at -70°C and cultured on Buffered Charcoal Yeast Extract (BCYE) following standard microbiological procedures. All organisms were identified using MALDI-TOF technology to confirm culture purity, prior to spiking bulk sterile water test portions. Additionally, *Legionella pneumophila* isolates were characterized serologically to belong to one of two groups: Serogroup 1 (SG1) or Serogroup 2-15. Specific serological identification within SG2-15, such as SG3 or SG6, was not determined as this information was not a focus of the study.

Legionella cultures selected to represent "dead" cells were heat killed by immersing the culture suspension test tubes in boiling water for approximately 60 minutes. Selected cultures were serially diluted and plated onto BCYE agar before and after this lethality process to establish the initial organism concentration of the suspension and to be certain the inoculating cells were rendered non-viable ("dead").

For each sample series a bulk volume of sterile water was inoculated according to the study design previously described. The bulk volume of inoculated water was homogenized thoroughly and distributed

equally into 100 mL portions in sterile containers for each of the three PCR assays and the culture method. Inoculated samples were held at 2 - 8°C for 24 to 48 hours prior to initiating testing to allow the organisms to equilibrate to their new environment.

Sample Analysis

For Method A, per the manufacturer's package insert, a 25 mL aliquot from the 100 mL sample was concentrated via centrifugation at 3000 x *g* for 10 min. For Methods B and C, each 100 mL water sample was filter concentrated. All PCR sample preparations, and DNA extraction procedures conducted, were specifically written for *Legionella* testing of potable waters. If the manufacturer's insert provided instructions for detecting all *Legionella* present (live and dead), only the live detection procedures were followed.

For the culture method, each 100 mL water sample was filter concentrated and resuspended (washed), in a small volume of sterile water. From the wash suspension an aliquot was plated onto BCYE, BCYE containing Polymyxin B Sulfate, Vancomycin and Cycloheximide (PCV) and PCV with Glycine (GPCV) agars. Additionally, an aliquot of the filtrate was treated for 3 minutes with an equal volume (1:1 ratio) of HCl-KCl acid buffer, pH 2.2, and plated onto BCYE and PCV agars. Agar plates were incubated in a humidified atmosphere at 35 °C for 3 to 4 days and examined under magnification for suspect *Legionella* colonies. If no suspect *Legionella* colonies were observed, agar plates were incubated for up to an additional 10 days before concluding the sample to be negative for *Legionella*. Periodic examination of the plates was conducted during the 10-day incubation time.

Suspect isolates from any agar plate were identified for confirmation using MALDI-TOF technology. Isolates identifying as a *Legionella* species within the acceptable confidence range of the instrument were regarded as a confirmed *Legionella* species. Isolates identified as *L. pneumophila* were further cultured and serotyped using a commercially available rapid *Legionella* latex slide agglutination test.

For samples containing *Legionella*, typical colonies were enumerated.

Results

Low level L. pneumophila SG1 and SG2-15

Ten samples were artificially contaminated with a reference culture, *Legionella pneumophila* SG1 ATCC 33152, at a low level, 0.77 CFU/mL. The mixed culture of interfering organisms was inoculated at a medium level, 5.2 CFU/mL.

As expected, as the inoculation level was below the claimed LOD of the assay, Method A was unable to detect *Legionella* in any of the ten test samples.

Interestingly however, despite the fact that the inoculum was also below the claimed LOD of Methods B and C, both methods did show detection of *Legionella*. Method B detected *Legionella* in five of the ten test samples. Using this assay for accurate *Legionella* quantification requires two PCR wells with valid reactions for each sample or control analyzed. Of the five samples determined to be positive, all five were interpreted by the PCR analysis software as lower than the LOQ. PCR Method C detected *Legionella* in eight of the ten samples tested. Correct characterization into groups (LP SG1, LP SG2-15, *Legionella* spp.) was accurate (LP SG1) for 30% of the samples. Quantification was interpreted as less than the LOQ for each positive sample.

Table 4 – Relative Efficiency at Low Inoculation

Method	Manufacturer Stated LOD (*Legionella* spp.)	Detection Efficiency at 0.77 CFU/mL	Qualitative Accuracy
A	10 CFU/mL[a]	0%	N/A
B	5 GU/reaction	50%	100%[b]
C	3 GU/reaction	90%[c]	100%[c]

[a] Method A employs non quantitative PCR. Thus, the LOD was determined via serial dilution with amplicon verification via gel electrophoresis.
[b] Method B accurately reported 100% of samples as "*Legionella* spp." positive (it does not have further characterization capabilities)
[c] 30% called specifically as LP SG1, 30% as LP, and 30% as *Legionella* spp. (90% correct call to varying degrees of characterization, the other sample experienced PCR inhibition).

For the *Legionella* cultural method one of the ten samples was positive. With only one colony observed for the positive sample the *Legionella pneumophila* SG1 concentration was calculated as 0.5 CFU/mL.

Ten additional samples were artificially contaminated with a wild-type culture, LP SG2-15 (QL0515290-8), at a medium level, 4.8 CFU/mL. The mixed culture of interfering organisms was inoculated at a medium level, 52 CFU/mL.

Method A qualitatively detected Legionella species in three of the ten test samples.

Method B detected *Legionella* in nine of the ten test samples. Of the nine samples determined to be positive, eight were interpreted by the PCR analysis software as lower than the LOQ.

PCR Method C detected *Legionella* spp. in all ten of the samples tested. Correct characterization into groups (LP SG1, LP SG2-15, *Legionella* spp.) was accurate for 100% of the samples. Quantification was interpreted as less than the LOQ for each positive sample except one.

For the *Legionella* culture method, none of the ten samples were positive.

Low level Legionella pneumophila SG2-15

Ten samples were artificially contaminated with a wild-type culture, *Legionella pneumophila* SG2-15 QL0515290-6, at a low level, 0.9 CFU/mL. The mixed culture of interfering organisms was inoculated slightly lower than previous medium level spiked samples, 3.7 CFU/mL.

PCR Method A detected *Legionella* in zero of the ten test samples.

PCR Method B detected *Legionella* in all ten of the test samples. Valid PCR reactions took place in each well though the *Legionella* concentrations in all ten were interpreted by the PCR analysis software as lower than the LOQ.

PCR Method C detected *Legionella* in all ten of the samples tested. Correct characterization into groups (LP SG1, LP SG2-15, *Legionella* spp.) was accurate (LP SG2-15) for 100% of the samples. Quantification was interpreted as less than the LOQ for each positive sample except one yielding a concentration of 190 GU/100 mL.

For the *Legionella* culture method, five of the ten samples were positive. The calculated *Legionella* concentrations for the five positive samples varied from 0.5 CFU/mL in four of the samples to 1 CFU/mL for the remaining sample.

Inoculation with Dead Cells

Ten samples were artificially contaminated with a heat-killed (dead) wild-type culture, LP SG1 (QL0566619), at 2.4 CFU/mL. Enumeration was conducted prior to the lethality step as conducted in this study. The mixed culture of interfering organisms was inoculated at high level, 37 CFU/mL.

Method A correctly excluded the dead *Legionella* in all ten of the test samples.

Method B did not exclude the dead *Legionella* cells in all ten of the test samples. Valid PCR amplification reactions took place in each well however the dead *Legionella* concentrations in all ten were interpreted by the PCR analysis software as lower than the LOQ. Verification of possible live *Legionella* in these samples was not performed due to steps in the method workflow that effectively kills live cells. This result was investigated, and it was determined that the stock viability solution provided with Method B removes free DNA from lysed cells, but does not suppress amplification from dead, intact cells. The assay was repeated with addition of EMA which resulted in accurate suppression of amplification in all ten test samples. It was determined at this point that Method B would require the addition of EMA to exclude amplification of intact dead cell DNA, though this was not performed in all sample sets.

Method C correctly excluded the dead *Legionella* in all ten of the samples tested as a result of a viability reagent incorporated into the sample processing workflow.

As expected for the culture method, *Legionella* was not recovered from any of the ten samples tested.

Low Level L. feeleii and L. oakridgensis

Ten samples were artificially contaminated with a wild-type culture, *Legionella feeleii* QL021116-1, at 1.4 CFU/mL. The mixed culture of interfering organisms was also inoculated at a medium level, 4 CFU/mL.

PCR Method A detected *Legionella* in zero of the ten test samples.

PCR Method B detected *Legionella* in all ten of the test samples. Valid PCR reactions took place in each well with the mean *Legionella* concentration as 370 GU/100 mL.

PCR Method C detected *Legionella* in all ten of the samples tested. Correct characterization into groups (LP SG1, LP SG2-15, *Legionella* spp.) was accurate (*Legionella* spp.) for 100% of the samples. Quantification was interpreted as less than the LOQ (<180 GU/100 mL) for each positive sample.

For the *Legionella* culture method, one of the ten samples was positive. With only one colony observed for the positive sample the *Legionella* spp. concentration was calculated to be 0.5 CFU/mL.

Ten additional samples were artificially contaminated with a wild-type culture, *L. oakridgensis* (QL14522-2A), at a low level of 0.2 CFU/mL. The mixed culture of interfering organisms was inoculated approximately 200 times higher at 40 CFU/mL.

PCR Method A detected *Legionella* in zero of the ten test samples.

Method B detected *Legionella* in all ten of the test samples. Valid PCR reactions took place in each well with the mean *Legionella* concentration as 2800 GU/100 mL.

Method C detected *Legionella* in all ten of the samples tested. Correct characterization into groups (LP SG1, LP SG2-15, *Legionella* spp.) was accurate (*Legionella* spp.) for 100% of the samples. Quantification was interpreted as less than the LOQ (<180 GU/100 mL) for five of the positive samples. The mean GU/100 mL for the remaining five positive samples was 270.

For the *Legionella* culture method, none of the ten samples were positive.

High level Legionella dumoffii dead and live

Ten samples were artificially contaminated with a heat-killed (dead) reference culture, *Legionella dumoffii* ATCC 33279, at a high level 9800 CFU/mL. The mixed culture of interfering organisms was inoculated at a medium level, 6 CFU/mL.

Method A correctly excluded the dead *Legionella* in all ten of the test samples.

Method B did not exclude the dead *Legionella* cells in all ten of the test samples. Valid PCR amplification reactions took place in each well however, no EMA had been added to ensure suppression of amplification of dead *Legionella* cells, and concentrations in all ten were interpreted by the PCR analysis software with a mean of 5000 GU/100 mL. Verification of possible live *Legionella* in these samples was not performed due to steps in the method workflow that effectively kill live cells.

Method C did report detection below the LOQ for one channel (*Legionella* spp.) for dead *Legionella* cells in all ten of the test samples. However, final software interpretation accurately reported "Negative" for all samples. Furthermore, characterization into groups (LP SG1, LP SG2-15, *Legionella* spp.) was accurate (*Legionella* spp.) for 100% of the samples. Verification of possible live *Legionella* in these samples was not performed due to steps in the method workflow that effectively kill live cells.

As expected for the culture method, *Legionella* was not recovered from any of the ten samples tested.

Ten additional samples were artificially contaminated with a live, wild-type culture, *L. dumoffii* (QL584231-6), at a high level, 7400 CFU/mL. The mixed culture of interfering organisms was inoculated at a medium level of 47 CFU/mL.

Method A detected *Legionella* in zero of the ten test samples.

Method B detected *Legionella* in all ten of the test samples. Valid PCR reactions took place in each well with the mean *Legionella* concentration as 51,000 GU/100 mL.

Method C detected *Legionella* in all ten of the samples tested. Correct characterization into groups (LP SG1, LP SG2-15, *Legionella* spp.) was accurate (*Legionella* spp.) for 100% of the samples. The mean quantification value was interpreted as 53,000 GU/100 mL.

For the *Legionella* culture method, nine of the ten samples were positive with a mean *Legionella* concentration of 190 CFU/mL.

High level L. micdadei and mixed L. pneumophila SG2-15 + L. oakridgensis

Ten samples were artificially contaminated with a live, wild-type culture, *Legionella micdadei* QL14522-1A, at a high level, 1300 CFU/mL. The mixed culture of interfering organisms was also inoculated at a high level, 64 CFU/mL.

Method A qualitatively detected *Legionella* in ten of the ten samples tested.

Method B detected *Legionella* in all ten of the test samples. Valid PCR reactions took place in each well with the mean *Legionella* concentration as 13,000 GU/100 mL.

Method C detected *Legionella* in all ten of the samples tested. Correct characterization into groups (LP SG1, LP SG2-15, *Legionella* spp.) was accurate (*Legionella* spp.) for 100% of the samples. The mean quantification value was interpreted as 94,000 GU/100 mL.

For the *Legionella* culture method, ten of the ten samples were positive with a mean *Legionella micdadei* concentration of 82 CFU/mL.

Ten additional samples were artificially contaminated with two live, wild-type cultures, *Legionella pneumophila* SG2-15 QL346945-6, at a medium level, 0.9 CFU/mL, and *L. oakridgensis* QL14522-2A at a high level, 1200 CFU/mL. The mixed culture of interfering organisms was inoculated at a medium level, 28 CFU/mL.

Method A qualitatively detected *Legionella* in five of the ten samples tested.

Method B detected *Legionella* in eight of the ten test samples. Valid PCR reactions took place in each well with the mean *Legionella* concentration as 5500 GU/100 mL.

Method C detected *Legionella* in all ten of the samples tested. Correct characterization into groups (LP SG1, LP SG2-15, *Legionella* spp.) was accurate (LP SG2-15 and *Legionella* spp.) for 100% of the samples. The mean quantification value for eight of the positive samples for LP SG2-15 was interpreted as 590 GU/100 mL. The remaining two samples were correctly detected however the interpreted concentration was below the LOQ. The mean quantification value for all ten of the positive samples for *Legionella* spp. was interpreted as 42,000 GU/100 mL.

For the *Legionella* culture method, nine of the ten samples were positive with a mean *Legionella oakridgensis* concentration of 3 CFU/mL. *Legionella pneumophila* SG2-15 was recovered from only one of the ten samples at a concentration of 1 CFU/mL.

High level Legionella micdadei and L. dumoffii

Ten samples were artificially contaminated with a live, wild-type culture, *Legionella micdadei* QL14522-1A, at a high level, 111 CFU/mL. The mixed culture of interfering organisms was also inoculated at a high level, 49 CFU/mL.

Method A qualitatively detected *Legionella* in one of the ten samples tested.

Method B detected *Legionella* in ten of the test samples. Valid PCR reactions took place in each well with the mean *Legionella* concentration as 930 GU/100 mL.

Method C detected *Legionella* in all ten of the samples tested. Correct characterization into groups (LP SG1, LP SG2-15, *Legionella* spp.) was accurate (*Legionella* spp.) for 100% of the samples. The mean quantification value was interpreted as 3300 GU/100 mL.

For the *Legionella* culture method, one of the ten samples were positive. With only one colony observed for the positive sample the *Legionella micdadei* concentration was calculated as 0.5 CFU/mL

Ten additional samples were artificially contaminated with a live, wild-type culture, *Legionella dumoffii* QL584231-6, at a high level, 114 CFU/mL. The mixed culture of interfering organisms was inoculated at a medium level, 4.9 CFU/mL.

Method A detected *Legionella* in zero of the ten test samples.

Method B detected *Legionella* in nine of the ten test samples. Valid PCR reactions took place in each well with the mean *Legionella* concentration as 980 GU/100 mL.

Method C detected *Legionella* in all ten of the samples tested. Correct characterization into groups (LP SG1, LP SG2-15, *Legionella* spp.) was accurate (*Legionella* spp.) for 100% of the samples. The mean quantification value was interpreted as 2300 GU/100 mL.

For the *Legionella* culture method, three of the ten samples were positive with a mean *Legionella dumoffii* concentration of 7 CFU/mL.

Summary of Results

These results indicate that modern PCR methods more accurately and reliably detect low levels of *Legionella* spp. Indeed, at low levels (<10 CFU/mL), the cultural method was at best only able to accurately detect the presence of *Legionella* spp. 10% of the time. Methods B and C demonstrated incredible qualitative accuracy in detection of *Legionella* across the inoculation range.

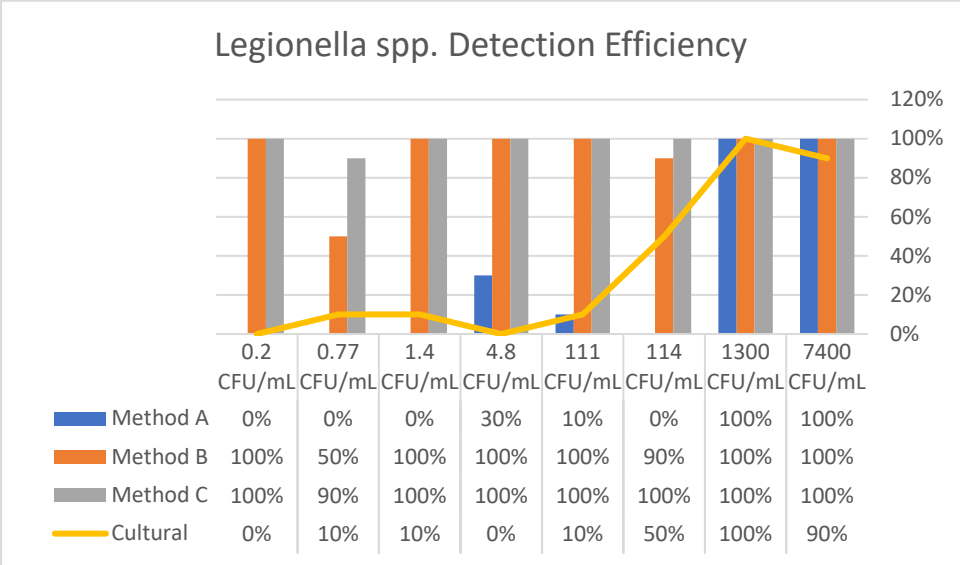

Fig. 1 Artificial contamination levels compared to detection efficiency. Method A showed no recovery until 4.8 CFU/mL Method B demonstrated 50-100% efficiency at low and medium contamination levels, and consistently showed 100% detection cy at high contamination levels. Method C consistently showed 90-100% efficiency across the contamination range.

In comparison, the cultural method could not be relied on to consistently recover *Legionella* spp. until 3 Log CFU/mL of *Legionella* spp. were present. This is particularly concerning given that these were artificially contaminated water samples; it may reasonably be expected that this phenomenon would be even more pronounced in stressed, field samples.

Analysis of the efficiency of the two quantitative PCR methods (Methods B and C) suggests that both are superior to the cultural method both in terms of relative detection efficiency of the inoculum, and consistency across sampling events. At the “high” inoculation level, Methods B and C were comparable to

each other in efficiency. Moreover, the data appear to suggest a mathematically consistent relationship between GU and CFU whereby Log (GU+1) = Log CFU, though more work is needed to evaluate the statistical significance of this observation.

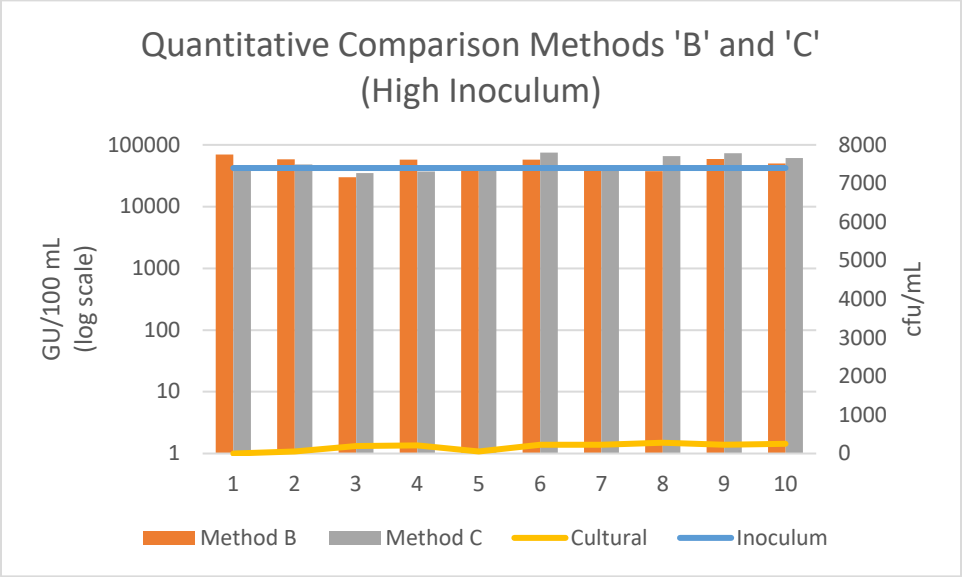

Fig. 2 Comparison of the quantitative results of methods B and C. Both methods showed very similar quantitative results (in GU/100 mL) and showed a high response consistent with the high inoculum level (7400 CFU/mL). The cultural method showed an average of only 2.3% recovery of the inoculum.

Table 5 – Log Comparison of GU to CFU

Method	Average Log GU/mL	Log CFU/mL[a]	Log Difference
B	2.71[b]	3.86	1.13
C	2.69[c]	3.86	1.16

[a]7400 CFU/mL
[b]st. dev. = 0.12
[c]st. dev. = 0.11

Discussion

The results of this study clearly demonstrate that modern PCR methods are more sensitive, reliable, and accurate, than traditional cultural methodology as outlined by the CDC and ISO 11731.

While all the modern PCR methods evaluated met or exceeded their label claims, with Methods B and C demonstrating superior reliability in detection at all levels of *Legionella* spp. contamination compared to the traditional cultural method, the additional qualitative capabilities of Method C, suggest that it may be a superior method option for routine *Legionella* spp. monitoring in premise water systems.

Additionally, historical PCR methods have been observed by many to have high negative predictive value (NPV), and they have been frequently suggested for use as a negative screening tool for routine sampling (Collins, Jorgensen, Willis, & Walker, 2015); (Collins, Stevenson, Walker, & Bennett, 2017); (Chen & Chang, 2010) (Guillemet, et al., 2010) (Lee, et al., 2011). While using PCR as a negative screening tool could certainly be helpful in building confidence in the microbial health of a water system, particularly after an excursion that required intervention, the practitioner is left with several issues. Most importantly, any

positive result must be “confirmed” with a traditional cultural method. As already discussed, attempting to confirm the result of a molecular assay by cultural means is fraught with pitfalls. Chiefly, given that as little as 53% of *Legionella* spp. present may be recovered (Boulanger & Edlestein, 1995), and with this subpopulation being dramatically decreased further by selective measures such as acid and heat treatment as recommended in CDC/ISO 11731 cultural methods (Roberts, August, & Nelson, 1987), one should not reasonably expect for results even among parallel cultural examinations of the same sample to be consistent (Boulanger & Edlestein, 1995). In practice, this means that reporting a “no growth” cultural result as part of a “confirmation” scheme following a positive PCR detection is not justifiable.

In contrast, our results indicate that the modern PCR methods evaluated may have a high positive predictive value (PPV). Treating the artificially contaminated waters as true positives (as confirmed by parallel inoculation verification), Method C demonstrates a PPV of 90.4%. While these results are preliminary and limited in applicability based on the limited scope, such a high PPV would indicate that a positive result from the modern PCR assay makes routine cultural confirmation of the PCR result redundant. Moreover, Method C provides significant qualitative characterization which should be sufficient in most cases of routine monitoring when a positive result is encountered. If so, culturing *Legionella* spp. may only be necessary for serious outbreak investigations for source tracing purposes.

Q Labs is now executing a second phase of this study wherein water treatment professionals are being recruited to submit water samples from their existing WMPs. These samples will be evaluated with Method C and the cultural method in parallel to determine if the results obtained are consistent with those observed in the artificially contaminated waters. Examination of the PPV as calculated from “field” samples will be of particular interest.

The data generated here further demonstrate the inadequacy of the cultural “gold standard” method. The cultural method cannot be reliably counted on to quantify *Legionella* spp., accurately nor precisely, in water samples. While historically, there perhaps were not superior alternatives, the industry should now closely examine the role that modern PCR methods should play in WMPs. The rapid time to result, and higher sensitivity when compared to cultural methods, may allow industry to be more agile in quickly responding to *Legionella* recoveries. To wit: modern PCR technologies may be a new “gold standard” for *Legionella* monitoring to maintain public health and safety.

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Conflicts of Interest

No conflicts of interest declared.

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