Next Day *Legionella* PCR: a highly reliable negative screen for *Legionella* in the built environment

Katherine E. Fisher, Leah P. Wickenberg, Lesley F. Leonidas, Anna A. Ranz, Michelle A. Habib, Rafael M. Buford and William F. McCoy

ABSTRACT

The opportunistic, waterborne pathogen *Legionella* caused 9,933 cases of Legionnaires' disease in 2018 in the United States (CDC.gov). The incidence of Legionnaires' disease can be reduced by maintaining clean building water systems through water management programs (WMPs). WMPs often include validation testing to confirm the control of bacteria, but the traditional culture method for enumerating *Legionella* requires 10–14 days to obtain results. A rapid DNA extraction developed by Phigenics and a real-time PCR negative screen for the genus *Legionella* provided results the day after sampling. This study evaluated the Next Day *Legionella* PCR (Phigenics, LLC) compared with the traditional culture method (ISO 11731) on 11,125 building water samples for approximately 1 year. Two DNA extraction methods (Methods 1 and 2) were compared. The negative predictive value (NPV) of the Next Day *Legionella* PCR in comparison to traditional culture for Method 1 was 99.95%, 99.92%, 99.85%, and 99.17% at >10, >2, >1, and >0.1 CFU/ml limits of detection, respectively. The improved DNA extraction (Method 2) increased the NPV to 100% and 99.88% at >1 and >0.1 CFU/ml, respectively. These results demonstrate the reliability of the genus-level *Legionella* PCR negative screen to predict culture-negative water samples.

Key words | *Legionella*, negative predictive value, negative screen, polymerase chain reaction (PCR), validation, water management program

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INTRODUCTION

Legionella bacteria are opportunistic pathogens that can cause respiratory infections in humans. These infections range in severity from Pontiac fever to Legionnaires' disease (LD). Pontiac fever is less severe with symptoms lasting approximately 1 week and does not develop into pneumonia (CDC 2016a). In contrast, LD is an extreme form of pneumonia, with symptoms lasting weeks, and has the potential to cause long-term debilitating effects including fatigue, memory loss, muscle pain, and post-traumatic stress disorder (Lettinga *et al.* 2002). LD can be treated with antibiotics, but has an overall mortality rate of 1 in 10 (CDC 2016b). *Legionella* enters the respiratory tract via the inhalation of aerosolized water droplets from contaminated water or by aspiration. The detection of these bacteria before infection is critical, especially in healthcare buildings with a high risk of exposure for immunocompromised persons (Marston 1994). Specifications for the actionable concentration of culturable *Legionella* vary by country and there is no worldwide consensus; the Centers for Disease Control and Prevention (CDC) has stated that there is no known safe concentration of *Legionella* in building water systems. Traditional microbiological methods of detection such as ISO 11731:2017 (ISO 2017), take up to 10 days of incubation; results are often not available for up to 14 days. In order to overcome the

doi: 10.2166/wh.2020.004

limitations of the culture method, real-time monitoring of flushing events and water temperature have been shown to correlate with heterotrophic bacteria counts and can be used to assess water quality immediately (Whiley *et al.* 2019). Dipslides used in-field for *Legionella* and heterotrophic bacteria have also reduced the time for culture results to 4 days (McCoy *et al.* 2012). A more specific, sensitive and rapid test, however, would enable infection preventionists and facility managers to assess the performance of control measures and identify possible problems before infections occur.

Validation (confirmation of hazard control) is required to be a part of every water management program (WMP) for all building water systems (CDC 2017). The CDC Toolkit is a guide for the development of a *Legionella* WMP that references the ASHRAE/ANSI Standard 188: Legionellosis: Risk Management for Building Water Systems (ASHRAE/ANSI 2018). Developing the WMP involves establishing a water management team (WMT). Then, a flow diagram of the water system is made to show how water is processed in the facility. This enables the WMT to determine where *Legionella* may grow in their building water systems. Next, control measures with critical control limits are established and routinely verified. Once the WMP is in place, it is necessary to confirm that the program is effective by performing validation testing. Environmental testing for *Legionella* can be undertaken to validate (confirm) that hazardous conditions have been controlled. Prior to the CDC Toolkit and ASHRAE 188, the Veterans Health Administration (VHA) issued VHA Directive 1061 to reduce the risk of healthcare-acquired LD. The VHA Directive 1061 requires quarterly validation testing for *Legionella* and recommends increasing the frequency of testing based on local risk assessment (Veterans Health Administration 2014). The World Health Organization (WHO) describes the creation and surveillance of water safety plans for the control of drinking water-associated hazards in all building types (World Health Organization 2011). The WHO water safety plan is based on the seven principles of Hazard Analysis Critical Control Point (HACCP). These same principles are the foundation of the WMP (McCoy & Rosenblatt 2015). By utilizing risk management principles, including validation testing, WMPs can reduce the risk of disease.

A molecular screen is one of the most rapid approaches to detect waterborne pathogens. A polymerase chain reaction (PCR) is a technique in molecular biology that detects the genetic material of the target organism. As a highly sensitive technique, the PCR provides a low limit of detection. PCR has been shown to be more sensitive than the traditional culture method for detecting *Legionella pneumophila* in hot water and cooling tower samples (Yaradou *et al.* 2007). Using the PCR as a *Legionella* negative screen strategy has been shown to be highly effective (Collins *et al.* 2017). The CDC has practiced a *Legionella* negative screen strategy where only positive PCR samples are cultured after the negative screen in certain non-outbreak situations or in research projects (Llewellyn *et al.* 2017). A significant limitation of PCR is that it cannot differentiate between viable and non-viable cells without additional processes (Delgado-Viscogliosi *et al.* 2009).

This study is comprised of *Legionella* validation test samples from across the United States analyzed from April 2018 to July 2019 at Phigenics Analytical Services Laboratories (PASL, Warrenville, IL and Fayetteville, AR) that are CDC Environmental *Legionella* Isolation Techniques Evaluation (ELITE)-certified. In addition to the PCR, all samples were cultured by the ISO 11731 method in 10–14 days and by the Phigenics Validation Test (PVT)–TimeZero™ in 4–10 days. The Next Day *Legionella* PCR by Phigenics, LLC allows the negative screen results to be reported to the WMT 1 day after sampling. The WMT can make defensible decisions based on results within 24 h based on which sampling locations are *Legionella* negative.

A negative predictive value (NPV) describes the ratio of PCR-negative results to culture-negative results; the higher the NPV the more reliable the screen is. The goal of this study was to quantitatively determine the NPV of the Next Day *Legionella* PCR in comparison with the ISO 11731 culture method and to assess its effectiveness as a negative screen for validation testing in WMPs.

METHODS

Sample collection

Eleven thousand one hundred and twenty-five (11,125) water samples were collected across the United States for

Legionella testing. From April to December 2018, 9,113 samples were collected and analyzed with Method 1. The remaining 2,012 samples were collected from May to July 2019 and analyzed with Method 2. All samples were collected in 0.25–1 L sterile containers containing sodium thiosulfate tablets sufficient to neutralize the residual oxidant contained in the sample volume. Each sample was also tested, per the WMP, for total and free residual oxidant levels by the DPD method (Hach, Loveland, CO) and for total heterotrophic aerobic bacteria (THAB) counts using the TimeZero™ dipslide. The samples were shipped overnight at ambient temperature to PASL in Warrenville, IL or Fayetteville, AR. On the day of receipt, a volume of 0.1 or 1 L of potable water was filter-concentrated on a 0.2 μm, 47 mm track-etched polycarbonate membrane (GE Healthcare, Chicago, IL). The membrane was resuspended in 10 ml of filtrate and vortexed for 30 s in a sterile 15 ml conical tube. For non-potable water samples, analysis was performed directly on the sample without concentrating.

Culture method

All samples were spread plated in accordance with the ISO 11731:1998 method. Spread plate results were compared with results from the *Legionella* PCR analyses for every sample. In short, 100 μl of potable water concentrate or 100 μl of unconcentrated non-potable water was spread plated onto Buffered Charcoal Yeast Extract (BYCE) agar supplemented with Glycine, Vancomycin, Polymyxin B, and Cycloheximide (GVPC) manufactured in-house (Phigenics, LLC, Fayetteville, AR). The cultures were incubated at 35 ± 1 °C for 7–10 days. Suspect *Legionella* colonies that displayed cysteine auxotrophy on a BYCE Biplate (Bio-Rad, Hercules, CA) were considered *Legionella* species (*L.* spp) and further characterized by the *Legionella* latex agglutination test (Thermo Scientific, Waltham, MA) to determine *L. pneumophila* serogroup (sg) 1, sg 2–14 or *L.* spp. The theoretical limit of detection (LOD) for this method was between 0.1 and 1.0 colony forming units per milliliter (CFU/ml) depending on the volume of potable water filtered and 10 CFU/ml for non-potable water.

All samples were also cultured in-field with TimeZero™ dipslides according to McCoy *et al.* (2012). The field culture test provides culture results in 4 days with an LOD of 10 CFU/ml. The TimeZero™ results were not compared with the PCR negative screen due to differences in holding time, but this test was critical in detecting *Legionella* after a water main breach (see Case study: Use of the Next Day *Legionella* PCR in a Crisis).

DNA extraction: Method 1

Method 1 was used to extract the DNA from the first 9,113 water samples. Two milliliters of the filter-concentrated potable sample or 2 ml of the unconcentrated non-potable sample were pelleted by centrifugation, and 1.7 ml of the supernatant was discarded. The remaining pellet and 300 μl of supernatant were used for DNA extraction with the proprietary Phigenics Ultra Rapid DNA Extraction (P.U.R.E.™) method.

DNA extraction: Method 2

After the main study was completed, a 2-month trial was done with an improved DNA extraction method to increase the NPV. Method 2 was used on the final 2,012 samples. The new method was as follows: 2 ml of the potable water concentrate or 2 ml of the non-potable water sample was pelleted by centrifugation and 2 ml of the supernatant was discarded. The remaining pellet was used for DNA extraction with the proprietary P.U.R.E.™ method. The PCR and the culture method remained the same.

Laboratory experiment comparing DNA extraction methods

A laboratory experiment was performed by the Phigenics Research and Innovation Team to compare Methods 1 and 2. Three *Legionella* isolates, one *L. pne* sg 1, 2–14, and species, were diluted to OD_{600} 0.1 and serial diluted to 10^{-5}. The three lowest dilutions were used in the experiment and 100 μl of each cell suspension was diluted in 9.9 ml of PBS to simulate a 10 ml filter concentrate. Each sample was plated on GVPC (100 and 500 μl) and extracted with Methods 1 and 2. The DNA extracts were analyzed with the Next Day *Legionella* PCR. The plates were incubated at 35 ± 1 °C for 6–10 days. The CFU/ml reported in

the results relates to the hypothetical 100 ml sample that the simulated 10 ml filter concentrate came from.

Real-time PCR

An ISO 12869:2012 compliant commercial *Legionella* real-time PCR kit was used for the Next Day *Legionella* PCR. This method detects 21 *Legionella* species, including *L. pneumophila*, by targeting a specific sequence of the 16 s rRNA gene. It also contains manufacturer provided quality control measures including an internal control, a positive control and a negative control. Validation for exclusivity against THAB strains is also reported by this method. Sixteen species of common heterotrophic bacteria were included in the validation. The manufacturer's instructions were followed for reaction set-up, thermocycling and data analysis. In the occurrence of PCR inhibition, the DNA sample was diluted 1:10 and re-analyzed.

Data analysis

The PCR results show a detect or non-detect for *Legionella* DNA in the water sample. These data were compared with the culture results. The data were grouped by (1) PCR-positive (PCR+)/Culture-positive (ISO+); (2) PCR-negative (PCR−)/ISO + ; (3) PCR + /Culture-negative (ISO−) and (4) PCR − /ISO−. The NPV and positive predictive value (PPV) of the test were calculated accordingly:

$$\mathrm{NPV} = (\mathrm{PCR}-, \mathrm{ISO}-)/[(\mathrm{PCR}-, \mathrm{ISO}+) + (\mathrm{PCR}-, \mathrm{ISO}-)]$$

$$\mathrm{PPV} = (\mathrm{PCR}+, \mathrm{ISO}+)/[(\mathrm{PCR}+, \mathrm{ISO}+) + (\mathrm{PCR}+, \mathrm{ISO}-)]$$

Additional statistics of accuracy, specificity and sensitivity were calculated as follows:

$$\mathrm{Accuracy} = [(\mathrm{PCR}+, \mathrm{ISO}+) + (\mathrm{PCR}-, \mathrm{ISO}-)]/\mathrm{Total}$$

$$\mathrm{Specificity} = (\mathrm{PCR}-, \mathrm{ISO}-)/[(\mathrm{PCR}-, \mathrm{ISO}-) + (\mathrm{PCR}+, \mathrm{ISO}-)]$$

$$\mathrm{Sensitivity} = (\mathrm{PCR}+, \mathrm{ISO}+)/[(\mathrm{PCR}+, \mathrm{ISO}+) + (\mathrm{PCR}-, \mathrm{ISO}+)]$$

RESULTS

Water samples

A total of 11,125 water samples were analyzed in this two-part study. Of these samples, 10,094 (90.73%) were potable and 1,031 (9.27%) were non-potable (mostly cooling tower water samples). There were 10,487 (94.18%) samples collected from healthcare facilities, 98 (0.88%) collected from hotels and the remaining 540 (4.85%) collected from other facilities such as universities, offices and commercial buildings. All residual oxidant measurements for potable samples complied with the Safe Drinking Water Act. For potable water samples, 77.72% were within Phigenics' validation criterion for THAB (<1,000 CFU/ml). Among the 22.28% of samples that were above the THAB validation criterion (≥1,000 CFU/ml), there was a higher percentage of culture-positive and PCR-positive results compared with samples within the validation criterion. Within the whole dataset, there were 895 *Legionella* culture-positive isolates; 377 (42.12%) isolates were *L. pneumophila* sg 1, 212 (23.68%) isolates were *L. pneumophila* sg 2–14, and 306 (34.19%) isolates were non-pneumophila *Legionella* spp. (Figure 1).

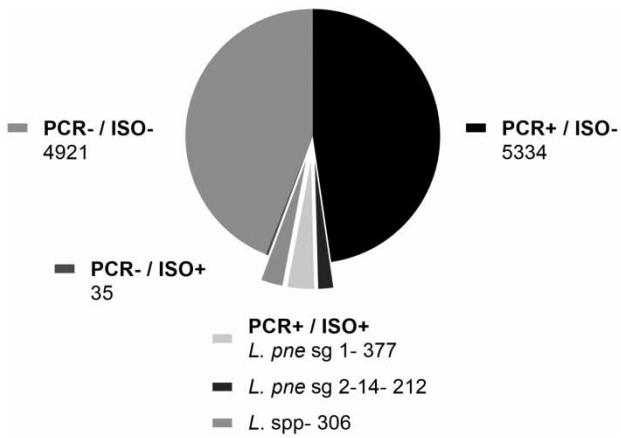

Figure 1 | Next Day *Legionella* PCR negative screen sample overview. Of 11,125 samples, 870 were culture-positive samples, 835 of which were positive by the PCR. There were 25 water samples with isolates cultured from multiple groups, *L. pneumophila* (*pne*) sg 1, *L. pne* sg 2–14, or non-pneumophila species (*L.* spp.), so the total number of positive isolates obtained from these samples was 895. The specific number of isolates from each group is shown in the expanded window. The pie chart shows the number of water samples in each category.

Only 40 (0.36%) water samples caused PCR inhibition out of the combined sample set. There were 35/10,094 (0.35%) potable inhibited samples and 5/1,031 (0.48%) non-potable inhibited samples. Data for these samples were able to be collected by the PCR on the 1:10 dilution of the DNA extract.

Method 1 NPV

Of the 9,113 samples analyzed with DNA extraction Method 1, there were 4,107 (45.07%) PCR-negative samples including 48.02% of potable samples and 13.94% of non-potable samples. The NPV of the Next Day *Legionella* PCR at >10 CFU/ml was 99.95% (Figure 2). At >2 CFU/ml, the NPV was 99.92% and at >1 CFU/ml the NPV was 99.85%. Table 1 shows the NPV for all 9,113 water samples; for potable and non-potable samples, the overall NPV was 99.17% at an LOD of 0.1 CFU/ml. The contingency table in Table 2 was used to calculate the NPV of the Method 1 dataset. A small portion of the samples were invalid (0.37%, $n = 34$) because the samples were culture-positive but PCR-negative (false-negative). The sensitivity of the test was 94.88% due to these false-negative results (Table 1). The majority of the invalid results occurred at 1 CFU/ml and originated from 100 ml water samples. Figure 3 shows the percentage of these invalid results out of the total number of culture-positive samples at the same CFU/ml. Twenty-seven samples (15.08% of 1 CFU/ml positives) were invalid at the 1 CFU/ml level, and three samples (3.41%) were invalid at the 2 CFU/ml level. Similarly, one sample (2.82%) and two samples (0.63%) were invalid in the ranges 3–10 and >10 CFU/ml, respectively (Figure 3).

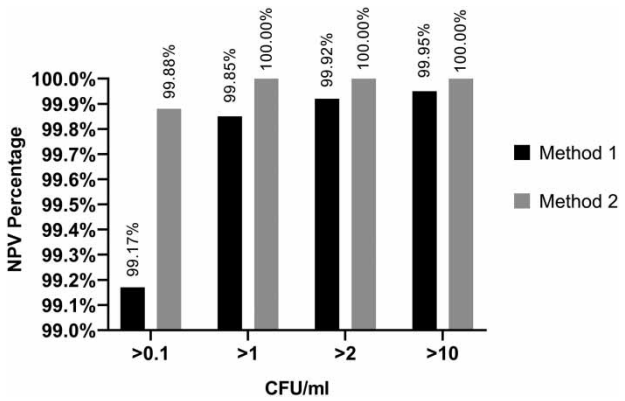

Figure 2 | NPV of the Next Day *Legionella* PCR. The NPV is shown for each method tested. Method 1 ($n = 9,113$) consisted of a DNA extraction with a lower sensitivity than Method 2 ($n = 2,012$). The CFU threshold was used to calculate the NPV at different limits of detection.

Method 1 PPV

Legionella DNA was detected in 5,006 (54.90%) samples using Method 1. The PPV was 14.06% for the entire sample set of 9,113 water samples. For potable and non-potable samples, the PPVs were 15.23% and 7.37%, respectively (Table 1). The accuracy of this test (52.40%) and the specificity of this test (48.63%) were very low, due to the

Table 1 | Binary statistics for the Next Day *Legionella* PCR negative screen calculated in comparison to culture results (ISO 11731)

	Total		Potable		Non-Potable	
Statistic	**Method 1 (%)**	**Method 2 (%)**	**Method 1 (%)**	**Method 2 (%)**	**Method 1 (%)**	**Method 2 (%)**
NPV	99.17	99.88	99.17	99.88	99.09	100.00
PPV	14.06	11.26	15.23	11.81	7.37	8.74
Accuracy[a]	52.40	48.66	55.63	52.26	20.27	22.31
Specificity[a]	48.63	45.11	52.00	49.03	14.91	16.07
Sensitivity	95.39	99.24	95.32	99.12	98.04	100.00

The binary statistics were calculated from setting ISO culture results to 'True'. These results were calculated for each sample set (Method 1: $n = 9,113$), (Method 2: $n = 2,012$) with an LOD of 0.1–1.0 CFU/ml depending on the sample volume.

[a]The data show low accuracy and specificity because with ISO culture set to 'True' in the binary statistic, and this PCR is not expected to yield high accuracy or specificity because it cannot distinguish between viable, viable but not cultural (VBNC) or non-viable (dead) *Legionella*.

Table 2 | Contingency table for Method 1

		Next Day *Legionella* PCR		
		Positive	**Negative**	**Total**
ISO11731:1998	Positive	620	34	654
	Negative	4,386	4,073	8,459
Total		5,006	4,107	9,113

This table shows the PCR results compared with the ISO 11731 results for the 9,113 samples analyzed with Method 1.

Table 3 | Contingency table for Method 2

		Next Day *Legionella* PCR		
		Positive	**Negative**	**Total**
ISO11731:1998	Positive	131	1	132
	Negative	1,032	848	1,880
Total		1,163	849	2,012

This table shows the PCR results compared with the ISO 11731 results for the 2,012 samples analyzed with Method 2.

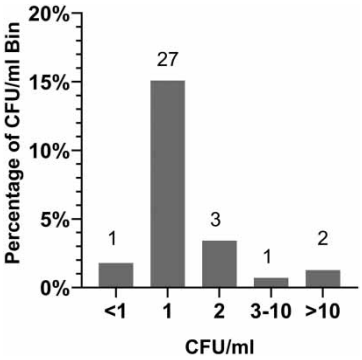

Figure 3 | Next Day *Legionella* PCR invalid results breakdown for Method 1. Of 9,113 potable and non-potable samples, 34 were invalid (PCR −, ISO+). The results are binned according to CFU/ml determined by culture and shown as a percentage of the total number of samples in each bin: <1 CFU/ml ($n = 56$), 1 CFU/ml ($n = 179$), 2 CFU/ml ($n = 88$), 3–10 CFU/ml ($n = 141$), and >10 CFU/ml ($n = 158$).

high number of PCR-positive, culture-negative samples; this is especially evident in non-potable samples (Table 1). However, there were 110 (13.94%) PCR-negative non-potable samples in this dataset. As expected for the PCR, which detects amplified genes, the PPV was very low because this assay detects any DNA with the target sequence regardless of its origin. These results confirm that the PCR did not differentiate between non-viable (dead), viable (living) or viable but not culturable (VBNC) cells in these samples (see Discussion).

Method 2: improved method to increase sensitivity

For approximately 2 months, the improved DNA extraction protocol was tested on 2,012 water samples. This included 1,770 (87.97%) potable and 242 (12.03%) non-potable samples. The NPV of this dataset was 100% at a threshold of >1 CFU/ml and 99.88% at >0.1 CFU/ml (Figure 2). Table 3 shows the contingency table that was used to calculate the NPV and the PPV. There was one (0.05%) PCR-negative, culture-positive result, and this sample had a concentration of 1 CFU/ml. Out of 132 culture-positive results, 56 were ≤2 CFU/ml and 38 were ≤1 CFU/ml. There were 848 (42.15%) PCR-negative, culture-negative samples. There were 1,032 (51.29%) PCR-positive, culture-negative samples. The PPV for this dataset was 11.26% (Table 1).

The Phigenics Research and Innovation Team performed an experiment comparing Method 1 and 2 using dilutions of laboratory strains of *Legionella*. All samples were positive on the negative screen. The nine DNA extracts from Method 2 consistently had lower cycle threshold (Cq) values (average 1.3 Cq less) than Method 1, meaning an increase in the DNA template. There was a greater decrease in the Cq value (average 2.48 Cq less) for the samples at <1 CFU/ml (Figure 4).

Case study: use of the Next Day *Legionella* PCR in a crisis

Due to a water line breach at a healthcare facility, dark colored water appeared throughout the potable water distribution system. The WMT decided to restrict potable water access in the building and required use of bottled water until the problem was resolved. An emergency flushing program was implemented. Each day, the Next Day *Legionella* PCR was performed along with PVT-TimeZero™ and other diagnostics for heterotrophic aerobic bacteria, heavy metals and coliforms to determine water quality and water safety.

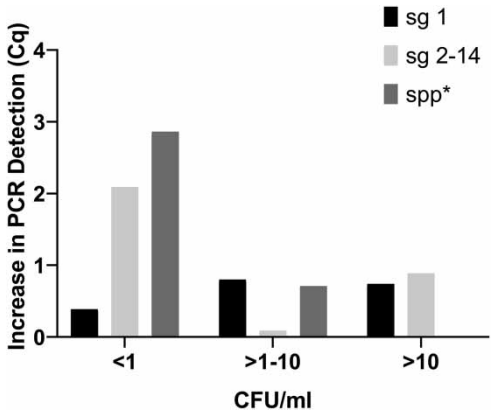

Figure 4 | Laboratory experiment comparing DNA extraction Method 1 and 2. Dilutions of three different *Legionella* isolates were made and DNA was extracted from each using both methods. The extracts were analyzed with real-time PCR. The difference in the Cq value between Method 1 and Method 2 is shown (Method 1–Method 2). The CFU/ml of each sample was calculated from culture plates.

On the first day of testing, 88% of the samples were *Legionella* PCR-positive. After flushing on the second, third, fourth and fifth days, 24%, 21%, 19% and 10% of samples were PCR-positive, respectively (Table 4). These results were available the next day after sampling, approximately 10 days before culture results were ready. After the first round of TimeZero™ results came back negative on day 4 (Table 4), the WMT decided to lift the restriction on day 5 rather than wait for further culture results. Confirmation was obtained from culture results (available 10–14 days after sampling); zero culture-positive results were reported. This defensible decision to lift the water restriction on day 5, based on results from the Next Day *Legionella* PCR and PVT-TimeZero™, saved substantial time and resources (see Discussion: The Next Day *Legionella* PCR allowed a rapid response to a crisis).

Table 4 | Example of the Next Day *Legionella* PCR negative screen used in a crisis

Test method (LOD)	Day 1	2	3	4	5	Days required to obtain results
Next Day *Legionella* PCR (+ve)	88%	24%	21%	19%	10%	1 day
Total Aerobic Heterotrophic Bacteria ($>10^3$ CFU/ml)	0%	4%	1%	0%	1%	3 days
PVT-TimeZero™ *Legionella* (>10 CFU/ml)	0%	0%	0%	0%	0%	3 days
ISO Spread Plate *Legionella* (>1 CFU/ml)	0%	0%	0%	0%	0%	10 days

A water line breach occurred at a healthcare facility and the water quality was monitored with the negative screen. The facility restricted water use and remediated with emergency flushing. The water was unrestricted after 5 days based on the results from the *Legionella* negative screen and the TimeZero™ PVT method.

Case study: negative screen use in a chloraminated system

The monthly trend of water sampling results from a healthcare facility over the 9-month period is shown in Figure 5 ($n = 1334$ samples). This example consisted of 4% non-potable and 96% potable water samples. Overall, there was an upward trend in both PCR-positive and culture-positive samples from April to July, and a downward trend from July through November as the weather got warmer and colder, respectively. The PCR-positive rate was on average 40% higher than the culture-positive rate throughout the year. This facility maintained water quality with free residual chlorine until December when chloramination was implemented. Figure 5 shows that in December, the PCR-positive rate was 96%, approximately 50% higher

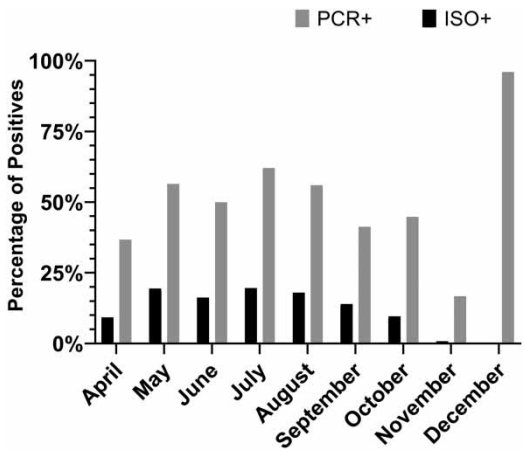

Figure 5 | Trends detected by the Next Day *Legionella* PCR based on supplemental disinfectant. Monthly trend of validation test results from a healthcare facility that switched from free residual chlorine to chloramine treatment of their water system in December 2018 is shown. Samples of 0.1 and 1 L of water were filter-concentrated and cultured via ISO 11731. Then, the concentrate was analyzed with the Next Day *Legionella* PCR negative screen. PCR-negative screen positives (PCR+) and culture-positive (ISO+) samples are shown as the percent of the total amount of samples collected in that month.

than the average of the 8 months before and 80% higher than November (16% PCR+). Additionally, after chloramination was implemented, water quality degradation was observed as indicated by higher THAB counts, higher free ammonia concentrations and detections of *Mycobacterium* in more than 50% of samples (data not shown). However, after chloramination was implemented, the *Legionella* culture-positive rate dropped to 0% in December from 0.79% in November and 9.6% in October. Chloramination of potable water has been associated with inducing the VBNC state in *Legionella* and enriching *Mycobacterium* (Baron *et al.* 2014); these results are consistent with those observations.

Case study: high PCR-positive rates in non-potable systems

The above cases focus on potable water samples, which make up the majority of samples in the entire study. This case study focuses on non-potable samples collected from April to December from a commercial facility ($n = 198$) (Figure 6). These non-potable water samples are from cooling towers, hydrants and small canals. The PPV for this dataset was 12.0%, which is approximately the median of the total potable and non-potable PPVs. The PCR-positive rate was approximately 82% higher than the culture-positive rate and 40% higher than the potable PCR-positive rate in the case study above (Negative screen use in a chloraminated system). There was a distinct increase in culture-positive samples in the warmer months of July, August, and September, but the PCR-positive rate remained high. This pattern can be indicative of a VBNC state where the cells are VBNC in the colder months and are resuscitated in the warmer months. WMTs should be cautious of extremely high PCR-positive rates because of these implications.

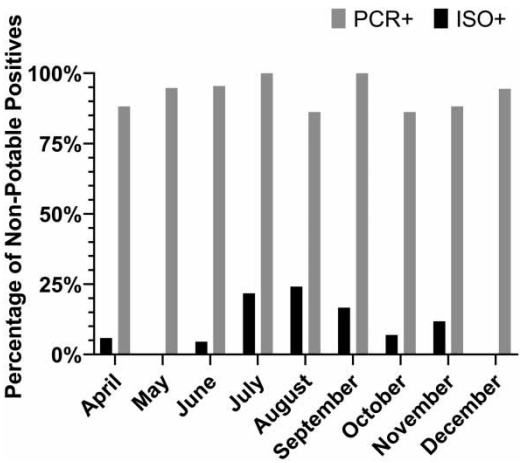

Figure 6 | The Next Day *Legionella* PCR used in a non-potable system. The monthly trend of PCR-positive (PCR+) and culture-positive (ISO+) non-potable samples from a commercial facility, April through December 2018, is shown. Samples of 0.1 and 1 L of water were filter-concentrated and cultured via ISO 11731. Then, the concentrate was analyzed with the Next Day *Legionella* PCR negative screen. PCR+ and ISO+ samples are shown as the percent of the total amount of samples collected in that month.

DISCUSSION

Water samples

The majority of the water samples in this study were potable and collected from healthcare facilities. This type of sampling location is in high proximity to at-risk patients; therefore, there is an increased risk of acquiring LD if there is *Legionella* contamination in these building water systems. It is important to maintain clean and pathogenic bacteria-free water in the healthcare setting through the use of a WMP that includes verification (confirmation that the program was implemented as planned) and validation (confirmation of hazard control) testing. The Centers for Medicare and Medicaid Services (CMS) require all hospitals or long-term care facilities that accept Medicare or Medicaid to reduce the risk of nosocomial infections by implementing a WMP to inhibit the growth of *Legionella* and other potentially pathogenic microbes in building water systems (CMS 2017). The Next Day *Legionella* PCR is a rapid test that can provide accurate information about water quality trends throughout the facility in order to confirm that control methods are working.

For both DNA extraction methods, using P.U.R.E.™, data were gathered for all 11,125 samples. Especially in non-potable samples, PCR inhibition can be a huge problem that detracts from its ease of use. Only 5 (0.48%) non-potable samples in this large dataset had PCR inhibition. This is a very low number out of 1,031 non-potable samples. These results show that the DNA extraction by Phigenics, LLC (P.U.R.E.™) is a robust method that produces inhibitor-free DNA 99.64% of the time.

The Next Day *Legionella* PCR detects *Legionella* at the genus level; therefore, it does not differentiate between *L. pneumophila* and other *L.* spp. This was not considered a limitation of the method because all *Legionella* species were treated as potentially pathogenic. Serotyping isolates from cultures revealed that 68.04% were *L. pneumophila* and 34.96% were non-pnuemophila *L.* spp.

Method 1 NPV

The NPV for the entire sample set was 99.85% when calculated with an LOD of >1 CFU/ml. An LOD of >1 CFU/ml is very conservative for validation testing. Jinadatha *et al.* support an LOD of 10 CFU/ml for validation testing. This LOD is scientifically defensible because approximately 100 times more water would have to be inhaled or aspirated in order for 1 CFU to be absorbed by an alveolus (Jinadatha *et al.* 2018). There was a highly defensible probability that a sample with a negative PCR result will also be culture-negative at an LOD of >1 CFU/ml. A sample under this LOD has a very low chance of causing disease. Currently, the practice for validation testing is to culture all samples independent of the PCR result. With an NPV of over 99%, it is feasible to only plate the PCR-positive samples and trust the negative screen result.

Out of the 9,113 samples in the first part of the study, there were 34 PCR-negative, culture-positive results. Most of which were equal to 1 CFU/ml and from 100 ml samples. These invalid results consisted of 33 potable samples and 1 non-potable sample. Most of the invalid samples were non-pneumophila species (51.28%), and the remaining samples were *L. pneumophila* sg 2–14 (15.38%) and *L. pneumophila* sg 1 (33.33%). The majority of the invalid isolates were analyzed with the Next Day *Legionella* PCR and all were detected (data not shown). This shows that the PCR primers can detect the strain. It is possible that this CFU/ml range was not detected in 100 ml samples because there was only a 10× concentration compared with a 100× concentration in the 1 L samples. In addition, the DNA extract for the PCR was collected from a small volume of the concentrated water sample. Sampling bias or other error could also account for a small number of the invalid results.

Originally, there were 38 invalid results, but 4 of these isolates were incorrectly identified as *Legionella* by the traditional culture method. These isolates were presumed to be *Legionella* after culturing, testing cysteine auxotrophy, and performing the latex agglutination test. Speciation by DNA sequencing was performed and the assay was negative for any *Legionella* spp. This shows that the negative screen PCR-negative result was correct. These results confirm inherent limitations in the traditional culture method. There is a need for genomic analysis of *Legionella* from validation testing due to situations like this. The PCR results can be confirmed through genomic analysis, so that isolate confirmation would not rely solely on the latex agglutination test (Thermo Scientific, Waltham, MA) to determine *L. pneumophila* sg 1, sg 2–14, or *L.* spp. Furthermore, the latex agglutination reagents only detect seven species (Thermo Fisher 2016) out of the >50 species of *Legionella*. It is possible to determine these groups with more specific PCR, but genomic analyses can provide more accurate information such as the exact species of *Legionella* or the sequence type of *L. pneumophila*. Genomics will greatly improve the defensibility of a WMP if there is a case of disease. Additionally, genomics will allow the WMT to take specific action depending on the virulence of the pathogen. The CDC speciates all *Legionella* isolates via the mip gene PCR and the European Study Group for *Legionella* infections has built a database for *L. pneumophila* sequence types. The *Legionella* spp. negative screen should be the first step in the validation of *Legionella* management in building water. This diagnostic has allowed facility managers to decide that it was safe to return water recirculation loops back into commission the day after remediation. Results from PCR negative screening provide a focused approach to culturing and verifying *Legionella* isolates.

Method 1 PPV

The PPV for the *Legionella* negative screen was quite low at 15.23% potable and 7.37% non-potable for the 9,113 sample set (Table 1). This means that the probability is very low that PCR-positive results will correlate to culture-positive results. There are many reasons for this discrepancy, one being the sensitivity of the PCR method. The culture method has a theoretical LOD of 1 CFU/ml, whereas the PCR has a much lower LOD of five genomic units. Often colonies are

formed by hundreds of cells from a lysed, infected amoeba or from a piece of biofilm in the water sample. These colonies contain hundreds of copies of the target DNA, but the PCR can detect down to five copies. Because of this inconsistency, it is common to have a culture-negative, PCR-positive result. Also, VBNC cells cannot be detected by the culture method because the bacteria have gone into a protective state and have lost the ability to form colonies (Oliver 2005). The PCR, however, detects the DNA from VBNC cells (Dusserre *et al.* 2008), which have been shown to exhibit pathogenic qualities (Alleron *et al.* 2013; Epalle *et al.* 2014). Molecular diagnostics are superior to traditional culture methods in this regard.

An important limitation of PCR is that it cannot differentiate between live and dead cells if the cell membranes are intact. Due to low dosage levels of disinfectants, *Legionella* cells are often metabolically dead with an intact membrane (ghost cell) (Virto *et al.* 2005). This causes the DNA from these ghost cells to be included in the DNA extraction. There is no way, except for metabolic/enzymatic methods, to differentiate live versus dead cells with intact membranes. Propidium monoazide (PMA) has been used to intercalate with DNA from dead cells with damaged membranes so that it cannot be amplified with PCR, but PMA cannot penetrate intact membranes (Ditommaso *et al.* 2014), therefore, making it an ineffective method to differentiate ghost cells. A novel viability assay is needed that possibly brings together the sensitivity of PCR and the ability to differentiate live and dead cells.

Method 2 provides increased sensitivity

Due to the fact that the NPV was less than 100%, there was room for improvement in this method. One area of optimization was the concentration of template DNA added to the PCR. A simple change to the DNA extraction protocol increased the concentration of the DNA by 2.5 times. The calculated LOD of the Next Day *Legionella* PCR was 2 CFU/ml, because 1 CFU/ml samples were not consistently detected. With the improved DNA extraction protocol, the theoretical LOD should decrease to approximately 0.8 CFU/ml. PASL changed to the new extraction protocol and after 2 months of validation testing, and the NPV was 100% at >1 CFU/ml and 99.88% at >0.1 CFU/ml (Figure 2).

The laboratory experiment (Figure 4) showed that there was an increase in the DNA template for low CFU samples using Method 2, especially samples ≤1 CFU/ml. The improved DNA extraction will help increase the NPV of the Next Day *Legionella* PCR by making it possible to more consistently detect 1 CFU/ml samples.

The Next Day *Legionella* PCR allowed a rapid response to a crisis

The Next Day *Legionella* PCR negative screen and PVT-TimeZero™ were used in a healthcare facility crisis where facility managers were able to use the data to assess water quality and bring the system back online in 5 days, compared with 10–14 days waiting for culture results. The percent of PCR-positive locations was above average at the beginning of the breach but were lowered to below average in 5 days. The facility was restricted to use bottled water during this time, which resulted in using approximately 20,000 bottles/day, costing approximately $5,000/day. The negative screen PCR results enabled the WMT to lift the restrictions 9 days before culture results, saving approximately $45,000 in bottled water use. A significant amount of time and resources were saved due to the negative screen. This case study shows one of the diverse ways this negative screen can be used. Because of the greater than 99% NPV, the WMT was confident to restore water access in half the time it would have taken to receive culture results.

The importance of the Next Day *Legionella* PCR in chloraminated systems

Looking closely at culture-positive and PCR-positive monthly trends from a healthcare facility, it was clear that temperature affected the culture-positive rate and the PCR-positive rate (Figure 5). The type of disinfectant also played a major role in the results from culture and the PCR. Free residual chlorine was used in this facility from April through November and was changed to monochloramines in December. The number of culture-positive samples was decreasing in November due to cold weather. In December, there were zero culture-positives; this could

have been due to the cold weather, but the PCR-positive results indicate that the amount of *Legionella* DNA increased. A significant increase in PCR-positive samples of 50% could suggest that chloramination induced a VBNC state in the *Legionella* present; indeed, the *Legionella* isolates were not culturable with the ISO 11731 method. Monochloramines have been shown to induce the VBNC state in *L. pneumophila* and VBNC *L. pneumophila* can remain viable for over 4 months after monochloramine treatment (Alleron *et al.* 2008). When *Legionella* cells are in the VBNC state, they pose a potential threat to human health because they can infect and replicate in human alveolar macrophage cell culture (Dietersdorfer *et al.* 2018). Because the traditional culture method cannot detect VBNC cells, molecular screens are useful for chloraminated water systems. The detection of potentially VBNC cells gives the WMT more accurate information about water quality in the system enabling more defensible decisions.

High PCR-positive rates in non-potable systems

The PPV for non-potable samples was 6.5% lower than for potable samples. This indicated that non-potable samples are more likely to be PCR-positive, culture-negative. A significant portion of the non-potable samples, however, were PCR-negative. A PCR-negative result in non-potable water is indicative of *Legionella*-free water and is very useful information for the WMT. A PCR-positive, culture-negative result could be due to dead cells in properly treated cooling tower water disinfected with much more toxic antimicrobials than potable water. In addition, non-potable water sources are likely to have much more organic matter than potable water. This has been shown to decrease the effectiveness of chlorine disinfectants (Virto *et al.* 2005). In this situation, a VBNC state or a ghost cell state can occur, thus increasing the PCR-positive rate and lowering the culture-negative rate. In the non-potable water case study shown in Figure 6, the PCR-positive rate was 40% higher than in the healthcare facility study, where the majority of samples were potable (Figure 5). This non-potable water system may have had VBNC cells or ghost cells that the Next Day *Legionella* PCR detected but cannot differentiate; therefore, a viability assay is particularly important for non-potable water.

CONCLUSIONS

The Next Day *Legionella* PCR negative screen provides results on the same day of sample receipt compared with the 10–14 day response time when using the traditional culture method. These rapid results allow WMTs to respond to crises efficiently. The innovation that allowed for this rapid turn-around time is the Phigenics proprietary DNA extraction, P.U.R.E.™. Not only was the extraction fast and high-throughput, but it also resulted in very few inhibited PCRs.

The robust dataset included 11,125 samples with a NPV at >1 CFU/ml of 99.85% and 100.00% using DNA extraction Methods 1 and 2, respectively. The extremely high NPV allows for a revolutionary change in *Legionella* testing – only plating the PCR-positive samples. Qualitative results from the *Legionella* negative screen can validate (confirm) that the WMP is effective. PCR-negative locations are *Legionella* DNA-free; therefore, the verified control limits are effective. PCR-positive locations require further tests (by the culture method). We have demonstrated the negative screen strategy to be plausible and if implemented a significant amount of time and resources can be saved.

There are many benefits to the negative screen strategy, but the inability to differentiate viable cells is a limitation. The Next Day *Legionella* PCR is a foundational test that can be built upon for future *Legionella* diagnostics including more accurate viability assays.

COMPETING INTERESTS STATEMENT

During this research study, all authors were employed by Phigenics, LLC.

FUNDING INFORMATION

Funding for this research was provided by Phigenics, LLC.

ACKNOWLEDGEMENTS

We thank Dr. Eugene T. Cloete (Stellenbosch University) and Aaron A. Rosenblatt (Gordon & Rosenblatt) for

helpful suggestions and comments on the manuscript. We also give special thanks to Dr. Claressa Lucas (CDC) for her feedback and expert suggestions for the manuscript.

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First received 6 January 2020; accepted in revised form 1 April 2020. Available online 6 May 2020