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On-filter direct amplification of *Legionella pneumophila* for rapid assessment of its abundance and viability

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Abstract

Guidelines and regulations to control *Legionella pneumophila* in cooling water systems of large buildings are evolving due to the increasing number of outbreaks. Rapid, on-site, simple, and sensitive quantification methods that are also able to assess viability may be extremely useful in monitoring and control. Culture-based methods for measuring *L. pneumophila* may take 4 to 10 days and qPCR-based methods are also slow, requiring at least a day from sample to result, albeit mainly due to the need for sample transport to a centralized laboratory. This study reports a rapid isothermal amplification method for *L. pneumophila* concentration and detection with live/dead differentiation under field conditions. Using an on-filter direct amplification (i.e., amplification of cells without DNA extraction and purification) approach with propidium monoazide (PMA), and a real time isothermal amplification platform (Gene-Z), *L. pneumophila* could be detected in 1 to 2 hr at ~1 cfu/100 ml of tap water. Signature sequences from 16S rRNA and *cadA* genes were used as genetic markers for *L. pneumophila* and loop-mediated isothermal amplification (LAMP) primers were designed using Primer Explorer V4. Result were also compared with direct amplification of cells spiked into distilled, tap, and cooling water samples as well as extracted DNA by qPCR. This method may be useful to managers of cooling water systems in large buildings for rapid detection of *L. pneumophila*. The overall approach of on-site sample concentration, on-filter amplification, and live/dead differentiation may be extended to other organisms where analytical sensitivity and speed are equally important.

Graphical abstract

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Keywords

Legionella pneumophila; direct on-filter amplification; Gene-Z; loop mediated isothermal amplification; water; sample processing; rapid detection; viable cells; Propidium monoazide

1.0 INTRODUCTION

Legionellosis, a disease caused by *Legionella pneumophila*, often present in cooling water systems of large buildings, is on the rise in the U.S. (Dooling et al., 2015). Data suggests that 9 out of 10 outbreaks of *Legionella* are preventable through better water management practices (Lucas et al., 2016). More than 60 species of *Legionella* are known but *L. pneumophila* serogroup 1 (*Lp1*) is the most common cause of legionellosis in the United States. Numerous organizations have issued guidelines to control and manage levels of *L. pneumophila* and *Legionella* spp in cooling water systems (Parr et al., 2015). *L. pneumophila* is also on the U.S. Environmental Protection Agency's Contaminant Candidate List 4 (CCL 4, USEPA, 2016) as one of 12 waterborne pathogens of concern. Prompted by a large outbreak of Legionnaires' disease responsible for 12 deaths and more than 100 cases of sickened patients (Toppo, 2015), the state of New York has adopted new regulations for control in building water systems (NYSDOH, 2016). As per this regulation, all cooling towers in the state must be registered with New York State's Department of Health and monitor for *Legionella* using a certified laboratory, every 90 days during the first year and annually thereafter. Moreover, the facility owner must "notify the local health department within 24 hours of getting a positive culture sample result exceeding 1,000 colony-forming units (cfu) per milliliter." As a result, more than 8,660 cooling towers are now registered in the database maintained by the State of NY (NYSDOH, 2015). Although similar databases are not available for the rest of the country, cooling towers are an integral part of the more than 53,554 hotels, 20,000 spa facilities, 15,401 long-term care facilities, and 5,627 hospitals located throughout the U.S. As part of their cooling tower management plan, these facilities must regularly monitor for *L. pneumophila* and *Legionella* spp. Similarly, in Flint, MI, a recent Legionnaires' disease outbreak killing at least 12 people (Anderson, 2016) has been linked to the corrosion in drinking water pipes near hospitals, which stimulated growth of *L. pneumophila* and *Legionella* spp (Schwake et al., 2016). Simple, quick, and cost-effective methods for assessing abundance and viability of *L. pneumophila* are expected to play a key role in implementing and monitoring better management practices.

In the State of New York, *Legionella* analysis must be carried by approved laboratories (NYSDOH, 2016). Though not yet in effect, Michigan is also considering implementing new *L. pneumophila* and *Legionella* spp testing regulations near hospitals (Associated Press, 2016). At present, culturing on buffered charcoal yeast extract agar (Feeley et al., 1979) and

qPCR (Krøjgaard et al., 2011; Mentasti et al., 2015; Vanyacker et al., 2014) are the two main techniques adapted for quantification. Culturing will assess viability but it is slow (4 to 10 days). qPCR is faster but must be done in a centralized location adding at least a day for sample transport and analysis. Targeting the 16S rRNA gene is more common but other genes (e.g., *cadA*, *mip*) have also been used. However, the traditional qPCR assay does not give information about viability. Because chemical or other form of disinfection is almost always a part of cooling water systems, assessment of viability is essential. The extracellular or dead cell DNA-binding reagents commonly demonstrated for viability assessment are propidium monoazide (PMA, Yáñez et al., 2011) and ethidium monoazide (EMA, Delgado-Viscogliosi et al., 2009). The latter, however, has been shown to also affect live cells to certain extent (Nocker et al., 2007). Because low levels of *L. pneumophila* and *Legionella* spp are ubiquitous in the environment, the State of NY regulations require notification if counts exceed 10^5 cfu/100 ml. Other guidelines have set a 30% positive sample threshold for notification (e.g., Herbers et al., 2013). Hence, methods with superior detection limits (e.g., 1–10 cfu/100 ml) are desirable.

The aims of this study were to demonstrate on-filter direct amplification for *L. pneumophila*, validate the same in vials for both isothermal and qPCR protocols, and integrate PMA with the isothermal approach to assess viability. To the authors' knowledge, there is no study demonstrating direct isothermal amplification of *L. pneumophila* cells, or one that integrates viability assessment under field conditions and can detect at 10 cfu/100 ml or higher. The on filter isothermal amplification method was also tested with tap and cooling water samples that had varying amounts of iron. Overall, this method may be broadly useful for many waterborne pathogens where rapid quantification at lower abundance and viability assessment are desired.

2.0 MATERIALS AND METHODS

2.1 *L. pneumophila* enumeration

L. pneumophila strain Chicago 2 (ATCC 33152) was purchased from the American Type Culture Collection (Manassas, VA) as lyophilized powder and grown on buffered charcoal yeast extract agar supplemented with Iron (III) pyrophosphate hydrate (Sigma-Aldrich, St. Louis, MO) and L-Cysteine hydrochloride monohydrate (Alfa Aesar, Ward Hill, MA). Inoculated plates were incubated at 37 °C for 4 days and colonies were harvested and re-suspended in 1 ml 1x Dulbecco's phosphate buffered saline. From this *L. pneumophila* suspension, 10-fold serial dilutions of bacterial cells were prepared in triplicate using Dulbecco's phosphate buffered saline to establish standard curves for quantitative analysis. Serial dilutions were enumerated for *L. pneumophila* colony forming units (cfu) by plating on buffered charcoal yeast extract agar plates and incubating at 37 °C for 4 days. All subsequent references to cfu are based on the plate counts obtained as described in this section.

2.2 Primer evaluation with extracted DNA

L. pneumophila DNA was extracted using MoBio PowerSoil DNA extraction kit and serially diluted in nuclease free water to obtain 10, 1, 0.1, 0.01, 0.001, or 0.0001 ng/μl DNA. For *L.*

pneumophila DNA, 0.0001 ng was considered equivalent to 23 genomic copies (GC) as previously estimated (Wellinghausen et al., 2001) based on the size of the genome. The loop-mediated isothermal amplification (LAMP; Notomi et al., 2000) reaction mixture per 10 µl contained 0.8 µl of large fragment of *Bst* DNA polymerase (New England Biolabs Inc., Ipswich, MA), 0.4 µl of Syto-82 (Invitrogen Corporation, Carlsbad, CA), 1 µl primer mix (targeting 16S rRNA or *cadA* gene), 1 µl BSA, 0.4 µl pluronic, 1X isothermal amplification buffer (New England Biolabs), 1.4 mM each dNTP (Invitrogen), 0.8 M Betaine solution (Sigma Aldrich), 6 mM MgSO₄ (New England Biolabs), 1.4 µl nuclease-free sterile water (Fischer Scientific, Pittsburgh, PA), 1 µl of sample template. LAMP primers for 16S rRNA gene were described previously (Lu et al., 2011), and *cadA* (Table S1) were designed using Primer Explorer software (<https://primerexplorer.jp/e/>). The *cadA* gene, part of the Cd²⁺ efflux system (Rankin et al., 2002), was selected to be species specific for *L. pneumophila*. Theoretical BLAST analysis verifies that the primers solely target strains of *L. pneumophila*. The loop primers (LF and LB) for the 16S rRNA gene assay, which was also designed to be specific to *L. pneumophila* (Lu et al., 2011) were included in this study to reduce time to amplification. Amplification was carried out isothermally at 63 °C using Chromo4™ real-time PCR equipment (Bio-Rad Laboratories, Hercules, CA) and increase in fluorescence was measured for 1 h.

2.3 Direct amplification of cells in vials

Direct amplification of *L. pneumophila* cells was carried out with *Bst* polymerase using 1 µl of serial dilutions (with *L. pneumophila* cell concentration in the range of 4 to 400,000 cfu/ml) and LAMP primers for the 16S rRNA gene. The 10 µl reaction mixture contained the same reagent mix as describe for extracted DNA except that the sample was cell suspension from serial dilutions. Amplification was carried out isothermally using Chromo4™ maintained at 63 °C and fluorescence measured every 1 min for up to 60 min. Positive calls were made if amplification was observed in two or more of three replicate reactions.

Similarly, a quantitative PCR (qPCR)-based direct amplification of *L. pneumophila* cells was carried out on Chromo4™ using Taq polymerase and the F3/B3 of LAMP primer pair listed in Table 1 but used as qPCR primer set. The reaction mixture (25 µl) contained 10 µl of sample (containing 1.84×10⁷ to 1.84 cfu/ml), 12.5 µl SYBR master mix, 1.25 µl primer mix, and 1.25 µl nuclease-free water. Eight replicates per dilutions were used to allow better statistical analysis at lower dilutions. Temperature cycling protocol used with was: 10-min denaturation at 95 °C followed by 50 cycles of denaturation (0 s at 95 °C), annealing (5 s at 57 °C), and amplification (15 s at 72 °C). *L. pneumophila* and PCR-grade water were used as positive and negative controls, respectively. The expected band size of target amplicons was confirmed by gel electrophoresis.

2.4 On-filter direct amplification in microfluidic chambers

Spiked water samples were prepared in triplicate by adding 10 µl of stock *L. pneumophila* serial dilutions in 100 ml of sterile distilled water in the range of 1 to 10⁷ cfu/100 ml. The spiked samples were then filtered using a 140-ml syringe (Medtronic Monoject 14032800) attached to a polycarbonate filter holder (Cole Palmer, EW29550-40) containing a 0.4 µm

isopore polycarbonate membrane filter (Millipore HTBP01300). When handling many samples, the syringe was connected to a filtration flask operated by a vacuum pump. Negative control consisted of 100 ml of sterile distilled waters. After sample concentration, filters were transferred to microfluidic cards fabricated using 1.6 mm thick acrylic sheets. Each card contained nine chambers; two of which were used as negative controls and six for samples. The microfluidic cards used with Gene-Z device was carved by a CO₂ laser to accommodate the 13 mm diameter filters. After the transfer of filters, the cards were sealed with a PCR film (MicroAmp Optical Adhesive Film; Applied Biosystems, Carlsbad, CA). Amplification reagents and primers were injected through an inlet leading to the filter chambers. Reagent mixture for LAMP was as described above and direct amplification was performed either using 16S rRNA or cad A gene primer sets or both (Table S1). Each reaction chamber had its own loading port and air vent that were sealed with epoxy (Pacer Technology, Rancho Cucamonga, CA) after addition of reaction mixture. The sealed microfluidic card was incubated at 63 °C either using a heater or Gene-Z - a real time isothermal amplification device described previously (Stedtfeld et al., 2012). When using a heater, the amplification signals were quantified using a CCD camera set up with excitation light and emission filter with exposure control described previously (Ahmad et al., 2011) by measuring fluorescence every 30 s for up to 60 min and exposure time of 1 s. Signal extraction and images analysis was carried out using Image J (Rasband, 1997) with signal-to-noise (SNR) ratio calculated as raw signal intensity minus the mean fluorescence signal for the first 10 min divided by the corresponding standard deviation. TTP was defined as the time at which the SNR reached an arbitrary threshold of 20. Average TTP values were calculated for triplicate amplification reactions. When using Gene-Z, temperature control, time for which fluorescent signals available from a photodiode are collected, plotting of fluorescence signals obtained from the filter chambers are automated as described elsewhere (Stedtfeld et al., 2012).

2.5 Viability assessment using PMA

Quantification of viable *L. pneumophila* cells was assessed by integration of propidium monoazide (PMA, Biotium) protocol (Zhao et al., 2013) with the microfluidic cards (Stedtfeld et al., 2015) and Gene-Z (Stedtfeld et al., 2012). Ten µl of heat treated (5 min at 95 °C) and non-heat treated stocks of *L. pneumophila* cells were spiked into 100 ml of autoclaved distilled water (in triplicate) resulting in 6×10^8 , 1×10^5 , and 1×10^2 cfu per 100 ml of spiked sample and filtered as described above. PMA (0.5 ml of 50 µM) was added to the filter cartridge using a 1-ml syringe, mixed by moving the syringe plunger 3x, and then by placing the syringe-filter cartridge assembly on a vortexer at minimal setting in the dark for 10 minutes. Next, the assembly was exposed to blue light (Zoomable Blue Light LED Flashlight, CrazyFire) for 15 minutes on a foil-wrapped ice-block to cross-link the DNA. This was followed by passing 3 ml of sterile PCR grade water through the filters to remove excess PMA. Filters were removed from the cartridge and placed in microfluidic cards. After LAMP reagent addition, the cards were incubated at 63 °C in Gene-Z and increase in fluorescence monitored by the iPod Touch as described above. For comparison, the method was also tested in conventional reaction vials, in which isothermal amplification reaction was carried out with DNA extracted after light induced cross-linking or directly amplified with LAMP reaction constituents with PMA in 1:10 ratio.

2.6 Collection and testing real water samples with direct isothermal amplification

Eight samples were collected from tap and cooling waters on the campus of Michigan State University in East Lansing in April, 2017. At the time of sampling, fresh water had recently been added to cooling towers. Using sterile 1 l plastic bottles, samples were collected from sources known to have varying levels of iron (Fig S1, Table 2). Iron levels were measured using a Hanna Instrument (HI 721 Iron High Range). Samples were divided into nine 100 ml portions, and 10 μ l of fresh *L. pneumophila* cultures were spiked into each 100 ml portion to yield 10^3 or 10^5 cfu/100ml. For the nine 100 mL portions, three replicates of each sample were handled as described for direct on-filter amplification with no pretreatment. Three replicates of each sample were passed through a 5- μ m pore nylon syringe filter (Cole Parmer) prior to direct on-filter amplification. Three replicates of each sample were incubated for 30 min with a 2:1 molar ratio of Na_3PO_4 to measured iron. This step was carried out prior to passing through a 5- μ m pore filter and direct on-filter amplification. Both treatment methods were used to reduce amplification inhibitors. In detail, the Na_3PO_4 was used to promote iron and manganese ions to remain dissolved in solution for passing through the 0.4 μ m filters (Cameron and Liss, 1984) used for on-filter amplification. The 5- μ m pore filter was used as previously described to remove larger solids (Stedtfeld et al., 2016) that may inhibit the reaction. Experiments were also performed using distilled water to ensure that prefiltration with 5- μ m filters did not influence levels of *L. pneumophila*. All direct on-filter isothermal amplification experiments with real samples were monitored in real time using the Gene-Z device.

3.0 RESULTS

3.1 Direct isothermal and qPCR amplification of *L. pneumophila* cells in vials

LAMP assays using the 16S rRNA gene primer for *L. pneumophila* was evaluated for direct isothermal amplification of cells in traditional 100- μ l PCR vials. *L. pneumophila* stocks containing 1.8×10^9 cfu/ml of *L. pneumophila* (as determined by plate count) was serially diluted 10-fold and 1 μ l aliquot of each dilution was used for direct isothermal amplification in vials. The T_t for the highest concentration of 1.8×10^9 cfu/reaction was 28 min (Fig 1A). For the lowest dilution that had at least one replicate amplified (2 cfu/reaction), the T_t was 51 min. For the three highest concentrations (1.8×10^8 to 1.8×10^6 cfu/reaction), there was some plateauing in T_t indicating saturation of target. Two most diluted samples (both less than 1 cfu/reaction) failed to amplify. Overall, the lower limit of detection by direct amplification in vials was between 2 and 18 cfu/reaction.

Prior to evaluating the direct cell-based amplifications, the 16S rRNA and *cadA* gene LAMP assays for *L. pneumophila* were also tested using extracted DNA of *L. pneumophila*. Direct cell amplification in vials matched the T_t obtained for extracted DNA amplification rather closely with 49 min of T_t for 27 genomic copies (Table 1). Amplification data using gDNA in the range of 27.3 to 273,000 genomic copies per reaction (0.0001 to 10 ng/ μ l) was linear with $R^2 = 0.94$, $R^2 = 0.75$ for 16S rRNA gene and *cadA*, respectively. For the same genomic copies, assay for *cadA* was faster than the assay for 16S rRNA gene. This was true at all concentrations except the 273 genomic copies per reaction. However, the assay using the 16S rRNA gene primer was more sensitive compared to the *cadA* gene.

Next, a direct qPCR assay for *L. pneumophila* cells was evaluated using the 16S rRNA gene primers (only F3/B3 primers were used). Amplification was carried out in eight replicates per dilution to maximize the chance of observing amplification especially at the higher dilutions. All 8 sample replicates amplified for cell concentrations ranging from 1.8×10^6 to 180 cfu per reaction. For 18 and ~2 cfu per reaction, 6 and 4 reactions out of 8 amplified, respectively. The next two highest dilutions having less than 1 cfu per reaction had only 2 and 1 (out of 8) positive results, respectively. These two dilutions will most likely yield negative results under routine analysis when only triplicates are used (Fig 1B).

3.2 Direct on-filter isothermal amplification of filtered *L. pneumophila* cells

Each run consisted of 8 microfluidic well containing the filters (one filter per well) used to concentrate *L. pneumophila* cells from 100 ml water. The eight wells could accommodate two samples in triplicate plus two negative controls (located on the right of each card). Upon incubation at 63 °C, the fluorescence increased with time (Fig 2 inset) indicating direct amplification of cells on the filter itself. Negative controls did not amplify within the time tested (40 min). T_t for sample having 1.8×10^6 cfu/100 ml of *L. pneumophila* cells on the filter was 16.5 min and for 2 cfu/100 ml - the lowest concentration for which at least 2 out 3 replicates amplified, T_t was 34.7 min. For the last dilution containing less than 1 cfu/100 ml, only one out of three wells amplified (Fig 2). Thus, direct amplification on filter can yield a lower detection limit of approximately 2 cfu/100 ml in approximately 34.7 min of amplification time. Even though the fluorescence in wells upon amplification was somewhat non-uniform (as seen in Fig 2), the integrated signal over the entire circular area was sufficiently robust to yield an amplification curve (SNR vs time plot) and calculation of T_t for each sample concentration. When the experiment was repeated using only the 16S rRNA gene primer set at 100, 40, and 4 cfu of *L. pneumophila* per 100 ml, the T_t increased to 37.5 ± 0.4 , 41.5 ± 3.7 , and 60 ± 0.0 , min, respectively.

3.3 Integration of PMA-based viability assessment

PMA treatment for testing viability of *L. pneumophila* was demonstrated using three separate dilutions of not lysed and heat-lysed cells spiked into 100 ml of water. On-filter direct amplification of *L. pneumophila* cells exhibited significant differences ($p < 0.05$) in T_t between unheated and heated cells exposed to PMA with a dilution of 6×10^8 cfu spiked into 100 ml (Fig 3). No amplification was observed for heat lysed cells treated with PMA using the dilution of 10^5 cfu spiked into 100 ml. Amplification was not observed in PMA treated cells (lysed and not lysed) with the lowest tested dilution of 10^5 cfu spiked into 100 ml.

Results also show the importance of removing PMA prior to the amplification reaction. No difference in T_t was observed between PMA treated heat-lysed and non-heat lysed samples without flushing water through filters (Fig S2C). This observation was also confirmed by isothermal amplification in vials using direct amplification of *L. pneumophila* cells after PMA cross-linking (Fig. S2A), and extracted DNA amplification after PMA cross-linking (Fig. S2B). Overall, these results suggest that PMA significantly hinders the LAMP reaction and must be removed by flushing filters with water after cross-linking.

3.4 Direct on-filter isothermal amplification of filtered *L. pneumophila* cells in Gene-Z

Direct on-filter amplification performed with Gene-Z, carried out in the range of 1 to 10^7 cfu/100 ml resulted in a Tt from 20 to 50 min (Fig 4). Dilutions containing less than 10 cfu per 100 ml and no template control did not show amplification. The amplification time and standard curves were similar to spiking the same number of cfu directly into LAMP reaction, tested using vials in a conventional thermocycler (under isothermal conditions). This results demonstrates utility of the filtration method for capturing a high percentage of the *L. pneumophila* cells, and the Gene-Z device.

3.5 Direct on-filter isothermal amplification of *L. pneumophila* cells spiked into tap and cooling waters

The method was further demonstrated using cooling and tap water samples with various levels of iron. Initial experiments with samples showed some inhibition in terms of shifting Tt even after pre-filtration with a 30-mm diameter 5- μ m pore syringe filters (Table 2). Thus, experiments with subsequent samples included 30 min incubation with phosphate, in an attempt to promote iron and manganese ions to remain dissolved and pass through the 0.4 μ m pore filters. Results showed that pre-filtration reduced the inhibition observed in cooling and tap water samples. An inhibitory shift in Tt did not appear to influence sensitivity for the dilutions tested (10^3 and 10^5 cfu/100 mL).

4.0 DISCUSSION

Amplification without DNA extraction and purification, referred to as direct amplification has been shown for several organisms and polymerases and reviewed recently (Williams et al., 2017) but to our knowledge, its performance for *L. pneumophila* was not yet validated. It is well known that at low cell abundance, recovery losses impact the lower detection limit. Hence, it was hypothesized that carrying out direct amplification “on the filter” itself will further enhance the limit of detection. In this study, *L. pneumophila* cells filtered and amplified directly on the filter eliminated the cell recovery and DNA extraction step(s), made the method more rapid, simplified the detection protocol, enhanced the lower limit of detection, and allowed the overall approach to be field-deployable. Although, the isothermal amplification was performed using Gene-Z- a real time isothermal device reported earlier by our group (Stedtfeld et al., 2012), any other isothermal amplification device may be used for this purpose using one of the protocols presented here.

The direct amplification approach using qPCR can be employed as part of existing protocols for *L. pneumophila* monitoring. The lower limit of detection for both culture-based and qPCR-based methods is in the range of 5 to 10 cfu/100 ml (Behets et al., 2007; Bonetta et al., 2010; Ditommaso et al., 2014). This is probably the rationale for WHO’s limit for *L. pneumophila* in tap water is below 10 cfu/100 ml (Füchslin et al., 2010; WHO, 2007). For qPCR, the limit of detection is mostly expressed as genomic copies per 100 ml. The infectious dose of *L. pneumophila* is unknown for healthy individuals but for immunocompromised patients 1 cfu is enough for onset of disease (USEPA, 2001). Even though the on-chip amplification method described here is somewhat better in terms of its limit of detection (2–10 cfu/100 ml), most likely due to the elimination of recovery and

extraction losses, the more significant benefit is the ability to carry out the rapid measurement under field conditions. Both - culture-based and qPCR-based methods, require sample transport and the former takes several days. The qPCR-based method, however, can be carried out within 90 min from receipt of sample to result including DNA extraction and confirmation of product identity (Ballard et al., 2000).

Use of two primer sets simultaneously often has some influence on the limit of detection. Joly et al., compared qPCRs method targeting the 16S rRNA gene specific for the genus *L. pneumophila* and the *mip* gene specific for the species *L. pneumophila* for a total of 223 hot water system samples and reported 3 genomic copies/100 ml for 16S rRNA gene primer and 30 genomic copies/100 ml for *mip* gene primer (Joly et al., 2006). A previous study did not observe a difference in limit of detection for the two genes (Wellinghausen et al., 2001). In this study, 16S rRNA gene primer alone was used for most of the method development and validation. This was based on the observation that the lower limit of detection desired for regulatory action (e.g., 1000 cfu/100 ml for the state of NY) was easily achievable using only the 16S rRNA gene primers and integration of viability and ease of filtration protocols are more important for a field-deployable method for *L. pneumophila*. However, when *cadA* was also added (e.g., for microfluidic cards shown in Fig 2), there was some enhancement in the lower limit of detection as well as T_t (from 42 min for 10 cfu/100 ml using only 16S rRNA primers to 34.7 min for 2 cfu/100 ml using 16S rRNA and *cadA* primers). In addition, the faster amplification with 16S rRNA primers compared to *cadA* was most likely due to *L. pneumophila* containing about three copies of 16S rRNA per genome (Pei et al., 2010) compared to only one copy of *cadA* (Chien et al., 2004).

The Gene-Z chip was designed to house filters while trying to minimize reaction volumes. Reactions required 135 μ l and Gene-Z chips could be run with nine separate reactions per chip in parallel. Volume was reduced by removing the tear drop shape that was previously required for loading wells without bubbles (Stedtfeld et al., 2016). The new well is designed to allow filters to be folded when placed into the chip, with a sharp edge near the outlet that eliminates bubbles formation during loading of reagents. Reducing the volume of the reaction wells helps decrease cost per reaction, with estimates comparable to qPCR with DNA extraction (Table 3), both of which are twice the cost of culturing. For replication, one can either filter sample three times and place each filter into an individual reaction well (as was done for experiments in this study), or divide the filter by cutting and placing into different reaction wells. Overall, the new chip serves as a more sensitive alternative to the 32 and 64 well Gene-Z chip described previously (Stedtfeld et al., 2015) when direct filtration amplification is performed. Although throughput is traded for sensitivity, this may not be drawback for some application such as field monitoring of *L. pneumophila*.

Nucleic acids amplification techniques are limited in their ability to differentiate between viable and dead bacterial cells. However, it is important to make this differentiation in the case of *L. pneumophila*. Extracellular DNA or non-viable cells can be present or persist after treatment, which can lead to an overestimation of *L. pneumophila* concentrations (Lee et al., 2011; Morio et al., 2008). To differentiate viable and dead cells, PMA has been previously described for viable-qPCR of *L. pneumophila* (Chang et al., 2010; Yáñez et al., 2011), and on filters (Slimani et al., 2012). The technique uses a light-induced cross-linking to reduce

amplification of extracellular DNA or cells with a non-intact cellular membrane. The light cross-linking step is typically performed prior to DNA extraction and purification, which is critical to remove PMA prior to amplification. Pretreatments of water samples with ethidium monoazide and PMA before extracting DNA inhibit the amplification of non-viable *L. pneumophila* cells (Delgado-Viscogliosi et al., 2009). However, our goal was to circumvent DNA extraction and purification so that the method could be used on-site. As such, the PMA technique was optimized for direct, on-filter LAMP with viability assessment. The lower limit of detection using extracted and purified genomic DNA was poorer than the cell-based amplification (27 genomic copies per reaction vs. 2–18 cfu per reaction). Such a discrepancy can be attributed to isolation and amplification of dead cells and extracellular DNA (Nocker and Camper, 2009). This seems to be the most likely explanation as supported by the amplification data with and without PMA. Without PMA, the T_t was 20 min which increased to 35 min upon the addition of PMA (Fig S2A).

In these experiments, a significant difference between heated and unheated samples was not observed at lower concentration of PMA (e.g. 30 μ M). The selected concentration of 50 μ M PMA was also described for *L. pneumophila* in previous studies (Ditommaso et al., 2014; ISO, 1998). Like our results, previous studies also observed differences in unheated samples tested with and without PMA, suggesting the presence of extracellular DNA or *L. pneumophila* cells with a compromised membrane in the culture suspension. Similar T_t values for unheated (therefore un-lysed) and heated (lysed) samples without PMA supports that for this cell concentration, heat lysis had negligible influence on amplification (Fig S2A). Thus, PMA negatively impacts *L. pneumophila* LAMP reaction and must be removed prior to amplification by flushing with water.

Testing the direct on-filter amplification with real samples showed that sample inhibition could influence T_t , and in some instances, amplification did not occur. This was surprising in that inhibition was not observed via direct amplification of groundwater samples (Stedtfeld et al., 2016). It should be noted that the real water samples were collected from sources known to have higher level of irons and perhaps other unknown discoloring constituents (Fig S1), thus a majority of tap and cooling water samples might not be expected to have this level of inhibition. Both the tested pre-treatment methods reduced the T_t , standard error of mean in the samples with a higher level of iron. Pre-treatment had a smaller influence on the inhibition observed in Tower K sample, which may be due to an elevated level of manganese or chlorine, both of which could be treated with additional levels of phosphate or sodium thiosulfate, respectively. Internal amplification controls may be required for accurate quantification of *L. pneumophila* in real samples with higher levels of discoloration.

5.0 CONCLUSION

In summary, direct amplification and PMA-based viability assessment may allow nucleic acids-based methods to be simple, rapid, and portable with lower limits of detection that are comparable to centralized lab analyses. Currently, testing charges for *L. pneumophila* is in the range of \$500 per test so four test per year for each facility may costs at least \$2,000 annually. While the actual cost of field testing may not come down substantially because of

personnel charges, using the approach presented here will help in better management of the facility because *L. pneumophila* test results will be available within 2 hr of sampling and include quantitative data with viability assessment. These on-filter amplification techniques may also be equally useful when large sample volume must be transported and processed. Results from this research will contribute to rapid, sensitive, and quantitative methods for monitoring *L. pneumophila* in cooling waters and allow viability assessment under field conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- A fieldable method is described for viable genetic detection of *Legionella* without DNA isolation.
- Following concentration, real-time amplification is performed directly on filters.
- The method was tested with propidium monoazide for live/dead differentiation.
- A sensitivity of ~1 cfu per 100 mL was obtained in 1 to 2 hr.

Fig 1.

Comparing direct LAMP with direct qPCR with *L. pneumophila* stock dilution series using 16S rRNA gene primers. **(A)** Direct LAMP of *L. pneumophila* stock dilution series using three replicate reaction per dilution. **(B)**. Direct qPCR amplification of *L. pneumophila* stock dilution series with 8 replicate reaction per dilution using only the F3/B3 primers for 16S rRNA gene as real time PCR primers. Error bars indicate standard error of all replicates per dilution. The number of positive reactions (out of 3 or 8) are shown next to each data point.

Fig 2.

On-filter direct isothermal amplification using primers for 16S rRNA and *cadA* gene simultaneously. Each sample was run in triplicate as shown in the insert inset. Two reaction wells were negative controls (right side of each image). Thus, each card had two samples in triplicate and two negative controls.

Fig 3. Optimization of PMA cross-linking for on-filter LAMP assay with viability for *L. pneumophila*. (A) Real-time amplification curves on filters used to capture 6×10^8 cfu spiked into 100 ml of water, and (B) real-time amplification curves on filters used to capture 1×10^5 cfu spiked into 100 ml of water, (C) average of three replicate reactions with 6×10^8 cfu spiked into 100 ml of water, and (D) average of three replicate reactions with 1×10^5 cfu spiked into 100 ml of water; in which heat lysed cells treated with PMA did not amplify. Amplification was not observed in lowest tested dilution (10^2 cfu per 100 ml) treated with PMA. SNR indicates signal to noise ratio. Stars indicate significant difference (p -value < 0.05) between heat-lysed (gray) and un-heated (open) markers. Bars indicates average and standard error of mean threshold time for three replicate reactions.

Fig 4.

On-filter isothermal amplification using primers targeting 16S rRNA gene of *L. pneumophila* and Gene-Z. Gray circles indicate *L. pneumophila* cfu spiked into 100 ml, filtered, and run on the Gene-Z device. Open circles indicate *L. pneumophila* cfu spiked directly into LAMP reaction, run in vials using a conventional thermal-cycler (under isothermal conditions). Lower tested dilutions and a no template control did not amplify. Error bars indicate standard error.

Table 1

Comparing *cadA* gene with *L. pneumophila* specific 16S rRNA gene LAMP assays using dilutions of gDNA tested in vials. Lower dilutions were tested but did not amplify.

Equivalent genomic copies per reaction	16S rRNA gene (Tt)	<i>cadA</i> (Tt)
273,000	22.0 ± 1.0	17.0 ± 1.7
27,300	25.3 ± 0.9	18.3 ± 0.3
2,730	30 ± 1.5	21.0 ± 1.15
273	37.6 ± 3.4	38.0 ± 10.6
27.3	49.0 ± 4.89	No amplification

Table 2

Testing direct on-filter amplification tap and cooling waters spiked with *L. pneumophila*. Iron measurements in collected samples are listed along with spiked cfu and threshold time (Tt). Two methods of sample pretreatment were tested to reduce inhibition. NT indicates not tested, and NA indicates no amplification.

Sample Name	Measured Iron (ppm)	<i>L. pneumophila</i> (cfu/100 ml)	No pre treatment Tt (min)	Pre-filtration Tt (min)	Phosphate & Pre-filtration, Tt (min)
Distilled	0.00	10 ⁵	22.3±1.2	23.2±1.4	NT
Tower I	2.26	10 ⁵	NA	45.8±3.2	NT
Tap 1	0.47	10 ⁵	33.7±3.8	28.9±0.5	NT
Tower B	1.62	10 ⁵	NA	35.2±2.8	36.3±3.2
Tap R	1.09	10 ⁵	NA	42.4±4.7	32.9±1.5
Tap 2	0.88	10 ⁵	34.4±4.3	27.7±1.9	27.7±1.2
Tower K	1.68	10 ³	NA	43.2±2.9	42.6±2.8
Tap 3	0.93	10 ³	48.1±2.2	41.5±5.0	34.7±2.7
Tower A	2.89	10 ³	56.4±5.2	31.6±1.8	33.6±0.1

Table 3

Initial and disposable costs for reagents (one reaction) for culturing, qPCR or the direct filter isothermal amplification method.

Cost	Culturing	qPCR	Direct filter isothermal amplification
Initial	\$500–\$3,000 Incubator	\$20,000 real-time cycler	\$3,000–\$8000
Culture plate	\$5 per plate	–	–
Filters	\$1	\$1	\$1
PMA for viability	–	\$1.51 (0.5 ml)	\$1.51 (0.5 ml)
DNA extraction	–	\$9.28	–
Reagents and holder	–	\$0.70 (20 µl SYBR-based)	\$9.82 (135 µl reaction)
Total Disposables	\$6	\$12.49	\$12.33
Time	2 to 5 days	2 to 5 hr	1 to 2 hr