Validation and in-field testing of a new on-site qPCR system for quantification of *Legionella pneumophila* according to ISO/TS 12869:2012 in HVAC cooling towers

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ABSTRACT

Legionella pneumophila, found in engineered water systems such as HVAC cooling towers, poses a significant public health risk. Culture, though routinely used to quantify *L. pneumophila*, has several disadvantages including long turnaround time, low sensitivity, and inter-laboratory variability. In this study, we validated the performance of an on-site quantitative polymerase chain reaction (qPCR) detection system for *L. pneumophila* in accordance with International Standards Organization Technical Specification 12869:2012. We evaluated specificity, limit of detection and quantification, and calibration curve linearity. Additionally, we evaluated whole system recovery and robustness using samples taken from taps and evaporative cooling towers. We then compared the system's performance against laboratory culture and laboratory qPCR across 53 cooling towers in a 12-week in-field study. We found that concordance between on-site qPCR and culture was both laboratory- and site/sample-dependent. Comparison of laboratory qPCR with on-site qPCR revealed that laboratory results were highly variable and showed little concordance. Some discordance may be explained by time delay between sample collection and testing ('shipping effect') which may lead to inaccurate reporting. Overall, our study highlights the value of on-site qPCR detection of *L. pneumophila*, demonstrates that laboratories are prone to misreporting results due to shipping effects, and reveals significant discordance between laboratory qPCR and culture.

Key words | cooling tower, HVAC, *Legionella pneumophila*, on-site qPCR, shipping effects, validation

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INTRODUCTION

Legionella is a common water-based pathogen in man-made engineered water systems in developed countries (Vinson 2012; Winn 2015; Lucas & Fields 2016) and represents a significant risk to public health. Infections by *Legionella* (Legionellosis) can cause Pontiac fever with respiratory flu-like symptoms, or Legionnaires' disease (LD) with more severe atypical pneumonia (Winn 2015; Lucas & Fields 2016). *L. pneumophila* strains are responsible for approximately 95% of all cases of LD (Walser *et al.* 2014; Kirschner 2016) and the sources of contamination have frequently been identified as HVAC (heating, ventilation, and air conditioning) evaporative cooling towers and domestic hot water systems (Walser *et al.* 2014; van Heijnsbergen *et al.* 2015).

Legionella is difficult to control due to its ability to replicate in protozoan hosts and its tendency to exist in biofilms, both of which contribute to its resistance to disinfectants (Kim *et al.* 2002; Abdel-Nour *et al.* 2013). Currently *Legionella* levels ≥100 CFU/mL in cooling

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towers represent a potential increased threat to human health (Occupational Safety and Health Administration Technical Manual Appendix III: 7–3 Water Sampling Guidelines). Disease prevention strategies focus on detection of *Legionella* and testing is mandatory in many countries (Bartram 2007; McCoy *et al.* 2012). Reporting false negatives or under-reporting of *Legionella* has significant consequences for public health, while reporting of false positives or over-reporting may increase the cost of system operation due to unnecessary treatment and cleaning. In the United States, the annual health-related economic cost is estimated to exceed $716 million (Giambrone *et al.* 2013; Winn 2015; Lucas & Fields 2016).

There are numerous methods available for *Legionella* detection, but the most widely used methods are culture and quantitative polymerase chain reaction (qPCR) (Lucas & Fields 2016; Whiley & Taylor 2016). The advantage of culture is it detects viable and culturable bacteria. In contrast, qPCR is significantly more sensitive than culture, but it is thought that this is due to detection of dead cells, extracellular DNA, and viable but non-culturable cells (VBNC) (Delgado-Viscogliosi *et al.* 2009; Whiley & Taylor 2016). The disadvantages of culture are that it is time-consuming (results are typically available in 10–14 days), labor intensive, and requires specialized expertise to correctly identify *Legionella*. The methodology is prone to both technical and sample-specific issues that can negatively impact its accuracy. Processes such as filtration, heat treatment, and acid washes, as well as interference from biocides, are all known to result in decreased cell culturability (Roberts *et al.* 1987; Boulanger & Edelstein 1995; Delgado-Viscogliosi *et al.* 2009; McCoy *et al.* 2012; Lucas & Fields 2016; Whiley & Taylor 2016). This was highlighted by the US Centers for Disease Control and Prevention (CDC) in a proficiency testing study of 20 US laboratories for the Environmental *Legionella* Isolation Techniques Evaluation (ELITE) Program. Overall, the certified laboratories underestimated actual *Legionella* concentrations by an average of 1.25 log (17-fold) and values differed between laboratories by an average of 0.78 log (6-fold) (Lucas *et al.* 2011). The study concluded that culture plating significantly underestimated *Legionella* counts, was highly variable between laboratories, and had a significant false negative rate (Lucas *et al.* 2011). This may be because culture cannot differentiate between various developmental forms and physiological states, such as cell doublets, filamentous forms, intracellular *Legionella* in amoeba and protozoa, and VBNCs (Hussong *et al.* 1987; Delgado-Viscogliosi *et al.* 2009; Ducret *et al.* 2014; Robertson *et al.* 2014; Lucas & Fields 2016; Kirschner 2016; Whiley & Taylor 2016).

Currently, both culture and qPCR are performed in a centralized laboratory location. The time delay between sample collection and processing (typically 24–72 hours) is primarily due to shipping. Issues with shipping include transportation of a human pathogen by mail, sample loss or mishandling, sample preservation, and *Legionella* growth or degradation during shipping. The effect of shipping on laboratory *Legionella* testing is unclear with some studies demonstrating a significant effect (McCoy *et al.* 2012), whereas others report minimal impact (Flanders *et al.* 2014). Therefore, on-site qPCR that enables simple and robust quantification of *Legionella* in the field may be useful in routine monitoring, developing an efficient treatment regimen, and facilitating rapid response and containment of infectious outbreaks (Kozel & Burnham-Marusich 2017). To this effect, Spartan Bioscience Inc. (Ottawa, Canada) has commercialized the first on-site qPCR detection system for quantifying *L. pneumophila* in water samples.

To validate the new system, performance was assessed under intermediate precision conditions (multiple analysts, reagent lots, equipment and days) following recommendations in the International Standards Organization Technical Specification (ISO/TS) 12869:2012 'Water quality – Detection and quantification of *Legionella* spp. and/or *Legionella pneumophila* by concentration and genic amplification by quantitative polymerase chain reaction (qPCR)' (International Organization for Standardization 2012). Two previously published papers validated laboratory-based qPCR tests in accordance with ISO/TS 12869:2012, and this study is modeled after those validations (Collins *et al.* 2015; Omiccioli *et al.* 2015). To further evaluate the performance of the on-site qPCR system, an in-field study was conducted with 53 cooling towers. On-site qPCR was used to monitor *L. pneumophila* levels in these HVAC cooling towers and the results were compared to traditional methodologies (laboratory qPCR and laboratory culture). This study adds to current literature and highlights the value of on-site qPCR detection of *L. pneumophila*.

MATERIALS AND METHODS

On-site qPCR using the Spartan *Legionella* detection system

On-site qPCR was performed using the Spartan *Legionella* Detection System (Spartan Bioscience, Ottawa, Canada). This system consists of a portable DNA analyzer called the Spartan Cube® and a single-use disposable concentration kit and test cartridge. Using this system, intact bacteria in the sample (including *L. pneumophila*) were concentrated and then *L. pneumophila* was quantified by qPCR.

Briefly, samples were introduced into the concentration kit through the use of a syringe. The samples were filtered across a 0.45 μm Polyethersulfone (PES) Millex-HP filter (Merck Millipore Ltd, Cork, Ireland). The filter was washed to remove unwanted contaminants, and then the captured intact bacteria were eluted from the filter by gentle homogenization (performed manually with a modified syringe, as part of the kit). The eluate was transferred to a test cartridge containing the Spartan *Legionella* detection reagents. Finally, the test cartridge was placed into the Spartan Cube® for quantification of *L. pneumophila*.

Each test cartridge includes qPCR primers and a probe that are designed against a highly conserved region of the *L. pneumophila* macrophage infectivity potentiator (*mip*) gene (Benitez & Winchell 2013). The test cartridge also contains an internal positive control to detect the presence of qPCR inhibitors in the sample, and to identify reagent degradation and contamination. Negative controls are performed during manufacturing of the sealed test cartridge, which is opened just prior to use.

Bacterial growth

Legionella pneumophila subsp. *pneumophila* strain Philadelphia-1 (ATCC 33152) was obtained from the American Type Culture Collection (ATCC, Manassas, VA). Bacterial stock was rehydrated, and maintained on Buffered Charcoal Yeast Extract (BCYE) agar plates (Bio-Media Unlimited Ltd, Toronto, ON) in an incubator at 37 °C. For liquid culture, *Legionella* was expanded in Yeast Extract Buffered (YEB) broth in a shaking incubator at 37 °C and 380 rpm. After an appropriate growth time in liquid culture, the bacterial concentration was measured at OD 600 nm and calculated as described by ISO/TS 12869:2012 (where an OD of 0.5 corresponded to 10^9 CFU/mL).

Verification of inclusivity and exclusivity

The on-site qPCR system was verified for analytical specificity according to Section 10.2 'Inclusivity and exclusivity of probes and primers' of ISO/TS 12869:2012. Specifically, this involved verifications for inclusivity (15 *L. pneumophila* serogroups) and exclusivity (25 non-target species recognized as not belonging to *Legionella* genus or *L. pneumophila* species and/or being phylogenetically close).

All 40 bacterial strains and corresponding media were obtained from ATCC. Bacterial strains were rehydrated and maintained in the appropriate culture growth media and conditions as recommended by ATCC. Bacterial concentrations were measured at an optical density (OD) of 600 nm and calculated such that an OD of 0.5 corresponded to 10^9 CFU/mL as described by ISO/TS 12869:2012.

For the inclusivity panel, bacterial strains were diluted to approximately 5 CFU/μL (100 CFU per reaction) in water. For the exclusivity panel, bacterial strains were diluted in water to approximately 500 CFU/μL (10,000 CFU per reaction). A positive result for *L. pneumophila* detection was characterized by a rise greater than 500 arbitrary units (AU). All samples were tested in triplicate.

In addition to testing against 40 bacterial strains, the specificities of the *L. pneumophila* primers and probe were assessed *in silico* for 15 serogroups of *L. pneumophila*. In brief, *mip* gene sequences were retrieved from NCBI GenBank (www.ncbi.nlm.nih.gov/genbank/) and primer and probe sequences were assessed for significant sequence homology using BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch).

Verification of linearity of the qPCR calibration curve

The calibration curve of the on-site qPCR system was verified according to recommendations in Section 10.3 'Verification of the calibration function of the quantitative PCR phase' of ISO/TS 12869:2012. Reproducibility was

assessed with three operators, over a range of 3 days, and using 20 Spartan Cube® devices.

Four concentrations of *L. pneumophila* were prepared from a secondary standard so that 20, 200, 2,000, and 20,000 GU of *L. pneumophila* were added per reaction. Concentrations of 20 GU/reaction were repeated 10 times per operator, while 200, 2,000, and 20,000 GU/reactions were repeated five times per operator.

The bias, precision, accuracy of linearity, and uncertainty of linearity were calculated as described in ISO/TS 12869:2012. The accuracy of linearity had to satisfy the requirement of $E_{lin} \leq 0.15$ for each concentration of the standard curve. For the overall result to be valid, the PCR efficiency was required to be between 75 and 125%, corresponding to a slope of regression between –4.115 and –2.839.

Verification of lower limit of detection and limit of quantification

The lower limit of quantification (LOQ) and limit of detection (LOD) for the on-site qPCR system were verified according to Section 10.4 'Verification of the PCR limit of quantification' and Section 10.5 'Verification of the PCR limit of detection' of ISO/TS 12869:2012, respectively.

A dilution from a secondary standard of *L. pneumophila* was made to 10^6 GU/μL, and then dilutions were made down to the LOD of 2 GU/reaction. LOD is defined as the concentration at which at least 90% of the results are positively detected. The dilution step was repeated by multiple operators. The LOQ was tested by multiple operators on multiple days at 20 GU/reaction.

Verification of the entire on-site qPCR *Legionella* detection system

The whole system (concentration and qPCR) was verified by assessing recovery and robustness using real-world water matrices from cooling towers. This verification addresses the objectives in Section 10.6 'Recovery method' and Section 10.7 'Robustness' of ISO/TS 12869:2012, respectively. Recovery was calculated as the percentage of qPCR fluorescence signal post concentration compared to the signal generated by directly amplifying the water sample without concentration (direct qPCR).

To verify that recovery was not affected by matrix, we tested distilled water, tap water, and cooling tower water that was known to be free of *L. pneumophila* DNA. These water samples were artificially contaminated with dilutions of a stock suspension of *L. pneumophila* (ATCC 33152). Three input concentrations were tested corresponding to 20, 100, and 250 GU/mL. Each concentration was made using different replicate serial dilutions from the same stock suspension. For each concentration, at least three separate 22-mL spiked samples were run by several operators.

Study design of the in-field assessment of *L. pneumophila* in HVAC samples

Samples to be externally evaluated were collected over a 12-week period from 51 HVAC cooling towers in the Canadian cities of Ottawa, Toronto, and Montreal. These samples were collected weekly from their designated system location on their scheduled day (Figure 1). Two out of the 51 towers were shut down due to operational issues and alternative towers were brought on-line in the same facility. As a result, a total of 53 towers were tested. Individual test results from these new towers were included in the weekly testing analyses (by culture and on-site qPCR). However, for the week-over-week analyses, the four towers affected were considered as discrete.

In-field water sample collection and preparation

Prior to starting this study, all operational towers were tested by building operators at start-up with qPCR, weekly with

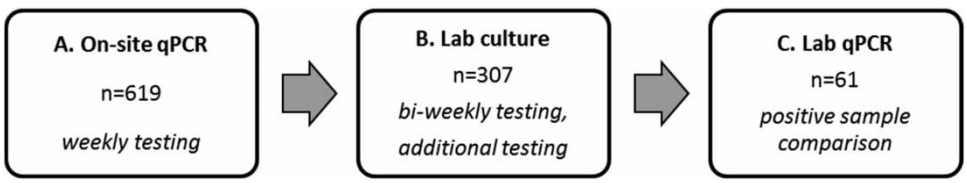

Figure 1 | Study design and water sample collection overview. Weekly on-site qPCR testing was performed on 53 HVAC towers (619 individual samples). Of these samples, 307 were sent for laboratory culture testing. Of the 307 samples sent for laboratory culture testing, 61 were also sent for laboratory qPCR testing.

dipslides, and every 4 weeks with laboratory culture testing (Public Works and Government Services Canada 2016). Since the on-site qPCR system was being evaluated against these existing practices, there was some heterogeneity in terms of culture laboratories (and culture methods) used by different buildings. During the in-field study, HVAC water samples were collected by site personnel using each building's standard collection procedure in accordance with one of the following sampling guidelines: ISO/TS 11731-2:2017, ISO/TS 12869:2012, or CDC culture procedure (Centers for Disease Control and Prevention (CDC) 2005; International Organization for Standardization 2012, 2017). For external laboratory analysis, samples were collected in sterile containers with sodium thiosulfate (provided by the external laboratories), then mailed in a Styrofoam cooler by express post for culture plating or qPCR in accordance with each laboratory's recommendations. A chain of custody form was required by each laboratory, and the sample's condition was assessed upon arrival for compliance. For on-site qPCR, no sodium thiosulfate was added to the water samples.

Quantification of *L. pneumophila* in HVAC samples by external laboratory culture

Culture samples were collected as described above and sent to the following laboratories: Mold & Bacteria Consulting Laboratories (MBL) (Mississauga, Canada), Pinchin (Mississauga, Canada), and EnvironeX (Quebec, Canada). These laboratories followed either ISO 11731:2004, CDC procedures, or Standard Methods for the Examination of Water and Wastewater (Centers for Disease Control and Prevention (CDC) 2005; International Organization for Standardization 2017; 9260 J Detection of Pathogenic Bacteria – *Legionella* Quantification and Identification). Individual samples may have been subjected to various pre-treatments such as heat and/or acid in order to eliminate other microbial flora that may confound the growth of *Legionella* spp. Results were presented as Colony Forming Units per milliliter (CFU/mL). Limits of detection reported by the external culture laboratories are shown in Table 1. In addition to these regularly scheduled monthly culture tests, extra samples were collected for culture testing, such that all towers were tested every 2 weeks on average. After week 5 of the study, a selection of samples that demonstrated a positive on-site qPCR result of >40 Genomic Units per milliliter (GU/mL) were sent for additional culture testing in an external laboratory.

Table 1 | Limit of detection reported by external culture laboratories (CFU/mL)

Laboratory	Limit of detection (CFU/mL)
Lab 1	<1
Lab 2	<1
Lab 3	<5, <100[a]

[a]Due to the presence of interfering microbial flora in samples.

Quantification of *L. pneumophila* in HVAC samples by external laboratory qPCR

During the study, 61 water samples were shipped to the following external laboratories after being collected as described above: Magnus (Boucherville, QC, Canada), Pinchin (Mississauga, ON, Canada), Sporometrics (Toronto, ON, Canada), and EnvironeX (Quebec, QC, Canada). These laboratories followed ISO/TS 12869:2012. The qPCR results were presented as GU/mL. This is the unit of measurement for estimating the number of bacterial DNA copies present in a sample and is synonymous with Genomic Equivalents per milliliter (GE/mL). The limits of detection reported by the external qPCR laboratories are summarized in Table 2.

Concordance between on-site qPCR, external laboratory qPCR, and culture for *L. pneumophila*-positive HVAC samples

To test the concordance of on-site qPCR against external laboratory methodologies, 19 water samples that had been reported positive by on-site qPCR were shipped to three

Table 2 | Limit of detection reported by external qPCR laboratories (GU/mL)

Laboratory	Limit of detection (GU/mL)
Lab 1	<0.5, <0.9[a], <2.5[a], <4.5[a]
Lab 2	<0.8
Lab 3	<1
Lab 4	<0.8

[a]Variable limit of detection due to presence of non-quantifiable legionella.

different laboratories (two for culture and one for qPCR) and compared.

Evaluating shipping effects on *L. pneumophila* quantification

To investigate the potential change in *L. pneumophila* quantification due to shipping time, 70 representative samples from 20 unique HVAC towers (a mix of those that were originally reported as positive or negative) were spiked with two different concentrations of *L. pneumophila* (15,000 or 4,000,000 GU/mL) in the log phase of growth. The biocide-neutralizing agent sodium thiosulphate, which is routinely utilized by external laboratories for sample preservation, was added to the samples (0.2% final concentration). A control group of HVAC samples was also tested without the addition of sodium thiosulphate to determine if this agent had an impact on sample preservation. Samples were held at room temperature and direct qPCR was performed at time zero and 72 hours. In order to simulate the shipping effect on in-field samples tested with on-site qPCR, 32 different HVAC samples were retained for an additional 24–72 hours and re-tested to monitor changes in quantification over that time period.

Categorization of test results

Test results for qPCR and culture were categorized as either positive or negative. Concentrations <10 GU/mL were considered to reflect cooling towers under control (described as negative in our results). This is also reflective of current standards for *Legionella* monitoring as a properly controlled tower (Public Works and Government Services Canada 2016). Samples with a concentration of ≥10 GU/mL were considered as positive, which would require additional monitoring or action such as potentially shutting down the tower. This threshold was also selected to normalize the results from external laboratories and to account for their variable limits of detection (Tables 1 and 2).

Statistical analysis

For statistical analysis, the GU/mL values were expressed as decimal logarithms. Statistical analysis was performed according to the recommendations in ISO/TS 12869:2012. Linear correlation between datasets generated by the three different methodologies (on-site qPCR, laboratory culture, and laboratory qPCR) was performed using the Pearson correlation coefficient. Chi-square (χ^2) tests were performed to compare multiple populations to determine if there was a statistical difference (p-value < 0.05).

RESULTS

Verification of inclusivity and exclusivity

All 15 *L. pneumophila* serogroups in the inclusivity panel were positively detected by the Spartan Cube® (Table S1). All 25 microbial species in the exclusivity panel were not detected (Table S2). (Tables S1 and S2 are available with the online version of this paper.)

Verification of calibration curve

Analysis of the on-site qPCR system's calibration curve resulted in a linear regression of $y = -3.516x + 38.664$, which corresponded to an efficiency of 92.5%. This meets the requirement of 75–125% efficiency and slope of –4.115 and –2.839. From the linear regression, accuracy of linearity values met the requirement of $E_{lin} < 0.15$ for each level tested (Table S3, available online).

Verification of lower limit of detection and limit of quantification

The limit of detection (LOD) of the on-site qPCR system was verified at 2 GU/reaction (Table S4). Similarly, the limit of quantification (LOQ) was verified at 20 GU/reaction (Table S5) with an accuracy at the LOQ (E_{LQ}) of ≤0.15. (Tables S4 and S5 are available online.)

Verification of the entire on-site qPCR system

Results showed that recovery of the on-site qPCR system was not affected by matrix conditions in the tested samples (Table 3). Specifically, the input bacterial concentrations were similar to the values determined by the on-site

Table 3 | Recovery and robustness of the entire on-site qPCR system in tap water, distilled water and HVAC matrix

Water source	Bacterial input (GU/mL)	Mean quantification (GU/mL)	Standard deviation of quantification	Mean quantification (Log GU/mL)	Log SD
Distilled water	20 ($n = 16$)	17.48	12.72	1.15	0.29
	100 ($n = 17$)	93.77	42.74	1.94	0.18
	250 ($n = 16$)	200.88	72.46	2.27	0.17
Tap water	20 ($n = 16$)	16.99	8.05	1.18	0.23
	100 ($n = 15$)	84.90	19.19	1.92	0.10
	250 ($n = 18$)	201.27	46.37	2.29	0.10
HVAC 1	20 ($n = 10$)	15.16	7.33	1.13	0.23
	100 ($n = 10$)	70.34	25.95	1.82	0.18
	250 ($n = 10$)	187.55	80.73	2.24	0.18
HVAC 2	20 ($n = 7$)	23.80	12.28	1.33	0.21
	100 ($n = 9$)	103.87	30.99	2.00	0.13
	250 ($n = 8$)	277.28	70.99	2.43	0.12
HVAC 3	20 ($n = 3$)	34.03	9.49	1.52	0.13
	100 ($n = 5$)	74.29	15.80	1.86	0.09
	250 ($n = 5$)	255.00	87.85	2.39	0.15

qPCR system. The low log standard deviation (LogSD) indicates robustness and reproducibility of the system as compared to intra-laboratory testing (Baume *et al.* 2013).

In-field study results for the on-site qPCR system and laboratory culture

Water samples were collected from 53 cooling towers for 12 weeks, resulting in a total of 619 on-site qPCR tests. Of the 619 tests, 93% produced a conclusive result, with 80% being negative (<10 GU/mL) and 13% positive (≥10 GU/mL) for *L. pneumophila*. Of the 79 positive samples, 14 had a level of *L. pneumophila* >100 GU/mL (Table 4). In terms of results by tower, 60% of towers were negative for the entire study, but 40% were reported as positive at least once during the study period, including four towers showing levels >100 GU/mL. Of the 307 tests performed by laboratory culture, 97% produced a conclusive result. Overall, 88% of culture results were negative and 9% positive. By culture, 85% of the towers were negative throughout the study and 15% were positive (Table 5). In comparison to on-site qPCR, laboratory culture under-reported *Legionella* levels in terms of positive tests (9 versus 13%) and under-called the number of positive towers (15 versus 40%).

Table 4 | Categorization of on-site qPCR results organized by test and by maximum value obtained in each HVAC cooling tower

	By test		By tower	
Level (GU/mL)	N	(%)	N	(%)
No result	44	(7.1)	–	–
<10	496	(80.1)	32	(60.4)
10–100	65	(10.5)	17	(32.1)
101–1,000	13	(2.1)	3	(5.7)
>1,000	1	(0.2)	1	(1.9)

Table 5 | Categorization of laboratory culture results organized by test and by maximum value obtained in each HVAC cooling tower

	By test		By tower	
Level (CFU/mL)	n	(%)	N	(%)
No result	9	(2.9)	–	–
<10	271	(88.3)	45	(84.9)
10–100	19	(6.2)	6	(11.3)
101–1,000	8	(2.6)	2	(3.8)
>1,000	0	(0)	0	(0)

(a)

	On-site qPCR (<10 GU/mL)	On-site qPCR (≥10 GU/mL)
Lab culture (≥10 CFU/mL)	3 (1%)	24 (8%)
Lab culture (<10 CFU/mL)	209 (70%)	62 (21%)

(b)

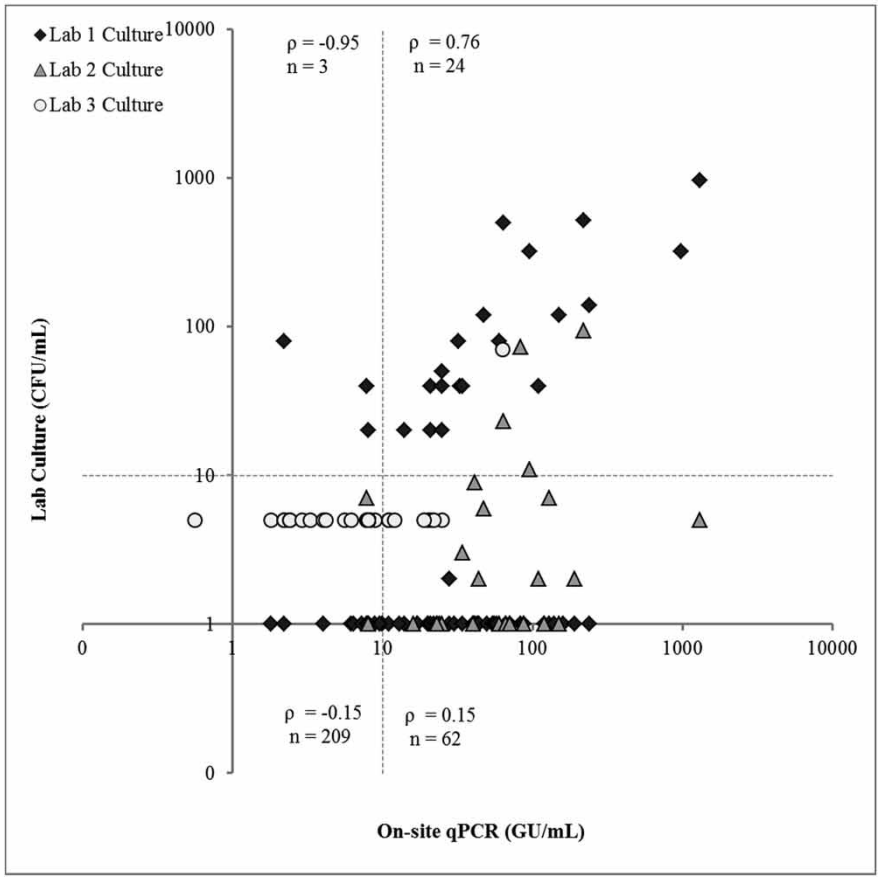

Figure 2 | Concordance between on-site qPCR and laboratory culture. (a) Concordance between on-site qPCR and laboratory culture categorized by positive (≥10 GU/mL or CFU/mL) or negative (<10 GU/mL or CFU/mL) results. (b) Comparison of on-site qPCR with culture performed by three laboratories. Pearson correlation was determined for data within each quadrant. The threshold for defining quadrants was set at 10 GU/mL and 10 CFU/mL.

Concordance between on-site qPCR and laboratory culture

By the end of the 12-week study, a total of 298 HVAC water samples had both an on-site qPCR result and a corresponding laboratory culture result. Overall, there was 78% concordance between on-site qPCR and laboratory culture (8% double positive, 70% double negative), and only 1% gave a laboratory culture positive result that was negative by on-site qPCR (Figure 2(a)). The majority of discordant results (21% of total) consisted of a positive on-site qPCR result that was negative by laboratory culture. Overall, concordance between results was laboratory-dependent (Figure 2(b)).

(a)

	On-site qPCR (<10 GU/mL)	On-site qPCR (≥10 GU/mL)
Lab qPCR (≥10 GU/mL)	4 (10%)	5 (12%)
Lab qPCR (<10 GU/mL)	7 (17%)	25 (61%)

(b)

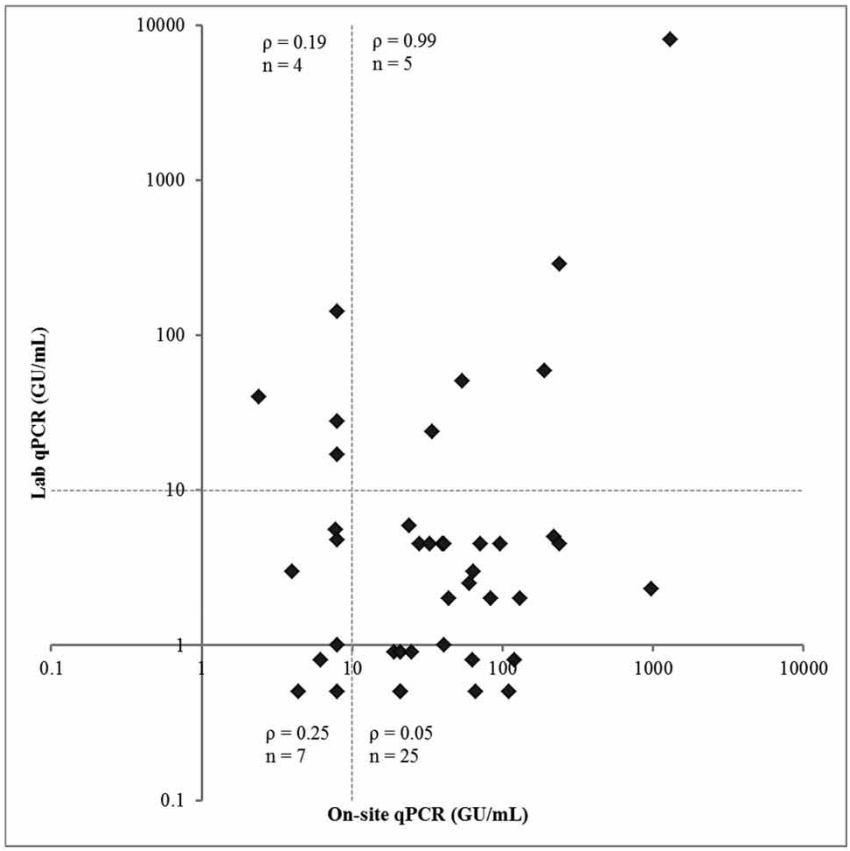

Figure 3 | Concordance between on-site qPCR and laboratory qPCR. (a) Concordance between on-site qPCR and laboratory qPCR categorized by positive (≥10 GU/mL) or negative (<10 GU/mL) results. (b) Comparison of on-site qPCR with laboratory qPCR. Pearson correlation was determined for data within each quadrant. The threshold for defining quadrants was set at 10 GU/mL.

Concordance between on-site qPCR and laboratory qPCR

A subset of 41 samples (41/619) was tested by both on-site qPCR and laboratory qPCR. Concordance was poor between the two methods (12% with positive samples and 17% with negative samples). The majority of samples (61%) were positive by on-site qPCR but negative by laboratory qPCR. Only 10% were positive by laboratory qPCR but negative by on-site qPCR (Figures 3(a) and 3(b)). All samples tested by laboratory qPCR experienced a shipping delay of 24–72 h.

Concordance between laboratory culture and laboratory qPCR

A subset of 61 samples (61/307) was tested by both laboratory culture (three different laboratories) and laboratory qPCR (four different laboratories). Concordance was poor

(a)

	Lab qPCR (<10 GU/mL)	Lab qPCR (≥10 GU/mL)
Lab culture (≥10 CFU/mL)	15 (25%)	3 (5%)
Lab culture (<10 CFU/mL)	34 (56%)	9 (15%)

(b)

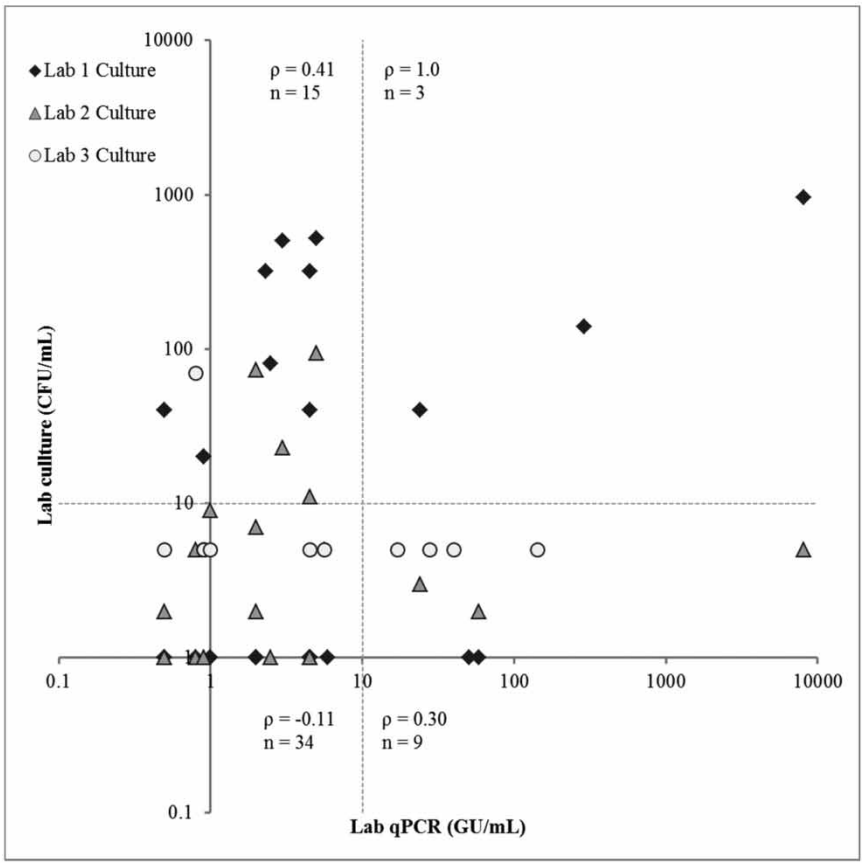

Figure 4 | Concordance between laboratory culture and laboratory qPCR. (a) Concordance between laboratory qPCR and laboratory culture categorized by positive (≥10 GU/mL or CFU/mL) or negative (<10 GU/mL or CFU/mL). (b) Comparison of laboratory qPCR with culture performed by three laboratories. Pearson correlation was determined for data within each quadrant. The threshold for defining quadrants was set at 10 GU/mL and 10 CFU/mL.

between the two methods (5% with positive samples and 56% with negative samples). Of these samples, 40% were positive by one method but negative by the other (25% positive by culture, 15% positive by qPCR) (Figures 4(a) and 4(b)). Overall, there was no correlation between the two methodologies. All samples tested by laboratories experienced a shipping delay of 24–72 h.

Comparison of matched samples evaluated using on-site qPCR, laboratory culture, and laboratory qPCR

Over the duration of the study, 19 matched samples were tested independently by three different methodologies: on-site qPCR, laboratory culture (by two different laboratories), and laboratory qPCR. Based on positive results, on-site

Table 6 | Comparison of matched samples evaluated using on-site qPCR, laboratory culture, and laboratory qPCR

Lab 1 culture (CFU/mL)	On-site qPCR (GU/mL)	Lab 2 culture (CFU/mL)	Lab qPCR (GU/mL)
520	**220**	**94**	5
500	**64**	**23**	3
320	**96**	**11**	<4.5
120	**47**	6	–
80	**60**	1	<2.5
60	**150**	<1	–
40	**110**	<1	<0.5
40	**34**	1	**24**
20	**25**	1	<0.9
<1	190	2	N/A[a]
<1	130	7	2
<1	120	<1	<0.8
<1	88	<1	–
<1	71	<1	<4.5
<1	44	2	2
<1	**41**	**73**	2
<1	41	1	1
<1	40	<1	<4.5
<1	16	<1	<0.5

[a]Delayed result (>3 days) due to shipping issues. Result excluded from analysis. *Note*: bold text indicates positive (≥10 GU or CFU/mL) results that were obtained by more than one method for a given sample.

qPCR was concordant with Laboratory 1 culture for 9/19 samples, but only 4/19 samples with Laboratory 2 culture. Of these four concordant samples, three corresponded to the highest positive results from Laboratory 1 culture and were the only samples concordant across both culture laboratories and on-site qPCR. Laboratory qPCR displayed poor concordance with laboratory culture (1/19 with Laboratory 1 culture and 0/19 with Laboratory 2 culture) and poor concordance (1/19) with on-site qPCR (Table 6).

Correlation of on-site qPCR and laboratory culture by HVAC cooling tower

Testing of *L. pneumophila* by on-site qPCR and culture revealed that some towers showed strong correlations while others did not. An analysis of six representative towers showed that three towers had a strong correlation between Laboratory 1 culture and on-site qPCR (Figures 5(a)–5(c)). In the other three towers, Laboratory 1 culture was negative for all tests, whereas there were dynamic changes in *L. pneumophila* levels ('growth events') reported by on-site qPCR (Figures 5(d)–5(f)).

Shipping effects in contrived *L. pneumophila* HVAC samples by direct qPCR

To investigate the impact of a shipping effect on the HVAC samples collected during this study, direct qPCR was performed on 70 samples derived from 20 unique HVAC towers that had been artificially spiked with *L. pneumophila*. After a 72-hour delay, the HVAC samples that had been spiked showed degradation relative to their time zero measurement in 66% of all samples tested (2-fold or greater decrease in quantification), 23% showed no change (less than 2-fold change), and 11% displayed growth (2-fold or greater increase in quantification). Furthermore, sodium thiosulphate effects on sample preservation were not statistically significant (χ^2 p-value = 0.70) (Table 7). These findings clearly indicate that there is a significant 'shipping effect' and that the time delay between sample collection and analysis can have a large impact on quantification.

Shipping effects on in-field samples

In order to confirm the shipping effect on *L. pneumophila* quantification, samples that were identified as positive by on-site qPCR were evaluated with three different methodologies: delayed on-site qPCR, laboratory qPCR, and laboratory culture. Relative to the original on-site qPCR result, the 'shipping effect' was again found to be substantial and was consistent across methodologies. Approximately 72% of samples displayed degradation, 15% showed no change, and 13% showed growth (Table 8). There were no statistically significant differences between the three methodologies (χ^2 p-value = 0.89) confirming the universality of the 'shipping effect' in these samples.

Week-over-week *L. pneumophila* growth

The potential significance of weekly versus monthly testing was evaluated. From weekly testing, it was observed that rapid *L. pneumophila* growth events occurred in 11/20

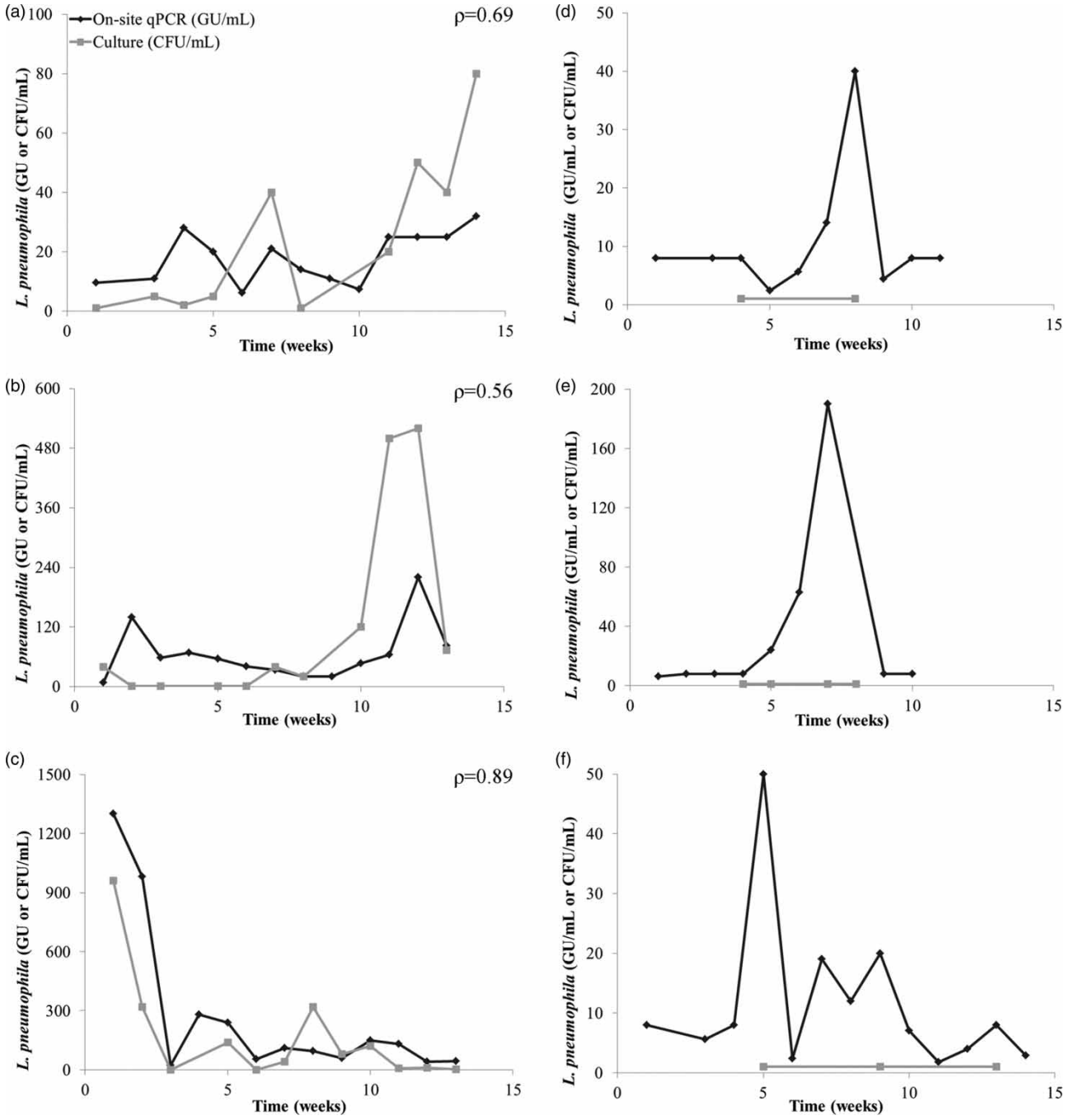

Figure 5 | Evaluation of select cooling towers by on-site qPCR and Laboratory 1 culture over time. (a)–(c) Individual cooling towers that demonstrate correlation ($\rho > 0.50$) between on-site qPCR and Laboratory 1 culture results. (d)–(f) On-site qPCR and Laboratory 1 culture results from individual cooling towers that do not track together.

positive cooling towers. These towers experienced between 3- and 21-fold growth over 7 days. Furthermore, the effect of testing weekly, bi-weekly, every 3 weeks, and monthly was analyzed to identify the number of transitional events from <10 to ≥ 10 GU/mL that would be missed with reduced testing frequency. Testing every 3 or 4 weeks would miss half of the events, whereas biweekly testing would miss approximately one-third (Table 9).

Table 7 | Effects of sodium thiosulphate on HVAC water samples spiked with *L. pneumophila* after 72 h incubation period relative to time zero

Relative change after shipping time delay	Sodium thiosulphate treated ($n = 48$) (%)	Untreated ($n = 22$) (%)	Overall ($n = 70$) (%)
≥2 Fold degradation	63	73	66
No change	25	18	23
≥2 Fold growth	13	9	11

(χ^2 p-value = 0.70).

Table 8 | Effects of shipping (1–3 days) on *Legionella* quantification in a subset of positive samples by three different methodologies

Relative change[a] after shipping time delay	Lab 1 culture (CFU/mL) ($n = 60$) (%)	Delayed on-site qPCR (GU/mL) ($n = 32$) (%)	Lab qPCR (GU/mL) ($n = 41$) (%)
≥2-Fold degradation	68	72	77
No change	17	16	13
≥2-Fold growth	15	13	10

(χ^2 p-value = 0.89).
[a]Changes were determined relative to original on-site qPCR values.

Table 9 | Evaluation of positive on-site qPCR results that would be missed with reduced testing frequency

Frequency of testing (# weeks)	Missed positive events (%)
1	0
2	31
3	49
4	53

Positive events were determined as transitions from <10 to ≥10 GU/mL.

DISCUSSION

The Spartan *Legionella* Detection System is the first on-site qPCR test for *L. pneumophila* in water systems. It was designed to meet the objectives of ISO/TS 12869:2012 and verified accordingly. The PCR primers and probe were designed against a highly conserved region of the *mip* gene and as such the on-site qPCR system accurately detected 15 *L. pneumophila* serogroups and did not detect 25 other microbial species (inclusivity and exclusivity lists in ISO/TS 12869:2012).

The calibration curve of the on-site qPCR system was verified across a dynamic range of 20–20,000 GU/reaction. As reflected by in-field testing, this dynamic range covers concentrations that are relevant to real-world results, such as the threshold of 1,000 CFU/mL that is found in various *Legionella* testing regulations around the world (Peter *et al.* 2017). The lower limit of detection for the qPCR assay was verified to be 2 GU/reaction and 8 GU/mL for the entire system. This LOD is similar to the 5–10 GU/reaction in previously validated *L. pneumophila* qPCR assays (Collins *et al.* 2015; Omiccioli *et al.* 2015).

Verification of the entire system with water samples suggests that it is not affected by the matrix, which was further investigated through in-field testing. This is important because several previously published qPCR assays have been shown to be inhibited by substances in HVAC water samples (Joly *et al.* 2006; Diaz-Flores *et al.* 2015). The entire system was shown to be robust and reproducible, with standard deviations per water source <0.25 log for all but one condition near the LOD of the test. In contrast, proficiency testing revealed a standard deviation of 0.78 log between certified culture laboratories (Lucas *et al.* 2011). This finding suggests that the on-site qPCR system may be a superior detection method compared to culture (Whiley & Taylor 2016).

The in-field results for on-site qPCR testing of HVAC towers demonstrated a much higher number of positive results than would have been anticipated by regional historical data: 13% of 619 samples had levels of *L. pneumophila* >10 GU/mL and 8% of cooling towers reached a level requiring immediate attention (>100 GU/mL). All samples requiring immediate attention came from four towers which repeatedly tested positive by on-site qPCR (Figures 5(b), 5(c) and 5(f)). This result indicated that the problem was likely not a new contamination with *L. pneumophila* but an inability to detect and control established contamination. Interestingly, two of the four problematic towers were located in connected buildings suggesting potential cross-contamination or common failures of biocide treatment. By on-site qPCR testing, 60% of towers had levels of *L. pneumophila* <10 GU/mL across the entire study period, which is similar to data collected from a previous survey of 1,000 cooling towers (Occupational Safety and Health Administration 1999).

Laboratory culture results were similar but generally under-reported positives compared to on-site qPCR: 9% of

tests were positive and only 15% of towers had at least one positive result across the entire study (compared to 40% by on-site qPCR). A recent review summarizing results from 28 studies showed that qPCR was approximately 50% more likely to return a positive result, with the majority of studies also reporting higher levels of *Legionella* by qPCR than by culture (Whiley & Taylor 2016).

There are several reasons why a 1:1 correlation between qPCR and culture is not expected. For example, conventional culture plating would undercount any intracellular *Legionella* inside amoeba or protozoa (400–1,000 CFU per cell), vesicles expelled from amoeba (20–200 CFU per cell), cell doublets, chains and other cell aggregates (Hay *et al.* 1995; Robertson *et al.* 2014). qPCR is expected to detect more *Legionella* than culture because it detects genomic material, whereas culture detects only culturable cells or clusters of culturable cells as colony forming units (CFU). In this study, on-site qPCR correlated well with culture (especially from Laboratory 1). With an overall concordance of 78%, it presents itself as superior to laboratory qPCR, which essentially shows no concordance with culture. Additionally, the observation that correlations between laboratory culture and laboratory qPCR were highly dependent on the individual laboratories suggests that any comparative studies in the literature may be confounded by this degree of variability. Other explanations have been proposed for the discordance between qPCR and culture results (Lee *et al.* 2011; Collins *et al.* 2017). Common hypotheses include: (1) effect of sample processing, (2) detection of dead cells and external DNA, (3) creation of VBNC cells, and (4) effect of time delay on testing.

The first hypothesis addresses the fact that standard procedures for the recovery of *Legionella* for culture include: filtration, heat/acid enrichment, and plating on selective media, with each step known to lead to a loss of cell culturability (Roberts *et al.* 1987; Boulanger & Edelstein 1995; Leoni & Legnani 2001). The presence of other microflora in the sample has also been shown to inhibit *Legionella* detection by culture (Lucas *et al.* 2011; Diaz-Flores *et al.* 2015). The laboratory-to-laboratory culture differences evident in this study may be due, in part, to sample processing differences.

The second and third hypotheses purportedly explain the disparity between qPCR and culture as the result of the presence of dead cells/extracellular DNA from lysed cells, or VBNC cells which would all be detected by qPCR but not by culture (Al-Bana *et al.* 2014; Whiley & Taylor 2016). The on-site qPCR concentration system allows >99.9% of extracellular DNA to pass through the filter, which means there is minimal to no impact on the qPCR results (manuscript in preparation). Furthermore, analysis of contrived HVAC samples revealed that bacterial death and subsequent DNA degradation occurred at a significant level (≥50% loss) in the majority (65%) of samples over 72 hours (Table 7). In the presence of oxidizing biocides (such as chlorine), death of bacteria and DNA degradation will occur faster. For this reason, the chlorine-neutralizing agent sodium thiosulphate is added to laboratory samples. In this study, bacterial death and rapid genomic DNA degradation in most of the HVAC matrices suggested that the impact of genomic DNA from dead bacteria on qPCR may be less than previously assumed. The fourth hypothesis is the time delay effect of shipping on levels of *Legionella*. When a subset of samples was retained and re-tested by on-site qPCR, it was observed that a majority of samples (approximately 85%) across all methodologies displayed a significant change in *Legionella* quantification, with the majority displaying degradation (approximately 72%), some showing no change (approximately 15%), and the rest displaying growth (approximately 13%) (Table 8). Similar observations have been reported previously, but there is considerable variability in terms of the degree of the problem and the pattern of changes. The effects may be highly dependent on the particular water source and the specific biological and chemical composition of the sample (the matrix) (McCoy *et al.* 2012). In this study, the consistent response across all three methods suggests that time delay may be a significant contributor to the inaccuracy of *Legionella* enumeration independent of the method of quantification in the tested towers. Furthermore, this study showed that sodium thiosulphate may be ineffective as a preservative suggesting that degradation of the bacteria may occur via mechanisms other than those associated with chlorine-based oxidizing biocides. Testing by on-site qPCR with no time-delay is a new method for eliminating the shipping effect. Moreover, it was found that on-site qPCR correlated better with laboratory culture than laboratory qPCR (albeit in a laboratory-dependent manner). Overall, these factors may explain why on-site qPCR in this study

was more successful at quantifying *Legionella* in cooling towers compared to previous studies with laboratory qPCR (Joly *et al.* 2006).

The findings of this study strongly suggest that on-site qPCR is able to accurately detect and quantify *L. pneumophila* in HVAC cooling towers and has the potential to significantly reduce public health risk compared to existing testing methods. Given that the on-site qPCR system is comparable to culture, its test results can be used within existing standards and action levels and is an important addition to current testing methods.

CONCLUSIONS

A new on-site qPCR detection system for *L. pneumophila* has been developed that provides immediate results in less than 1 hour. This validation study has shown that the system meets the objectives of ISO/TS 12869:2012 and performs as well as previously published qPCR assays.

In the HVAC cooling towers monitored in this study, we found that on-site qPCR was more sensitive and detected more positive towers than culture. Furthermore, the concordance between on-site qPCR and culture was significantly higher than that observed between external laboratory qPCR and culture. However, the degree of concordance was both laboratory- and tower-dependent. Comparable results between positive on-site qPCR and culture suggested that the on-site detection system is not prone to over-quantification due to the presence of dead bacteria or free DNA. Additionally, we demonstrated that shipping time-delay had a significant impact on *Legionella* enumeration in HVAC water samples regardless of methodology. Furthermore, we showed that on-site qPCR was a more reliable and rapid method of *Legionella* quantification compared to laboratory culture and that increasing the frequency of testing greatly improved response time to elevated levels of *Legionella*.

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CONFLICTS OF INTEREST

At the time of the study, all of the authors were employed by Spartan Bioscience Inc.

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