Water Research 121 (2017) 162–170

Contents lists available at ScienceDirect

Water Research

journal homepage: www.elsevier.com/locate/watres

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

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ARTICLE INFO

Article history:
Received 17 January 2017
Received in revised form
8 May 2017
Accepted 12 May 2017
Available online 13 May 2017

Keywords:
Legionella pneumophila
Direct on-filter amplification
Gene-Z
Loop mediated isothermal amplification
Water
Sample processing
Rapid detection
Viable cells
Propidium monoazide

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 4–10 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 1–2 h 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.

1. 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 (*Lp*1) 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 Legionaires' 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 h of getting a positive culture sample result exceeding 1000 colony-forming units (cfu) per milliliter." As a result, more than 8660 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

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http://dx.doi.org/10.1016/j.watres.2017.05.028
0043-1354/

part of the more than 53,554 hotels, 20,000 spa facilities, 15,401 long-term care facilities, and 5627 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; Vanysacker et al., 2014) are the two main techniques adapted for quantification. Culturing will assess viability but it is slow (4–10 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. 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 1× 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, 1× isothermal amplification buffer (New England Biolabs), 1.4 mM each dNTP (Invitrogen), 0.8 M Betaine solution (Sigma Aldrich), 6 mM $MgSO_4$ (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 Cd^{2+} 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 4–400,000 cfu/ml) and LAMP primers for the 16S rRNA gene. The 10 μl reaction mixture contained the same regent 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 1.84×10^7 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 10^7 cfu/100 ml. The spiked samples were then filtered using a 140-ml syringe (Medtronic