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SCIENTIFIC BRIEFING :
ON-SITE LEGIONELLA DNA TESTING

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ON-SITE LEGIONELLA TESTING: SCIENTFIC BRIEFING

New award-winning Legionella testing technology detects water contamination in buildings

SUMMARY

- Legionnaires' disease is a common and potentially fatal pneumonia caused by inhaling water vapour contaminated by Legionella bacteria
- Cooling towers in buildings are frequently contaminated with Legionella
- Traditional Legionella testing methods are slow and inaccurate, and have proven ineffective at detecting Legionella contamination
- Spartan Bioscience has developed the first and only on-site Legionella test that is fast and accurate for detecting Legionella contamination before it grows out of control
    - The test is the 2018 winner of the HVAC industry's top innovation award for Indoor Air Quality
    - Customers include the U.S. Centers for Disease Control & Prevention (CDC), New York State Department of Health (NYS DOH), Virginia Tech (the team that solved the Flint, Michigan water crisis), and the majority of Canada's largest real estate companies

SCIENTIFIC BACKGROUND

LEGIONNAIRES' DISEASE IS COMMON AND UNDERDIAGNOSED

- Legionnaires' is a severe pneumonia with a mortality rate ranging between 5-20% that is caused by breathing in water vapor contaminated with Legionella bacteria[1]
- High-risk groups include smokers, people over the age of 50, and those who are immunocompromised, but healthy people of all ages can be infected[2]
- Legionnaires' in the United States
    - Community-acquired pneumonia (CAP) affects 5.6 million patients annually in the US[3]
    - It is estimated that Legionnaires' accounts for 2–9% of cases of CAP[2]
        - i.e., 100K-500K cases per year, with a mortality rate of 5-20%
        - For comparison, asbestos-related cancer kills only 2,579 people per year in the US[4]

- According to the Centers for Disease Control & Prevention (CDC), 8,000–18,000 patients are hospitalized with Legionnaires' each year[5]
  - However, cases reported to the CDC "probably represent less than 5% of actual cases."[2]
  - The reason for the underreporting of cases is that pneumonia patients are not routinely tested for Legionnaires'. When they are, studies have found that 3.7% of non-hospitalized patients and 14% of hospitalized patients tested positive for Legionnaires'.[6]

- Legionnaires' in Canada
  - Public Health Ontario (PHO) confirmed 203 cases of Legionnaires' in the province in 2017[7]
  - But PHO estimates that the true number of cases is 7,574 per year in Ontario[8] [i.e., confirmed cases probably represent less than 5% of actual cases] [this number corresponds to approximately 20,000 cases across Canada]
    - For comparison, asbestos-related cancer kills only 467 people per year in Canada[9]
  - In Summer 2018, Humber River Hospital (North York, ON) conducted a clinical study in which hospitalized pneumonia patients were tested for Legionnaires'.10 of 32 patients, 9 tested positive for Legionnaires' (28%). 4 of the 9 patients were so sick that they required admission to the Intensive Care Unit (ICU). Based on these findings, discussions are underway to replicate this study with hospitals in the Ottawa-Gatineau region.

LEGIONELLA CONTAMINATION OF WATER SYSTEMS IN BUILDINGS IS COMMON

- Based on investigations of Legionella outbreaks, the CDC found that the most common sources of Legionella infection were:[11]
  - Potable water (e.g., showers): 56%
  - Cooling towers: 22%
  - Hot tubs: 7%
  - Industrial equipment: 4%
  - Decorative fountains: 4%
- Also, the CDC found that the most common outbreak settings were:
  - Hotels and resorts: 44%
  - Long-term care facilities: 19%
  - Hospitals: 15%
  - Senior living facilities: 7%
  - Workplaces: 7%
  - Community: 7%

- Legionella contamination of water sources in buildings is common:
    - In a study of healthcare facilities, 16% of cold-water sources and 6% of warm-water sources had *Legionella pneumophila* at concentrations >10 bacteria/mL[12]
    - The CDC collected water samples from cooling towers across the US and found *Legionella pneumophila* growing in 27% of towers[13]
    - In a study of hot-water showers in swimming pools, 40% of samples tested positive for *Legionella pneumophila*[14]

GROWTH OF LEGIONELLA BACTERIA

- There are more than 50 Legionella species, but *Legionella pneumophila* is the cause of 90%-95% or more of Legionnaires' cases[15,16]
- Legionnaires' disease outbreaks typically occur when *L. pneumophila* concentrations reach 1,000 bacteria/mL. However, lower concentrations may be dangerous for the young, elderly, and immunocompromised. For example, CDC guidelines recommend maintaining undetectable levels in hospitals with transplant units.[1,17]
- In water systems in buildings, the doubling time of *L. pneumophila* is typically between 22–72 hours, although the doubling time can be as few as 150 minutes, as reported in a case to investigators from the American Society of Heating, Refrigerating and Air-Conditioning Engineers (ASHRAE).[18,19] This means Legionella can reach outbreak levels of 1,000 bacteria/mL in as few as 7 days.
- *L. pneumophila* grows quickly at temperatures between 25°C-42°C, with an optimal growth temperature of 35°C.[15]

LEGIONELLA TESTING METHODS

Testing methods	Time to result	Detects live not dead bacteria?	Avoids false negatives from bacterial degradation during shipping?	Accuracy
Dipslides	24-48 hours	Yes	Yes	Poor
Lab culture	10-14 days	Yes	No	Good
Lab qPCR	2-7 days	No	No	Good
On-site qPCR	45 minutes	Yes	Yes	Excellent

DIPSLIDES

- Dipslides are performed on-site by growing bacteria from a water sample on a nutrient strip
    - Results are available in 24-48 hours

- Dipslides do not distinguish between Legionella and other bacterial species
- Dipslides have a real-world limit of detection of 10,000 bacteria/mL[20,21]
    - i.e., this poor sensitivity means that dipslides fail to detect almost all cases of Legionella contamination

LAB CULTURE

- Culture tests are performed by shipping a water sample to a lab where it is grown on a Petri dish
    - Results are available in 10-14 days because it takes this long for the Legionella bacteria to grow
- The Petri dish technology which underlies culture and dipslide tests was invented in 1887
- Culture testing has a real-world limit of detection of approximately 10 bacteria/mL even if concentrating a 250-mL or 1,000-mL sample[22,23]
- The CDC found that certified culture tests undercounted actual Legionella concentrations by 1.25 log (17-fold) and values differed between labs by 0.78 log (6-fold)[23]
    - On average, culture had a false negative rate of 11.5% [and this was with proficiency samples which are cleaner and have less inhibitors than real-world water samples]
- Australian public health scientists found that culture tests completely failed to detect Legionella in an outbreak investigation, but Legionella was accurately detected by qPCR tests[24]
- In a head-to-head study of lab culture vs. on-site qPCR for cooling towers, on-site qPCR detected Legionella bacteria above the acceptable limit of 10 bacteria/mL in 40% of cooling towers vs. only 15% of towers with lab culture[25]
    - A major reason for culture's poor performance was that bacteria degraded in 72% of water samples shipped to the lab
    - This caused culture to fail to detect a tower with contamination >1,000 bacteria/mL
    - 21% of cooling towers experienced rapid Legionella growth of 3X to 21X over 7 days
- Several jurisdictions have implemented mandatory culture testing of cooling towers, but this has failed to prevent ongoing disease outbreaks due to the poor performance of culture
    - e.g., During the summer of 2015, a Legionella-contaminated cooling tower caused an outbreak of Legionnaires' disease in New York City that killed 16 people and sickened 138 others.[26] In 2016, NYC required culture testing for all cooling towers. Despite this mandatory testing, the city continues to experience outbreaks. For example, an outbreak on the Upper East Side killed 1 person and sickened 6 more.[27] This was followed by an outbreak that sickened 14 people in the Flushing neighborhood of Queens.[28]

LAB QPCR

- qPCR tests utilize a Nobel-Prize-winning chemistry called quantitative Polymerase Chain Reaction (qPCR) to detect the DNA of organisms such as Legionella bacteria
    - In medical diagnostics, qPCR tests have replaced culture tests because qPCR is significantly faster and more accurate
- A scientific review of 28 studies involving 3,967 water samples found that qPCR was significantly more sensitive than culture at detecting Legionella, and culture consistently underreported Legionella levels[29]
- Leading organizations such as the CDC, New York State Department of Health (NYS DOH), and New York City Department of Health (NYC DOH) use qPCR tests for investigations of Legionnaires' outbreaks[30,31]
- qPCR tests may be performed on-site or by shipping a water sample to a laboratory
- Lab qPCR tests suffer from the following limitations:
    - They detect both live and dead bacteria, which means they can over-call the amount of pathogenic bacteria, thus leading to overtreatment of the water source[32]
    - In a study that compared on-site qPCR with lab qPCR, Legionella bacteria degraded in 77% of water samples shipped to the lab, which led to underestimates of the true amount of Legionella[25]

ON-SITE QPCR

- Spartan Bioscience has developed the first and only on-site Legionella qPCR test
    - It provides results in 45 minutes, in contrast to 10-14 days with culture testing
    - It provides results immediately on site, which means there is no bacterial degradation or false negative results from shipping water samples to a lab
    - It has patent-pending technology that detects live and not dead Legionella bacteria[25]
    - It is calibrated to culture so that 1 Genomic Unit (GU)/mL with Spartan's test is equivalent to 1 Colony Forming Unit (CFU)/mL with culture, which means that positive test results may be disinfected according to existing industry action levels
    - The test is validated according to the ISO 12869 standard for Legionella qPCR and has a limit of detection of 8 bacteria/mL[25]
        - Risk analysis research shows that a limit of detection of 10 bacteria/mL is effective at preventing Legionnaires' disease even for high-risk patients such as those undergoing organ transplant, chemotherapy, or dialysis[22]
- Spartan's on-site test is the winner of AHR Expo's 2018 Innovation Award for Indoor Air Quality (this is the HVAC industry's most prestigious innovation award)
- Customers of the test include expert Legionella organizations such as the CDC, New York State Department of Health (NYS DOH), and Virginia Tech (the team that solved the Flint, Michigan water crisis)

EXAMPLES OF LEGIONELLA TESTING STANDARDS AROUND THE WORLD

CANADA: PUBLIC SERVICES AND PROCUREMENT CANADA (PSPC) - MD 15161 STANDARD

- Cooling towers
    - Test weekly with dipslides and monthly with culture
    - Test with qPCR when starting up a tower and to confirm disinfection following a positive culture result
- Open water systems e.g., decorative fountains
    - Test weekly with dipslides and every 2 months with culture
    - Test with qPCR when starting up the water system and to confirm disinfection following a positive culture result
- Humidifiers
    - Test non-steam humidifiers monthly with dipslides and every 3 months with culture
    - Test steam humidifiers every 3 months with culture
    - Test with qPCR to confirm disinfection following a positive culture result
- Domestic hot water systems
    - Test monthly with dipslides (for systems connected to shower facilities and with a tank <50°C)
    - Test every 6 months with culture (for the most remote shower facility)
    - Test annually for all other domestic hot water systems
    - Test with qPCR to confirm disinfection following a positive culture result

UNITED STATES: CENTERS FOR MEDICARE & MEDICAID SERVICES (CMS) - QSO-17-30 DIRECTIVE

- As of July 6, 2018, all Hospitals, Critical Access Hospitals (CAHs) and Long-Term Care (LTC) "must have water management plans and documentation that, at a minimum, ensure each facility: Specifies testing protocols and acceptable ranges for control measures, and document the results of testing and corrective actions taken when control limits are not maintained."

NEW YORK CITY: LOCAL LAW 77 AND CHAPTER 8 RULES

- Cooling towers must be tested weekly with dipslides and every 90 days with culture

UNITED KINGDOM: HSG282 (2017)

- Spa pools and hot tubs should be tested quarterly

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SPARTAN ISO 12869 VALIDATION PAPER.

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- 53 cooling towers were tested for 12 weeks by Spartan's on-site qPCR test and lab culture
- On-site qPCR detected Legionella above the acceptable limit of 10 bacteria/mL in 21 towers (40%)
- In contrast, lab culture only detected Legionella above the limit in 8 towers (15%), and completely missed a tower with contamination >100× the limit
- A major reason for culture's poor performance was that bacteria degraded in 72% of water samples shipped to the lab
- Lab qPCR showed no correlation with lab culture and suffered the same bacterial degradation effect during shipping
- 11 cooling towers (21%) experienced rapid Legionella growth of 3× to 21× over 7 days
- Spartan's on-site Legionella qPCR test was validated according to ISO 12869 and detects live not dead bacteria

IWA PUBLISHING

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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

Shaimaa Ahmed, Urszula Liwak-Muir, Danielle Walker, Agnes Zoldowski, Alan Mears, Sergey Golovan, Steve Mohr, Paul Lem and Chris Harder

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

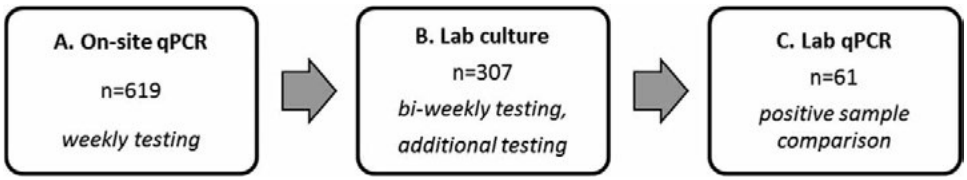

Figure 1 | Study design and water sample collection overview. Weekly on-site qPCR testing was performed on 53 HVAC towers (619 individual samples). Of these samples, 307 were sent for laboratory culture testing. Of the 307 samples sent for laboratory culture testing, 61 were also sent for laboratory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

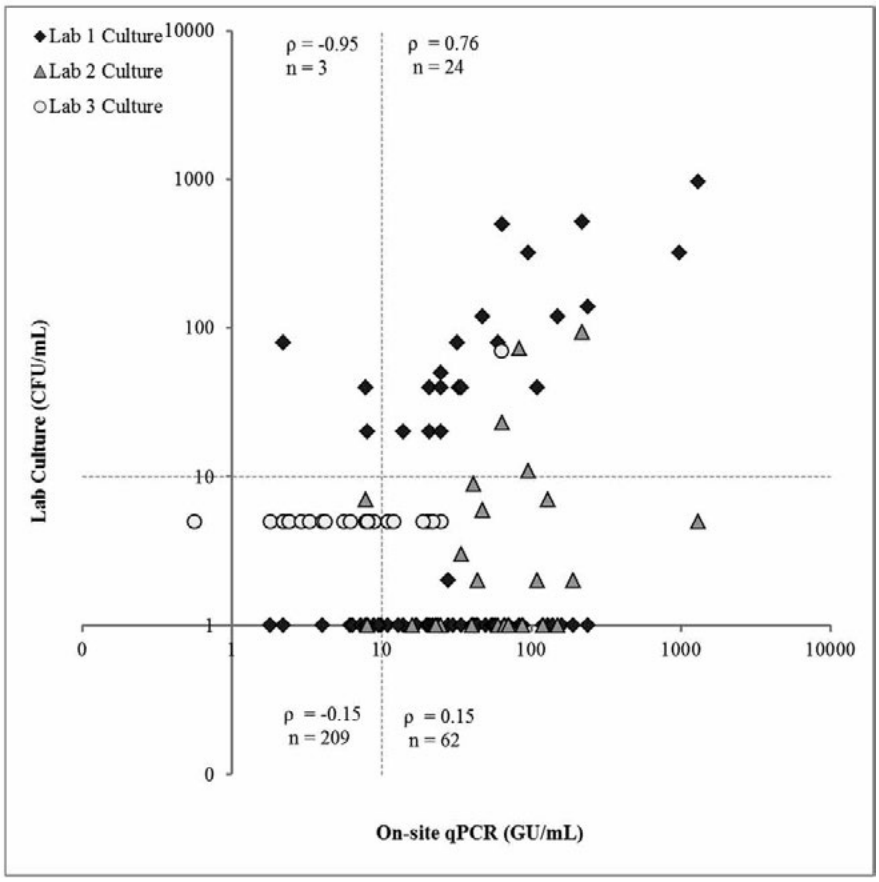

Figure 2 | Concordance between on-site qPCR and laboratory culture. (a) Concordance between on-site qPCR and laboratory culture categorized by positive (≥10 GU/mL or CFU/mL) or negative (<10 GU/mL or CFU/mL) results. (b) Comparison of on-site qPCR with culture performed by three laboratories. Pearson 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)

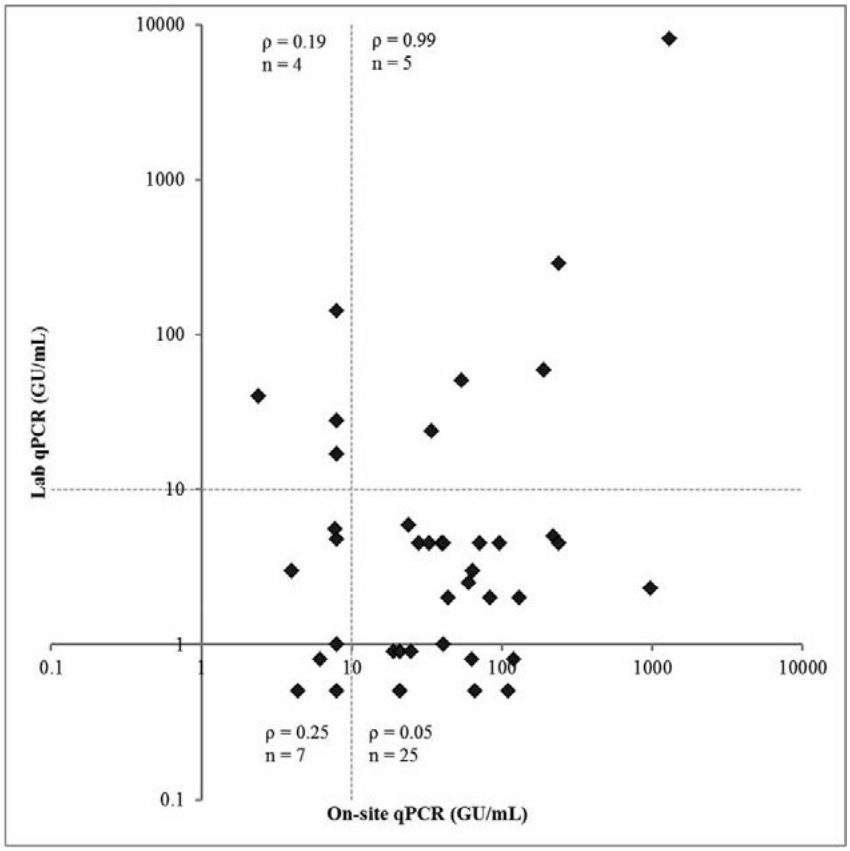

Figure 3 | Concordance between on-site qPCR and laboratory qPCR. (a) Concordance between on-site qPCR and laboratory qPCR categorized by positive (≥10 GU/mL) or negative (<10 GU/mL) results. (b) Comparison of on-site qPCR with laboratory qPCR. Pearson correlation was determined for data within each quadrant.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

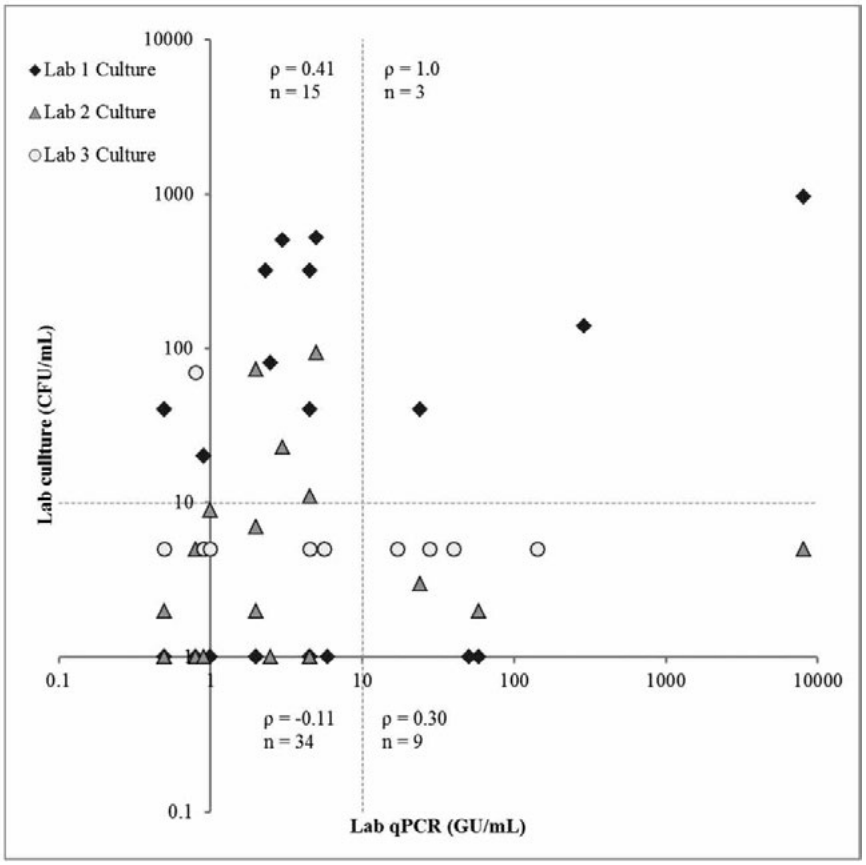

Figure 4 | Concordance between laboratory culture and laboratory qPCR. (a) Concordance between laboratory qPCR and laboratory culture categorized by positive (≥10 GU/mL or CFU/mL) or negative (<10 GU/mL or CFU/mL). (b) Comparison of laboratory qPCR with culture performed by three laboratories. Pearson 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

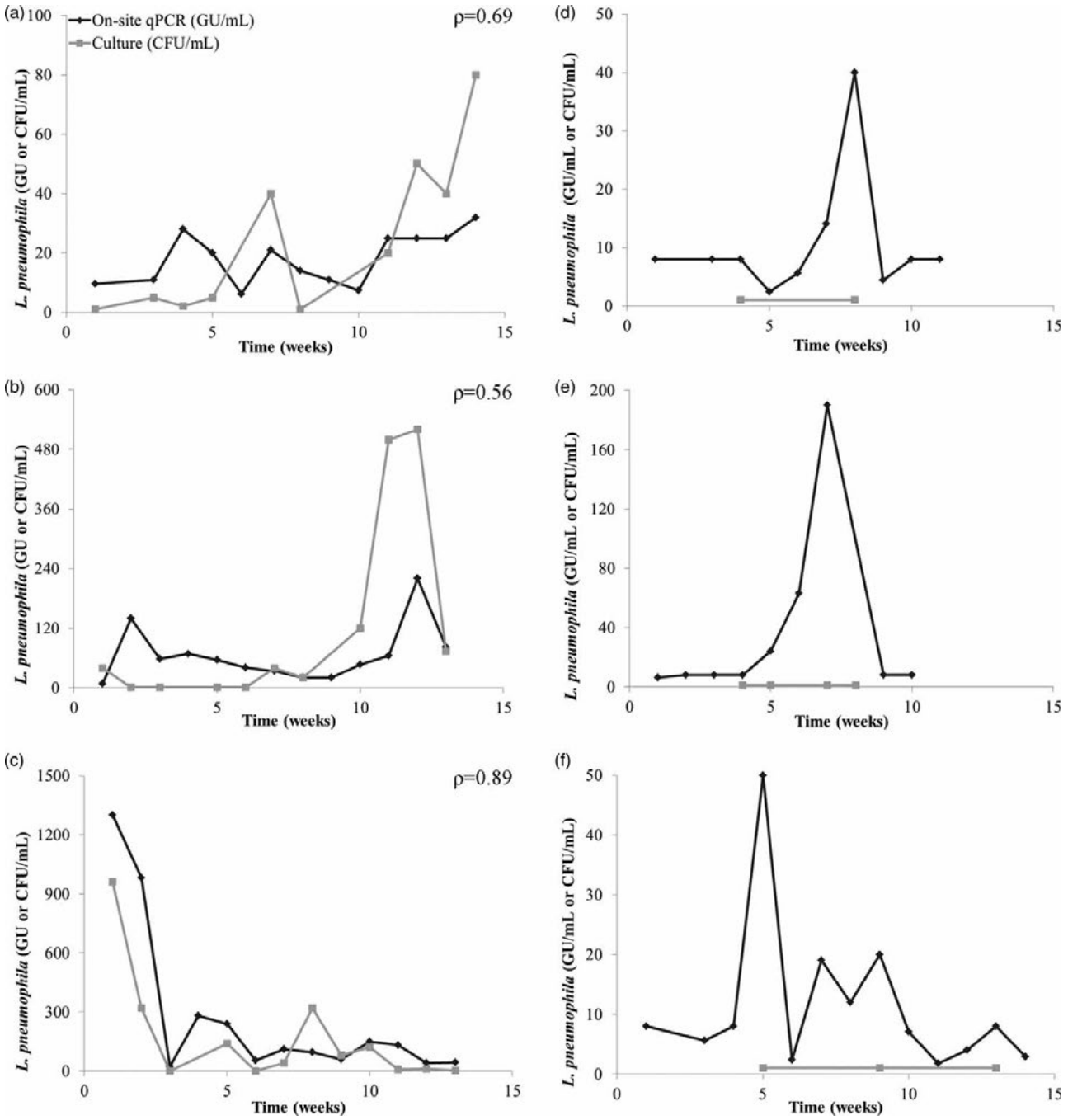

Figure 5 | Evaluation of select cooling towers by on-site qPCR and Laboratory 1 culture over time. (a)–(c) Individual cooling towers that demonstrate correlation ($\rho > 0.50$) between on-site qPCR and Laboratory 1 culture results. (d)–(f) On-site qPCR and Laboratory 1 culture results from individual 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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CDC STUDY SHOWS CULTURE TESTING IS INACCURATE.

WATER RESEARCH (2011). 45: 4428-4436.

- The CDC conducted a proficiency study of 20 ELITE-certified Legionella culture testing labs
- On average, the culture labs undercounted actual Legionella concentrations by 17-fold and values differed between labs by 6-fold
- On average, culture had a false negative rate of 11.5% [and this was with proficiency samples which are cleaner and have less inhibitors than real-world water samples]

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Accuracy and precision of *Legionella* isolation by US laboratories in the ELITE program pilot study

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ABSTRACT

A pilot study for the Environmental *Legionella* Isolation Techniques Evaluation (ELITE) Program, a proficiency testing scheme for US laboratories that culture *Legionella* from environmental samples, was conducted September 1, 2008 through March 31, 2009. Participants ($n = 20$) processed panels consisting of six sample types: pure and mixed positive, pure and mixed negative, pure and mixed variable. The majority (93%) of all samples ($n = 286$) were correctly characterized, with 88.5% of samples positive for *Legionella* and 100% of negative samples identified correctly. Variable samples were incorrectly identified as negative in 36.9% of reports. For all samples reported positive ($n = 128$), participants underestimated the cfu/ml by a mean of 1.25 logs with standard deviation of 0.78 logs, standard error of 0.07 logs, and a range of 3.57 logs compared to the CDC re-test value. Centering results around the interlaboratory mean yielded a standard deviation of 0.65 logs, standard error of 0.06 logs, and a range of 3.22 logs. Sampling protocol, treatment regimen, culture procedure, and laboratory experience did not significantly affect the accuracy or precision of reported concentrations. Qualitative and quantitative results from the ELITE pilot study were similar to reports from a corresponding proficiency testing scheme available in the European Union, indicating these results are probably valid for most environmental laboratories worldwide. The large enumeration error observed suggests that the need for remediation of a water system should not be determined solely by the concentration of *Legionella* observed in a sample since that value is likely to underestimate the true level of contamination.

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1. Introduction

Legionellaceae are ubiquitous in moist environments and a frequent contaminant of building water systems (Fields et al., 2002). Inhalation of aerosolized water contaminated with legionellae by susceptible individuals results in legionellosis that may present as the acute pneumonia, Legionnaires' disease, or the less severe Pontiac Fever. *Legionella pneumophila* is the most common etiological agent, however all species of legionellae are presumed to be capable of causing disease (Alli et al., 2003; Palusinska-Szysz and Cendrowska-Pinkosz, 2009). Legionellosis cannot be spread person-to-person and is only acquired from environmental sources. The widespread presence of legionellae precludes their removal from the environment so that disease prevention methods instead focus on reducing transmission of bacteria to susceptible hosts (Fields et al., 2002; Sehulster and Chinn, 2003; Freije, 2004; Tablan et al., 2004; Fields and Moore, 2006).

Monitoring levels of legionellae in building water systems by routine environmental sampling has been employed by some as a means of controlling transmission, though the relationship between the presence of legionellae and

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incidence of disease remains unclear (O'Neill and Humphreys, 2005; Den Boer et al., 2007). Environmental sampling for *Legionella* spp. as an approach for primary control can provide useful information to institutions that house persons at high risk for disease, such as chronic care and transplant facilities (Anonymous, 1997; Butler et al., 1997; Yu, 1997; Fiore et al., 1999; Fields and Moore, 2006). However, routine culture in the absence of documented cases of legionellosis is an area of considerable controversy, mostly because there is no generally accepted protocol for the choice of sampling sites, frequency of sampling events, or endpoints for remediation (Den Boer et al., 2007; Stout et al., 2007; Ditommaso et al., 2010). Recommendations that suggest the use of routine sampling for primary control acknowledge that the acceptable limits of contamination, either measured in cfu/ml or as a percentage of positive sites sampled, are based on limited data (Force, 1997; Sehulster and Chinn, 2003; Stout et al., 2007). Another confounding factor that is not frequently mentioned in the discussion on routine sampling is the considerable variability in recovery of legionellae from repeated sampling of sites or even seeded tap water suggesting that interlaboratory enumeration error may be high (Boulanger and Edelstein, 1995; Bentham, 2000; Napoli et al., 2009). The Centers for Disease Control and Prevention (CDC) guidelines for the reduction of legionellosis advise that there is no acceptable level of legionellae contamination and that if *Legionella* spp. are detected a plan to prevent transmission to susceptible individuals should be employed (1997).

Domestic and international organizations have published procedures for the recovery of *Legionella* bacteria from environmental samples but the application of these techniques can still require considerable expertise from the laboratorian (Anonymous, 1998, 2000, 2004, 2005a,b, 2008). Numerous variables contribute to effective recovery including properly taking and transporting samples, the application of sample pre-treatments intended to increase the representation of viable legionellae, and the use of suitable selective media (Reeves et al., 1981; Tesh and Miller, 1981; Shahamat et al., 1991; Lee et al., 1993; Ta et al., 1995; Leoni and Legnani, 2001; Wiedenmann et al., 2001; Bartie et al., 2003; Luck et al., 2004). Characterization of isolates entails the ability to distinguish *Legionella* colony morphology from autochthonous microbiota and serological methods for confirmation of identity, both of which determinations are subjective and sometimes difficult to interpret without considerable prior experience by the laboratorian (Benson and Fields, 1998; Helbig et al., 2007; Wagner et al., 2007). PCR-based methods of isolate identification offer greater sensitivity and specificity but are often cost prohibitive for laboratories with a low volume of tests for legionellae and do not yet provide enough discrimination between strains for epidemiological surveillance (Thurmer et al., 2009; Tronel and Hartemann, 2009).

In the European Union (EU) laboratories that culture environmental samples for *Legionella* spp. are required to participate in a proficiency testing (PT) scheme to ensure baseline quality standards throughout the industry (2005). The EU scheme, in operation since June 2004, requires participants to process PT products as would be performed for potable water and report results to the program administrators for scoring according to ISO standards (1997; 1997). The EU scheme has been successful in assessing the status of the industry and publishes quarterly reports of their trends and performance distributions over time. A new PT scheme, the Environmental *Legionella* Isolation Techniques Evaluation (ELITE) program was created by CDC to capture similar information for US laboratories. A Pilot Program was performed September 1, 2008–March 31, 2009 to determine industry limits of detection and ascertain the accuracy of legionellae enumeration by participating laboratories. The results indicate that US laboratories are generally capable of a qualitative assessment of environmental samples for the presence of legionellae but that quantitation displays significant inter- and intra-laboratory variability.

2. Methods and materials

2.1. Sample creation, panel composition, quality control, and re-tests

Legionellae and heterotrophic bacteria were grown from freezer stocks of less than three passages on BCYE media (BBL agar base plus 10 g/L L-cysteine) for 3–5 days at 35 °C with 2.5% (v/v) CO_2. Samples distributed for PT were removed from plates by suspension in sterile de-ionized water. Bacterial suspensions were diluted in 10% (v/v) AYE broth prior to lyophilization. The formula for full strength AYE broth is as follows: 5 g bovine serum albumin fraction V, 10 g ACES, 10 g yeast extract, 0.4 g L-cysteine, 0.25 g iron pyrophosphate. Bring volume to 1 L with sterile de-ionized water, adjust pH to 6.9 with 1 N potassium hydroxide, and filter to sterilize. The AYE broth helped to stabilize lyophilized cells during storage and formed a consistent pellet regardless of sample composition. Samples were made in batches monthly with 20–25 aliquots per lot. A representative sample from each lot was examined for quality control (QC) one week after lyophilization by plating serial dilutions from the vial reconstituted in 1 ml sterile de-ionized water on BCYE in triplicate at each serial dilution. Plates were incubated 5 days at 35 °C with 2.5% (v/v) CO_2 prior to being read. The total bacterial count and *Legionella*-specific count per plate were determined. At least one representative *Legionella*-like colony per plate was confirmed as a cysteine auxotroph.

PT panels consisted of 6 samples, one of each sample type: pure positive, mixed positive, pure negative, mixed negative, pure variable, and mixed variable (see Table 1). Positive samples were either *Legionella* in pure culture or mixed with a low ratio of heterotrophs. Negative samples contained no viable legionellae, though they might contain heat killed legionellae. Variable samples consisted of either low levels of *Legionella* in pure culture or a mixture of organisms with a high ratio of heterotrophs to legionellae. Laboratories were scored on a pass/fail basis, required to correctly identify both positive and both negative samples for a passing score. Variable samples did not count toward the score but were included in each panel to assess lower limits of detection.

Panels identical to those sent to participants were shipped back to the program administrators for re-tests. Re-test samples were first reconstituted in the vial with 1 ml of sterile,

Table 1 – PT sample characteristics: All PT samples used during the Pilot Program (Panel column: Panels number I–VIII and Accelerated) are listed here with sample composition, QC, and re-test results. Samples were categorized into sample types according to QC results. Re-test treatments specific to each sample are abbreviated: D = direct plating, SD = serial dilutions, A = 15 min acid.

Sample ID no	Status	Panel	Sample composition	QC total cfu/ml ± SD	QC *Legionella* cfu/ml ± SD	Re-test[a] total cfu/ml ± SD	Re-test Legionella cfu/ml	Re-test protocol
A50-08081901	Negative – media	I	Blank media	0	0	0	0	D, BCYE
A49-08071519	Variable – mixed	I	*L. pneumophila* Sg1 + HTs	5 ± 0.3	0.6 ± 0.4	7 ± 0.3	1	D, GPCV
A32-08061701	Variable – low	I	*L. pneumophila* Sg3	Pure	53 ± 13	Pure	52	D, BCYE
A12-08041602	Positive – pure	I	*L. pneumophila* Sg8	Pure	211 ± 14	Pure	139	SD, BCYE
A31-08061718	Negative – mixed	I, A	HTs	1370 ± 374	0	861 ± 5	0	SD, BCYE
A25-08052219	Positive – mixed	I, A	*L. bozemani* + HTs	4400 ± 712	170 ± 11	≥3000	170	SD, PCV
A50-08081905	Negative – media	II	Blank media	0	0	0	0	D, BCYE
A30-08061719	Negative – mixed	II	HTs	703 ± 9	0	≥300	0	SD, BCYE
A49-08071520	Variable – mixed	II	*L. pneumophila* Sg1 + HTs	4 ± 1	0.7 ± 4	4 ± 1	1	D, GPCV
A32-08061707	Positive – mixed	II	*L. pneumophila* Sg3 + HTs	583 ± 26	48 ± 4	≥300	35	SD, A, PCV
A12-08041604	Positive – pure	II	*L. pneumophila* Sg8	Pure	254 ± 17	Pure	344	SD, BCYE
A32-08061702	Variable – pure	II, A	*L. cherrii*	Pure	7 ± 4	Pure	2	D, BCYE
A31-08061704	Negative – pure non-Legionella	III	HTs	3330 ± 471	0	ND	0	SD, BCYE
A21-08052203	Negative – mixed	III	HTs	83,000 ± 3740	0	ND	0	SD, BCYE
A48-08071517	Variable – mixed	III	*L. pneumophila* Sg1 + HTs	7870 ± 309	150 ± 39	ND	13	A, SD, PCV
A65-08091607	Variable – pure	III	*L. pneumophila* Sg1	Pure	593 ± 170	Pure	120	SD, BCYE
A67-08091619	Positive – mixed	III	*L. pneumophila* Sg8 + HTs	4730 ± 624	2000 ± 630	ND	311	A, SD, GPCV
A62-08091601	Positive – pure	III, A	*L. pneumophila*	Pure	7600 ± 1280	Pure	≥3000	SD, BCYE
A20-08052201	Negative – media	IV	Blank media	0	0	0	0	D, BCYE
A28-08052204	Negative – mixed	IV	HTs	15, 200 ± 2380	0	ND	0	SD, BCYE
A6508091615	Variable – pure	IV	*L. pneumophila* Sg8	Pure	61 ± 7	Pure	16	D, BCYE
A48-08071518	Variable – mixed	IV	*L. pneumophila* Sg1 + HTs	883 ± 58	257 ± 38	ND	193	A, SD, BCYE
A6708091617	Positive – mixed	IV	*L. pneumophila* Sg1 + HTs	18, 7000 ± 1250	4000 ± 1410	ND	412	A, SD, PCV
A62-08091609	Positive – pure	IV	*L. pneumophila* Sg8	Pure	3300 ± 804	Pure	≥3000	SD, BCYE
A50-08081904	Negative – media	V	Blank – broth only	0	0	ND	0	D, BCYE
A71-08111906	Negative – mixed	V	HTs	15,600 ± 1130	0	ND	0	SD, BCYE
A38-08061703	Variable – pure	V	*L. rubrilucens*	Pure	10 ± 0.4	Pure	3	D, BCYE
A25-08052218	Positive – mixed	V	*L. dumoffii* ± HTs	39,000 ± 5720	1670 ± 125	ND	1000	A, SD, GPCV
A84-08120201	Positive – pure	V	*L. feelei*	Pure	27,300 ± 5910	Pure	16500	SD, BCYE
A88-08120215	Variable – mixed	V, A	*L. pneumophila* Sg1 + HTs	600 ± 36	33 ± 5	ND	12	A, SD, BCYE
A50-08081908	Negative – media	VI	Blank – broth only	0	0	0	0	D, BCYE
A28-08052207	Negative – mixed	VI	HTs	13,600 ± 8740	0	ND	0	SD, BCYE
A89-08120213	Variable – pure	VI	*L. pneumophila* Sg10	Pure	183 ± 24	Pure	102	SD, BCYE
A14-08041609	Positive – mixed	VI	*L. rubrilucens* + HTs	16,200 ± 9420	1290 ± 173	ND	540	A, SD, PCV
A39-08061716	Variable – mixed	VI	*L. rubrilucens* + HTs	70,700 ± 7040	2400 ± 57	ND	2000	A, SD, BCYE
A86-08120217	Positive – pure	VI	*L. dumoffii*	Pure	74,200 ± 17,000	Pure	34300	SD, BCYE
A31-08061717	Negative – mixed	VII	HTs	7870 ± 141	0	ND	0	SD, BCYE
A89-08120211	Variable – mixed	VII	*L. rubrilucens* + HTs	205 ± 23	35 ± 4	ND	17	SD, BCYE
A62-08091607	Variable – pure	VII	*L. pneumophila* Sg1	Pure	59 ± 17	Pure	60	D, BCYE
A32-08061706	Positive – mixed	VII	*L. pneumophila* Sg3 + HTs	11,700 ± 5350	1480 ± 398	ND	200	A, SD, GPCV

A62-08091603	Positive – pure	VII	*L. pneumophila* Sg1	Pure	3100 ± 804	Pure	1410	SD, BCYE
A90-09010505	Negative – media	VII, A	*L. pneumophila* Sg1 [heat killed]	0	0	0	0	D, BCYE
A90-09010509	Negative – media	VIII	*L. pneumophila* Sg10 [heat killed]	0	0	0	0	D, BCYE
A71-08111907	Negative – mixed	VIII	HTs	7670 ± 858	0	ND	0	SD, BCYE
A89-08120212	Variable – mixed	VIII	*L. rubrilucens* + HTs	1130 ± 17	23 ± 12	ND	5	A, GPCV
A65-08091608	Variable – pure	VIII	*L. pneumophila* Sg1	Pure	≥300	Pure	45	SD, BCYE
A32-08061708	Positive – mixed	VIII	*L. pneumophila* Sg3 + HTs	4430 ± 377	183 ± 37	ND	200	A, SD, BCYE
A62-08091604	Positive – pure	VIII	*L. pneumophila* Sg1	Pure	1330 ± 557	Pure	835	SD, BCYE

a Heterotrophic plate counts were enumerated by re-test only for the first round (Panels I and II). Heterotrophic cfu/ml were not determined (ND) by re-test for panels III through VIII.

de-ionized water then diluted 1:1000 in a bottle of sterile, de-ionized water to yield a 1 L Test Sample. All Test Samples were filtered. Each Test Sample was then processed according to an individual protocol determined by sample composition, which was designed for maximum recovery and enumeration of legionellae (Table 1). Three replicates of each dilution were plated but only the highest count was used as the re-test value for data analysis. All re-test values were within one standard deviation of the mean of the re-test replicate plate counts (data not shown). Test Samples were subjected to combinations of pre-treatment with acid (18 parts 0.2 M KCl to 1 part 0.2 M HCl), serial dilutions prior to plating, and/or plating on selective media PCV (BCYE plus 13.22 mg/L polymixin B, 80 mg/L cyclohexamide, and 5 mg/L vancomycin) or GPCV (PCV plus 2 g/L glycine). QC results, re-test results, and re-test treatment protocols are listed for all samples in Table 1.

2.2. *Pilot participants*

Because current industry capabilities were unknown at the inception of the ELITE Program, a pilot study was conducted September 1, 2008 through March 31, 2009 to generate baseline values. The Pilot Program was limited to ten participating laboratories. A total of 20 laboratories enrolled in the program prior to the August 31, 2008 deadline for inclusion in the Pilot Program. Laboratories that could not be accommodated in the Pilot Program due to space limitations were enrolled as Accelerated Members. Commercial laboratories comprised the majority of enrollees but the Pilot Program was designed to include at least one of each type of laboratory as listed in Table 2. CDC reference laboratory personnel separate from those administering the ELITE Program participated in the pilot. To avoid confusion in the text, CDC reference laboratory Pilot Program participants are designated the “federal” laboratory in the text and figures, while administrators’ results are labeled “CDC”. For data analysis purposes, State, County, and Hospital laboratories were grouped into the category “Local Public Health.”

Participants were asked to process the panels according to their standard in-house protocol and return results as they would be given to clients. Participants were directed to rehydrate the lyophilized pellets in 1 ml sterile water then dilute the suspension 1:1000 in sterile water to yield a test sample of the volume dictated by the participant’s in-house protocol. Supplement 1 contains the complete Sample Handling Instructions available to all participants. Pilot Members processed four panels shipped September 3, 2008, November 3,

Table 2 – Pilot and accelerated program demographics: the number and type of each participant in the pilot or accelerated program.

	Pilot	Accelerated	Total
Commercial	6	6	12
Federal	1	0	1
State	1	3	4
County	1	0	1
Hospital	1	1	2
Total	10	10	20

2008, December 29, 2008, and March 2, 2009. Accelerated Members processed a single panel consisting of samples already tested by Pilot Members that was shipped March 20, 2009. All laboratories enrolled during this time were given two months to process a panel and report results.

2.3. Data collection and analysis

Combined results from Pilot and Accelerated Members were captured from the CDC SQL 2005 database June 12, 2009. Reported laboratory concentrations were tabulated and summarized. Differences were computed and tabulated between the reported concentrations and (a) the CDC-validated concentration (re-test values) and (b) the intra-laboratory mean among all laboratories reporting a value. In addition, tabulations of un-quantified identifications were made. Quantitative results were analyzed directly and through log transformations. Reporting laboratories were categorized, respectively, by: type of laboratory (state, local, federal, hospital, commercial); annual volume of *Legionella* tests performed; how long (years) the laboratory has tested for *Legionella*; number of full-time and *Legionella*-dedicated employees; and by protocol-related factors such as incubation time and temperature, water volume, use of CO_2, and standard protocol reference (ASM, ISO, or CDC). State, county, and hospital laboratories were grouped into the category "Local Public Health" for further analyses. For each categorization of laboratories or procedures, Chi-square tests were computed for dichotomous outcomes such as finding *Legionella* or not or being within a specified error, such as one log, of the respective (re-test or interlaboratory mean) reference concentration. In addition, for continuous measures, t-tests were performed comparing the errors of mutually-exclusive groups of laboratories, such laboratories which used less than 750 ml of water versus those who used 750 ml or more.

3. Results

3.1. PT sample qualitative identification

Concordance between expected positive or negative results, as determined by QC, and results returned by Pilot or Accelerated Members was high regardless of laboratory type (Table 3). The majority of positive samples (88.5%) were correctly identified. No false positives from a negative sample were reported by any member. In contrast, 36.9% of all variable samples tested ($n = 103$) were incorrectly identified (Table 4). The federal laboratory was most often correct, followed by commercial laboratories, and then local public health laboratories.

Correct identification of positive samples depended on the *Legionella* concentrations in the samples. Samples with less than 10 cfu/ml, as determined by re-test, were identified as negative in 93.1% of reports while samples with 10 cfu/ml or more were reported positive in 85.3% of reports. These results were independent of whether the sample was pure or mixed, positive or variable indicating that 10 cfu/ml is at or near the lower limit of detection. In addition to whether a sample was positive or negative for *Legionella* growth, participating laboratories had the option to report species and serogroup of legionellae recovered. All laboratories that provided optional results correctly identified the species and serogroup of recovered *Legionella*.

Table 3 – Concordance of positive and negative sample reports by laboratory type: displays the agreement between expected results and participants' reported results for all positive or negative samples, both mixed and pure. Variable samples' reported results are not included in this table. Definitions of column headings are: True Negative = expected negative and reported negative, False Negative = expected positive but reported negative, True Positive = expected positive and reported positive.

	True negative	False negative	True positive	Total results (*n*)
Commercial	56 (100%)	3 (6.3%)	45 (93.8%)	104
Federal	8 (100%)	0 (0%)	7 (100%)	15
Local public health	32 (100%)	7 (21.9%)	25 (78.1%)	64
Total results (*n*)	96 (100%)	10 (11.5%)	77 (88.5%)	183

3.2. Accuracy and precision of Legionella quantitation

Pilot and Accelerated Members were encouraged to also provide results for their observed concentration of legionellae in samples ($n = 128$). The expected concentration for each sample was determined by re-test (Table 1). The log error for each report is displayed graphically in Fig. 1. The majority (99.5%) of reported results underestimated the expected concentration by an average of 1.25 logs with a standard deviation of 0.78 logs, standard error of 0.07 logs, and range of 3.57 (−3.30 to 0.27) logs. Centering the results on the inter-laboratory mean forced an overall net difference of zero logs (Fig. 2). The standard deviation was 0.62 logs, standard error was 0.06 logs and the range was 3.22 (−1.89 to 1.33) logs. The standard deviation from the interlaboratory mean of the results from laboratories that entered at least three concentrations for evaluation ($n = 11$) ranged from 0.53 to 0.96 and standard error from 0.14 to 0.58. No sampling protocol, treatment regimen, incubation procedure, or organizational structure type analyzed produced significantly greater accuracy when compared to re-test values or precision when compared to the interlaboratory mean concentration (Table 5).

Table 4 – Accuracy of variable sample reports by laboratory type: Displays the reported results for all variable samples, both mixed and pure. No positive or negative sample results are included in this table.

	Reported positive	Reported negative	Total results (*n*)
Commercial	39 (62.9%)	23 (37.1%)	62
Federal	7 (77.8%)	2 (22.2%)	9
Local public health	19 (59.4%)	13 (40.6%)	32
Total results (*n*)	65 (63.1%)	38 (36.9%)	103

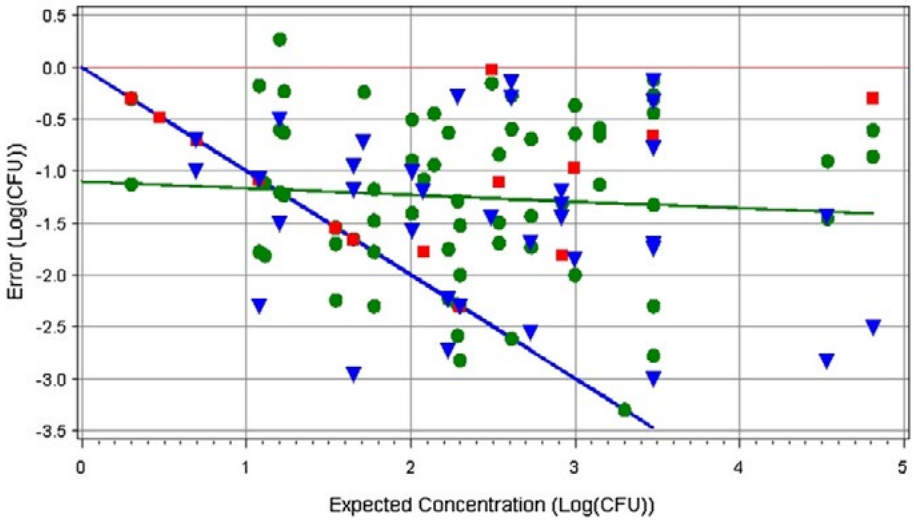

Fig. 1 – Accuracy of quantitative results relative to re-test values for all samples reported positive: Displays the log error of the difference between re-test values and reported concentrations for all samples reported positive. Each marker represents a sample reported positive by a participant ($n = 190$). Markers are coded by laboratory type: commercial (green circle), federal (red square), local public health (blue triangle). Enumeration error [Error(Log(CFU))] was calculated by taking the difference between the log value of the expected concentration in cfu/ml and the log value of the concentration reported by a participant in cfu/ml. Participant responses are plotted as enumeration error (y-axis) vs. expected concentration derived from re-test values (x-axis). Samples reported positive without a concentration value ($n = 62$) are indicated by markers connected with the blue line ($y = -x$) and are not included in calculations measuring accuracy. The green line depicts the mean enumeration error across the range of reported concentrations (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

The results from the ELITE Pilot Program indicate that most participating US laboratories are capable of qualitatively identifying *Legionella* spp. from a water sample and that the lower limit of detection is approximately 10 cfu/ml. According to 2009 EU Health Protection Agency external quality assessment for *Legionella* isolation from water samples reports, the accuracy of the European PT participants (Shah et al., 2009a,

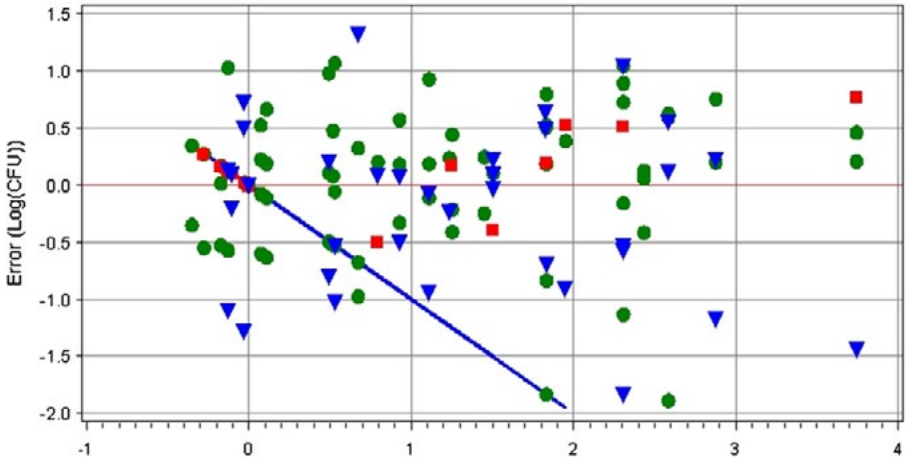

Fig. 2 – Precision of quantitative results relative to interlaboratory means for all samples reported positive: Displays the log error of the difference between interlaboratory mean values and reported concentrations for all samples reported positive. Each marker represents a sample reported positive by a participant ($n = 190$). Markers are color coded by laboratory type: commercial (green circle), federal (red square), local public health (blue triangle). Enumeration error [Error(Log(CFU))] was calculated by taking the difference between the log value of the interlaboratory mean cfu/ml and the log value of the concentration reported by a participant in cfu/ml. Participant responses are plotted as enumeration error (y-axis) vs. expected interlaboratory mean (x-axis). Samples reported positive without a concentration value ($n = 62$) are indicated by markers connected with the blue line (y = -x) and were not included in calculations to determine the interlaboratory mean (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 5 – Statistical analysis of accuracy as a function of procedural variables: The enumeration errors associated with treatment, protocol, and experience categories were compared with *t*-tests. The *p*-values are given when the baseline is calculated from either re-test values or the interlaboratory mean.

Measurement	Categories	*p* Value compared to re-test	*p* Value compared to interlaboratory mean
Incubation temperature	35.0 °C or >35.0 °C	0.297	0.311
Incubation with CO_2	<2.5% CO_2 or 2.5–5.0% CO_2 (v/v)	0.537	0.332
Water volume	<750 ml or 750–1000 ml	0.454	0.476
Incubation time	≤7 days or >7 days	0.318	0.562
Sample composition	Pure or mixed sample	0.065	>0.999
Sampling protocol	CDC or other	0.221	0.216
Years of testing	≤10 or >10 years	0.672	0.159
Number of dedicated employees	≤2 or >2 employees	0.993	0.960
Number of samples processed annually	≤200 or >200	0.919	0.565

2009b, 2009c, 2009d) averaged 93% concordance (range 78–98%). US laboratories in this study averaged 86% concordance with positive samples, well within the range of EU performance. On the other hand, with regard to precision, US laboratories demonstrated more extreme variability in enumerating bacteria in the PT samples both within and between participants. US labs underestimated the concentration of *Legionella* by an average of 1.25 (±0.78) logs when compared to re-test values. Further, US labs exhibited 0.62 logs variance around the interlaboratory mean. In contrast, EU laboratory results from 2009 clustered very close to the interlaboratory mean and FEPTU median (a reference standard comparable to re-test values), usually within a 0.5 log range of either score, with a mean standard deviation of 0.44 logs (Shah et al., 2009a, 2009b, 2009c, 2009d). Thus, EU and US laboratories demonstrate similar accuracy in identifying positive samples but US laboratories appear to be less precise than EU laboratories in enumerating viable bacteria.

The apparent greater precision of enumeration by EU laboratories compared to US laboratories is partially due to the bacterial concentrations used in the two sources of PT samples. EU sample concentrations spanned 0.2–110 cfu/ml while US PT samples contained between 1 and 34,300 cfu/ml (as determined by re-test), allowing the possibility for a larger range of enumeration error within US samples. However, a greater contribution to the disparity in variance between EU and US reported results was the different methodologies used to generate reference standards for comparison. US re-test values were calculated from the highest count recovered from a representative sample after treatment with an individualized protocol that took sample concentration and composition into account. Plating serial dilutions from the PT samples in triplicate, a feature of the majority of individualized re-test protocols (Table 1) but no referenced standard protocol (Anonymous, 1998, 2004, 2005a, 2005b, 2008, 2010), resulted in plates that had reduced numbers of obscuring heterotrophs and increased physical distance between colonies, making *Legionella* cfu easier to determine. In contrast, EU FEPT medians were generated from the results of 10 samples treated according to a single standard protocol without regard to sample concentration or composition, making the results inherently less variable. Both reference standards are valuable in assessing participant results but address different laboratory capabilities. Re-tests return the most accurate count of viable bacteria within a sample, independent of protocol bias while the EU FEPT median and ELITE interlaboratory mean values address the precision with which a sample can be enumerated using a (set of) standard protocol(s). Thus, comparison to the re-test value indicates how close a laboratory can come to a 'true' answer while FEPT medians and interlaboratory means illustrate how reproducible the results are between and within a laboratory.

Laboratories that submitted more than three concentration reports were statistically indistinguishable from each other in enumeration error and also demonstrated large intralaboratory variance (0.53–0.96). Two PT samples were quantified with significantly different log error from re-test values compared to the rest. These observations can be explained by sample composition. A62-08091603 (−0.71 vs. −1.27, $p = 0.002$, $n = 5$) was a pure sample with a concentration within the optimum range for allowing distinct colony growth on media from a single 10-fold dilution and so could be measured more precisely than other samples. In contrast, sample A32-08061706 (−2.19 vs. −1.21, $p = 0.006$, $n = 5$) was heavily mixed with heterotrophs that had a spreading colony morphology, obscuring legionellae growth, resulting in a sizeable difference between QC and re-test results (Table 1), and a substantial interlaboratory enumeration error. Enumeration of the other 46 samples was statistically indistinguishable. No sampling protocol, treatment, incubation, or experience level analyzed in this study affected the accuracy or precision of enumeration (Table 5) at the 0.05 significance level. Taken together, these results indicate that intralaboratory and interlaboratory variance in precision and accuracy were similar in degree and magnitude for all pilot study participants.

Two obvious caveats to these results are the small number of participating laboratories and their method of selection. ELITE Program participation is voluntary rather than mandated by legal statutes, as in the EU, and there was little promotion for the creation and implementation of the program. Most Pilot and Accelerated Members discovered the program through scientific meetings and/or word of mouth. Pilot Members were also likely to have a previous relationship with CDC, multiple years experience with *Legionella* isolation,

and/or a large number of well-trained personnel dedicated to *Legionella* testing. Thus, it is possible that Pilot Members do not accurately reflect the capabilities of all US laboratories that culture environmental samples but are a subset of those whose primary focus is *Legionella*.

5. Conclusions

Overall, *Legionella* PT qualitative and quantitative results were similar between US and EU laboratories in 2009 despite differences in sample composition, delivery, and reference standard determination. The observed variability in enumeration by both US and EU laboratories is probably due to the inherent inconsistency in assessing a sample by culture techniques. Given these data no protocol can be recommended to yield more accurate or precise results than any other. Responses from ELITE Program participants will continue to be monitored and analyzed to determine if more data can illuminate practices that contribute to increased accuracy and precision. However, the current findings have several implications for the use of routine sampling as a primary method of legionellosis control. Agreement between EU and US PT schemes suggest these results are applicable worldwide to environmental sampling laboratories. Since a sample qualitatively identified as positive could represent a 3 log cfu/ml range of viable legionellae it would be in the best interests of public health to consider any detectable level a hazard. Therefore, primary legionellosis prevention should consider the risk posed by an individual water system, assessing the likelihood of transmission and population affected, to determine if remediation is required rather than relying on a contamination cutoff level to take action.

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Appendix. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.watres.2011.05.030.

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