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REVIEW ARTICLE

Legionella detection by culture and qPCR: Comparing apples and oranges

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Abstract

Legionella spp. are the causative agent of Legionnaire's disease and an opportunistic pathogen of significant public health concern. Identification and quantification from environmental sources is crucial for identifying outbreak origins and providing sufficient information for risk assessment and disease prevention. Currently there are a range of methods for *Legionella* spp. quantification from environmental sources, but the two most widely used and accepted are culture and real-time polymerase chain reaction (qPCR). This paper provides a review of these two methods and outlines their advantages and limitations. Studies from the last 10 years which have concurrently used culture and qPCR to quantify *Legionella* spp. from environmental sources have been compiled. 26/28 studies detected *Legionella* at a higher rate using qPCR compared to culture, whilst only one study detected equivalent levels of *Legionella* spp. using both qPCR and culture. Aggregating the environmental samples from all 28 studies, 2856/3967 (72%) tested positive for the presence of *Legionella* spp. using qPCR and 1331/3967 (34%) using culture. The lack of correlation between methods highlights the need to develop an acceptable standardized method for quantification that is sufficient for risk assessment and management of this human pathogen.

Keywords

Culture, environmental samples, *L. pneumophila*, *Legionella* spp., PCR, public health, qPCR, risk assessment

History

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Introduction

Legionella is a genus of opportunistic pathogens of significant public health concern (Borges et al., 2012). It is the causative agent of Legionellosis, which collectively refers to Legionnaires disease and Pontiac fever (Fields et al., 2002). Legionnaires disease is a serious atypical bacterial pneumonia; whereas Pontiac fever is a self-limiting febrile illness (Neil & Berkelman, 2008). There have been no reports of human to human transmission of *Legionella* spp. (Khweek et al., 2013) and inhalation or aspiration of contaminated aerosols is the most commonly accepted mechanism of infection (Cianciotto, 2001). Community and nosocomial cases of Legionellosis are typically associated with cooling towers (Nhu Nguyen et al., 2006), hot water systems (Goetz et al., 1998; Leoni et al., 2005), potable water (Stout, 1992), spa pools (Benkel et al., 2000), decorative water fountains (Haupt et al., 2012) and potting mix (O'Connor et al., 2007).

Legionella spp. are difficult to control in environmental sources due to their resistance to disinfectants (Kim et al., 2002), association with biofilms (Murga et al., 2001) and parasitism of protozoan hosts (Thomas et al., 2004). Environmental surveillance and monitoring of *Legionella* spp. is crucial for evaluating risk and identifying control strategies (Cristino et al., 2012). This requires a quick and accurate method for detecting and enumerating *Legionella* spp. in environmental sources (Declerck et al., 2006).

Currently there are several methods for *Legionella* spp. detection and enumeration including: culture (Bopp et al., 1981); PCR (Mahbubani et al., 1990); qPCR (Behets et al., 2007); Fluorescent *in situ* hybridization (FISH) (Deloge Abarkan et al., 2007); solid phase cytometry (Aurell et al., 2004); optical wavelight spectroscopy (Cooper et al., 2009); Enzyme-Amplified Electrochemical Detection with DNA probe (Miranda-Castro et al., 2007) and Surface plasmon resonance immunosensor (Oh et al., 2003). Many national bodies have adopted guidelines which use culture methods as the standard; however, the development of more rapid techniques highlights the need for reassessment of guidelines. This review will compare culture and qPCR, which are the two methods which have gained prominence as the most widely used and accepted by analytical labs (Krøjgaard et al., 2011; Lee et al., 2011).

Detection of *Legionella* by culture methods

Due to the microbial complexity of environmental samples, isolating *Legionella* spp. by culture methods presents a range of challenges, which have been addressed by the development

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of specific agar formulations and sample treatments (Bopp et al., 1981). Sample collection protocols, sampling location and storage will not be specifically addressed in this manuscript; however, in its own right the sampling method used may exert a significant impact upon the likelihood of detection *Legionella* spp. in the environment (Asadi et al., 2011; CDC, 2005).

The requirement for sample treatment is generally contingent on the properties of the environmental source. Potable water and water from hot water reservoirs often requires less destructive sampling techniques than samples collected from cooling towers and potting mix as they are generally less microbially complex (Joly et al., 2006; Steele et al., 1990). For potable water, it may be sufficient to simply filter concentrated 1 L of water to ensure that sufficient microbial flora is present before plating (Fiume et al., 2005). Samples collected from chlorine treated water sources should be dosed with 0.5 ml of 0.1 N sodium thiosulfate per 1 L to neutralize residual disinfectants (CDC, 2005).

Samples from cooling towers, potting mix, reuse water distribution systems and non-potable sources generally require either heat or acid treatment to reduce the microbial content of the sample before plating (Bopp et al., 1981). As *Legionella* is thermally tolerant up to 63 °C (Fliermans et al., 1981) samples may be heat treated to reduce the content of competitive bacteria and fungi in a sample. Commonly this treatment comprises 30-min exposure to 50 °C (Leoni & Legnani 2001; Roberts et al., 1987). However, increasing exposure time or temperature further may reduce *Legionella* cultivability, particularly at temperatures above 60 °C (Rogers et al., 1994).

More commonly, water samples are acid treated using an adaptation of methods developed by Bopp et al. (1981). In this procedure water samples are either filter concentrated, or centrifuged and resuspended to produce a concentrate of the original sample. This concentrate is then diluted in a HCl–KCl buffer of pH 2.2 and incubated for ∼15 min before plating. To further reduce interfering microbial growth, plates may be incubated under a microaerophillic in a candle jar or under a 2.5% CO_2 atmosphere at 35 °C (CDC, 2005).

For laboratory culture, *Legionella* spp. requires relatively complex culture media in order to multiply. This requires specific additions to standard nutrient media, including L-cycseine, arginine, isoleucine, leucine, threonine, valine, methionine, phenylalanine, tyrosine and serine (Pine et al., 1979). The addition of trace elements iron, calcium, cobalt, copper, magnesium, manganese, molybdenum, nickel, vanadium and zinc has also been shown to stimulate the growth of *Legionella* species in culture (Reeves et al., 1981; Warren & Miller, 1979).

Several agar formulations exist, with slight differences in selectivity and growth characteristics for different *Legionella* species. Buffered charcoal yeast extract agar (BCYE) is the most commonly used for general growth and maintenance of *Legionella* spp. and contains 0.1% α-ketoglutarate and a range of supportive amino acids and micro-nutrients (CDC 2005; Feeley et al.; 1979; Pendland et al., 1997; Roberts et al., 1987; Ta et al., 1995). A modification of this agar containing 1% albumin (ABCYE) has been shown to slightly enhance the recovery and growth of *L. micdadei* and *L. bozemanii* (Morrill et al., 1990).

A range of antimicrobial compounds may be added to the BCYE agar base, which are designed to reduce the growth of competing bacteria and fungi without altering the growth of *Legionella* spp. This range of compounds incorporates vancomycin, polymyxin B and cycloheximide and will often include glycine to reduce the growth of glycine-sensitive Gram negative bacteria (Wadowsky & Yee, 1981). Treated environmental samples may also be plated onto selective media deficient in L-cysteine to serve as a negative control for *Legionella* spp. growth.

The most significant barriers to quantitative, reproducible enumeration of *Legionella* spp. using culture arises from two distinct problems; the growth of unwanted microorganisms which obscure identification (Bopp et al., 1981) and the presence of viable but non-culturable (VBNC) *Legionella* spp. (Shih & Lin 2006). *Legionella* which have replicated intracellularly (within marcophagic hosts) are morphologically distinct from other *Legionella* cells (Al-Bana et al., 2013). These cells have thickened outer membranes, greater resistance to environmental and chemical stresses, lower metabolic rates and readily enter a VBNC state in water. Hence, the number of *Legionella* cells detected using culture immediately post-parasitization of amoebic hosts may be distinctly lower than *Legionella* in different life-cycle stages. Chang et al. (2007) also demonstrated that *L. pneumophila* became VBNC after starvation in nutrient-free water for 33–40 days. They also demonstrated that heat disinfection at temperatures 60 °C or higher for between 5 and 30 min caused *L. pneumophila* to become completely unculturable but a large number of cells remained viable as determined by LIVE/DEAD BacLight bacterial viability kit (Molecular Probe, Eugene, OR). Also the longer a cell had undergone starvation the greater resistance it had to chlorine disinfection and heat treatment. A similar study by Alleron et al. (2008) demonstrated that *L. pneumophila* treated with 1–10 mg/L of monochloramine became unculturable on BCYE; however, 28.8–29.4% of cells were viable as determined by the LIVE/DEAD BacLight kit. *Legionella* recovery from environmental sources has been shown to be enhanced by passage through amoebic hosts (La Scola et al., 2001; Rowbotham, 1983). It has also been demonstrated that non-cultureable *L. pneumophila* can be resuscitated by co-culture with *Acanthamoeba polyphaga* (García et al., 2007) and *Acanthamoeba castellanii* (Steinert et al., 1997). However, this adds further time to the isolation process and at best only allows for a qualitative presence/absence assessment of *Legionella*'s presence in a sample. Sample holding time also exerts a significant impact upon *Legionella* recovery by culture, with enumerated *Legionella* changing by up to 50% within 6 h and up to 2 log difference after 24 h (McCoy et al., 2012).

If culturable *Legionella* is present in its slow growth rate often leads to plate overgrowth by competing organisms with more rapid generation times (Alary & Joly, 1992; Bopp et al., 1981; Steele, 1990). Plates often require at least 5–7 days before *Legionella* colonies become visible, at which point the density of competing organisms often either renders enumeration infeasible or completely obscures the surface of culture plates (Bopp et al., 1981, Leoni & Legnani, 2001). Once *Legionella* colonies are visible, their positive identification is often primarily visual and/or confirmed by latex agglutination

Table 1. Comparison of published studies from 2003 to 2013 using qPCR and culture enumeration of *Legionella* spp. from environmental samples.

Source	Detection method		Comments	Reference
	qPCR	Culture		
United Kingdom				
100 environmental samples (68 from various hospitals, 32 from private domestic water supplies and 4 from external sites).	4/100 (4%) were positive for *L. pneumophila.*	14/100 (70%) were positive for *L. pneumophila.*	All 10 samples positive for *L. pneumophila* by culture and not qPCR contained ≤200CFU/L. Also one culture negative sample was repeatedly qPCR-positive.	Levi et al. (2003)
20 hospital and environmental water samples known to be *L. pneumophila* culture-positive.	14/20 (70%) were positive for *L. pneumophila.*	All previously tested positive for *L. pneumohila* (however, this was not done concurrently).		
USA and Canada				
114 Water and biofilm samples from both warm and colder groundwater.		29/87 (33.3%) were positive for *Legionella* spp.	40/87 (46.0%) were positive for *Legionella* spp. using PCR. 61/87 (70.6%) samples were observed to contain PCR inhibitors.	Brooks et al. (2004)
Belgium				
46 water samples (25 shower water, 12 industrial water, 4 natural water, 5 tap water).		19/46 (41%) were positive for *Legionella* spp.	26/46 (56%) and 45/46 (98%) of samples were positive for *Legionella* spp. using PCR with LEG 225-LEG 858 and JFP-JRP primer, respectively.	Devos et al. (2005)
Italy				
124 tap water samples from hospitals and private residence.	111/124 (90%) were positive for *Legionella* spp. using TaqMan qPCR method.	77/124 (62%) were positive for *Legionella* spp.	88/124 (71%) were positive for *Legionella* spp. using nested PCR method.	Fiume et al. (2005)
Spain				
Water samples 40 water samples (spas, hotels, hospitals, residential tap water.)	17/40 (43%) were positive for *Legionella* spp. using qPCR. 15/40 (38%) were positive for *Legionella* spp. using PMA-qPCR.	12/40 (30%) were positive for *Legionella* spp.	qPCR and PMA-qPCR (propidium monoazide pre-treatment) compared.	Yáñez et al. (2005)
France				
Hot water system 128 samples tested in laboratory 1.	117/128 (91.4%) were positive for *Legionella* spp. and of these 89/128 (69.5%) were quantifiable. 94/122 (77%) were positive for *L.pneumophila* and 55/122 (45.1%) were quantifiable.	55/128 (43%) were positive for *Legionella* spp. and 27/128 (21.1%) of these had ≥250 CFU/L		Joly et al. (2006)
92 samples tested in laboratory 2.	76/92 (82.6%) were positive for *Legionella* spp. and of these 56/92 (60.9%) were quantifiable. 57/91 (62.6%) were positive for *L. pneumophila* and of these 31/91 (34.1%) were quantifiable.	41/92 (44.6%) were positive for *Legionella* spp. and 24/92 (26.1%) of these had ≥250 CFU/L		
36 Cooling tower samples.	36/36 (100%) were positive for *Legionella* spp. and 35/36 (97.2%) of these were quantifiable. 31/33 (93.9%) were positive for *L. pneumophila* and 19/33 (57.6%) were quantifiable.	9/36 were positive for *Legionella* spp. (25%) and of these 8/36 had ≥250 CFU/L (22.2%)		

(*continued*)

Table 1. Continued

Source	Detection method: qPCR	Detection method: Culture	Comments	Reference
The Netherlands				
68 commercial bottled mineral waters.		0/68 were positive for *L. pneumophila*	6/68 (9%) were positive for *L. pneumophila* using PCR.	Klont et al. (2006)
The Netherlands				
16 surface water samples,	14/16 (86%) were positive for *Legionella* spp.	0/16 were positive for *Legionella* spp.		Wullings & van der Kooij (2006)
97 treated surface and ground water samples.	97/97 (100%) were positive for *Legionella* spp.	0/97 were positive for *Legionella* spp.		
Belgium				
30 tap water samples (showers taps, eyewash stations, fire sprinklers and recirculation loops).	14/30 (46.7%) were positive for *L. pneumophila* at concentrations ranging from 4.4×10^3 to 3.1×10^5 GU/L.	10/30 (33%) were positive for *L. pneumophila* at concentrations ranging from 4.0×10^2 to 9.0×10^3 CFU/L.	12/30 (40%) contained qPCR inhibitors.	Behets et al. (2007)
Brazil				
River water.		*Legionella* spp. was not identified by culture.	24 sequences were identified as more closely related to *Legionella* spp. than any other genera. Some colonies present on *Legionella* selective medium had the typical ''ground glass'' appearance of *Legionella* but the results of the latex test kit and fluorescent antibody assay were negative for *Legionella* spp.	Carvalho et al. (2007)
The Netherlands				
357 water samples from 250 public buildings.	311/357 (87.1%) were positive for *Legionella* spp.	8/357 (2.2%) were positive for *Legionella* spp.		Diederen et al. (2007)
Spain				
25 potable water.	21/25 (84%) were positive for *Legionella* spp.	13/25 (52%) were positive for *Legionella* spp.	19/25 (76%) of potable water samples and 30/30 (100%) cooling tower samples were positive for *Legionella* spp using PCR.	Yáñez et al. (2007)
30 cooling tower samples.	28/30 (93%) positive for *Legionella* spp.	22/30 (73%) positive for *Legionella* spp.		
France				
136 hot water system samples collected form 55 sites.	87/132 (65.9%) *L. pneumophila* positive and of these 50/132 (37.9%) were quantifiable (>25 GU/reaction).	59/132 (44.7%) were positive for *L. pneumophila* and of these 40/132 (30.3%) were quantifiable (>250 CFU/L).	4 hot water system samples and 3 cooling tower samples contained inhibitors which would not be removed using dilution and were not used for the study.	Yaradou et al. (2007)
46 cooling tower water samples collected from 20 sites.	28/46 (60.9%) *L. pneumophila* positive and of these 20/46 (43.5%) were quantifiable (>25 GU/reaction).	15/46 (32.6%) were positive for *L.pneumophila* and of these 9/46 (19.6%) were quantifiable (>250 CFU/L)		
Japan				
130 Hot water samples from 40 public buildings (hotels, offices, schools, stores, assembly halls).	24/130 (18%) were positive for *Legionella* spp. In 21 of these samples concentrations of *Legionella* ranged from 1.7×10^5 to 2.6×10^{11} GU/L	5/130 (4%) were positive for *Legionella* spp. Concentration of *Legionella* ranged from 1.8×10^2 to 8.3×10^3 CFU/L	17 of the 40 sites were positive by qPCR	Edagawa et al. (2008)

Kuwait				
263 swabs from hospital faucets and showerheads. 20 water samples from hospital water storage facility.		61/263 (23%) were positive for *Legionella* spp. 0/20 (0%) were positive for *Legionella* spp.	61/263 (23%) were positive for *Legionella* spp. using PCR. 6/20 (30%) were positive for *Legionella* spp. using PCR.	Qasem et al. (2008)
France				
120 Hot water samples collected a hospital (including hot water tanks, showers and taps).	57/120 (47.5%) were positive for *L. pneumophila* (only 35/120 (29.1%) were quantifiable).	31/120 (25.8%) were positive for *L. pneumophila*	Samples taken from six different distribution systems within the hospital.	Morio et al. (2008)
USA				
276 environmental water samples.	138/276 (50%) tested positive for *L. pneumophila*	99/276 (35.8%) tested positive for *L. pneumophila*	97/140 (69.3%) positive samples tested both culture and qPCR positive. 41/140 (29.3%) tested qPCR-positve only. 2/140 (1.4%) tested culture-positive only.	Nazarian et al. (2008)
Switzerland				
46 brands of potting mix.	41/46 (89.1%) tested positive for *Legionella* spp.	21/46 (45.7%) tested positive for *Legionella* spp.		Casati et al. (2009)
Japan				
25 water samples (9 public spas and 16 from model spa systems).	16/25 (64%) tested positive for *Legionella* spp. 10/25 (40%) tested positive for *Legionella* spp after pre-treatment with 20 μg/ml of Ethidium monoazide (EMA).	14/25 (56%) were positive for *Legionella* spp.	Results with pre-treatment of 1, 5 and 10 μg/ml of Ethidium monoazide (EMA) are also presented.	Chang et al. (2009)
Taiwan				
34 samples from 13 spring resorts (source water, facility water and waste water).	5/34 (15%) tested positive for *Legionella* spp.	5/34 (15%) tested positive for *Legionella* spp.	No samples test both qPCR and culture positive. Therefore a total of 10/34 samples were positive by any method.	Hsu et al. (2009)
Italian				
76 water samples cold water tap, boiler room, shoers, hot water recycling) from 19 hotels.	56/76 (74%) were positive for *Legionella* spp. 37/76 (49%) were positive for *L. pneumophila.*	32/76 (42%) were positive for *Legionella* spp. 19/76 (25%) were positive for *L. pneumophila.*		Bonetta et al. (2010)
Spain				
20 cooling tower water samples.	10/20 (50%) were positive for *L. pneumophila* using *mip* Primers	8/20 (40%) were positive for *L. pneumophila*		
30 hot water system samples.	9/20 (45%) were positive for *L. pneumophila* using *dot* primers 19/30 63%) were positive for *L. pneumophila* using *mip* Primers 20/30 (67%) were positive for *L. pneumophila* using *dot* primers	14/30 (46.7%) were positive for *L. pneumophila*		Fittipaldi et al. (2010)
Canada				
101 samples from 95 whirlpool spas in semi-public establishments.	72/101 (72%) were positive for *Legionella* spp. 42/101 (42%) could be enumerated with concentrations ranging from 1000 (limit of detection) to 6.1×10^7 GU/L.	27/101 (27%) were positive for *Legionella* spp 14/101 (14%) could be enumerated with concentrations ranging from 250 to 3.5×10^5 CFU/L.		Guillemet et al. (2010)
France				
Water samples from the Tech River.	72/72 (100%) were positive for *Legionella* spp.	15/72 (20.8%) were positive for *Legionella* spp.	qPCR inhibitors were present in all non-diluted DNA extracts.	Parthuisot et al. (2010)

(*continued*)

Table 1. Continued

Source	Detection method: qPCR	Detection method: Culture	Comments	Reference
Indonesia				
9 cooling tower water samples (from 9 cooling towers).	7/9 (78%) were positive for *Legionella* spp.	0/9 were positive for *Legionella* spp.		Yasmon et al. (2010)
China				
51 samples from recreation spring facilities (source water, waste water and facility water).		3/51 (5.9%) were positive for *Legionella* spp.	5/51 (10%) were positive for *Legionella* spp. using PCR. 11/51 (22%) were positive using free living amoeba (FLA) culture method.	Huang et al. (2011a)
Taiwan				
47 Carbonate springs.		7/47 (15%) were positive for *Legionella* spp.	10/47 (21%) were positive for *Legionella* spp.	Huang et al. (2011b)
17 Mud springs.		0/17 were positive for *Legionella* spp.	5/17 (29%) were positive for *Legionella* spp.	
4 Sodium bicarbonate spring.		1/4 (25%) were positive for *Legionella* spp.	1/4 (25%) were positive for *Legionella* spp. Using PCR method.	
Denmark				
84 Hot water samples.	84/84 (100%) were positive for *Legionella* spp. 75/84 (89%) were positive for *L. pneumophila.*	43/84 (51%) were positive for *Legionella* spp.		Krøjgaard et al. (2011)
6 European Countries				
232 cooling tower water samples.	221/232 (95%) were positive for *Legionella* spp. and 114/232 (49%) were positive for *L. pneumophila.*	73/232 (31%) were positive for *Legionella* spp. and 62/232 (27%) were positive for *L. pneumophila.*	7 laboratories from 6 countries participated in this study. This study also discussed the potential of qPCR results predicting culture results.	Lee et al. (2011)
506 hot and cold water samples.	495/506 (98%) were positive for *Legionella* spp. and 417/506 (82%) were positive for *L. pneumophila.*	278/506 (55%) were positive for *Legionella* spp. and 249/506 (49%) were positive for *L. pneumophila.*		
France				
185 water samples from 9 cooling water systems at 4 different sites.	181/185 (98%) were positive for *Legionella* spp. and 111/185 (60%) were positive for *L. pneumophila.*	102/165 (62%) were positive for *Legionella* spp. and 101/164 (63%) were positive for *L. pneumophila*		Touron-Bodilis et al. (2011)
Spain				
25 samples from hotel potable hot water systems.	15/25 (60%) were positive for *Legionella* spp.	13/25 (52%) were positive for *Legionella* spp.	Regression analysis showed that average concentration of *Legionella* spp determined by qPCR were 20-fold higher that the culture determined concentrations.	Yáñez et al. (2011)
35 cooling tower water samples.	28/35 (80%) were positive for *Legionella* spp.	22/35 (63%) were positive for *Legionella* spp.		
The Netherlands				
Two un-chlorinated drinking water supplies (water and Biofilm samples).	*Legionella* spp detected at ranges of 7.6×10^1 to 3.9×10^2 GU/L.	No *Legionella* spp. were detected from culture in any of the water or biofilm samples.		Wullings et al. (2011)
Kuwait				
Domestic water samples.	45/82 (55%) were positive for *Legionella* spp.	27/82 (33%) were positive for *Legionella* spp.		Al-Matawah et al. (2012)
82 samples from bathroom faucets and showerheads.	19/51 (37%) were positive for *Legionella* spp.	15/51 (29%) were positive for *Legionella* spp.		

51 from kitchen taps. 71 from hot/cold water tanks.	21/71 (30%) were positive for *Legionella* spp.	6/71 (8.5%) were positive for *Legionella* spp.		
China				
216 cooling tower water.	67/216 (31%) were positive for *Legionella* spp.	57/216 (26%) were positive for *Legionella* spp.	43/216 (20%) cooling tower samples, 27/132 (20%) piped water and 64/90 (71%) of hot spring samples were positive for *Legionella* spp. using PCR.	Qin et al. (2012)
132 piped water samples.	32/132 (24%) were positive for *Legionella* spp.	25/132 (19%) were positive for *Legionella* spp.		
90 hot spring water samples.	84/90 (93%) were positive for *Legionella* spp.	49/90 (93%) were positive for *Legionella* spp.		
USA				
Chloraminated potable water distribution systems.	27/90 (30%) of water samples were positive for *Legionella* spp. at average concentration of 186.6 ± 458.2 GU/L and 4/90 (4%) were positive for *L. pneumophila* at average concentrations of 9.8 ± 4.4 GU/mL.	1/56 (2%) were positive for *Legionella* spp. at density of 2 CFU/mL.		Wang et al. (2012)
Taiwan				
Puzih River water samples.	59/150 (39%) were positive for *Legionella* spp. and 3/150 (2%) were positive for *L. pneumophila.*	14/150 (9%) were positive for *Legionella* spp. and 14/150 (9%) were positive for *L. pneumophila.*		Tung et al. (2013)

(Sathapatayavongs et al., 1983). However, these methods in their own right present limitations, as noted by Carvalho et al. (2007) who demonstrated that from 20 colonies presenting the characteristic *Legionella* ''ground glass'' appearance on BYCE GVPC agar, all 20 were negative when tested using latex agglutination and fluorescent antibody assays but were confirmed to be *Legionella* when sequencing of 16S rDNA was carried out.

As *Legionella* is a pathogen of public health concern, the consequences of reporting false negatives or underreporting the concentration of *Legionella* may be serious. The potential harm caused by the failure to detect and treat systems containing *Legionella* may be ultimately deemed greater than the cost of presumptively treating/cleaning systems where results are equivocal. As culture results tend to underestimate the presence of *Legionella* in water systems, it may be better suited to use culture as an adjunct to molecular detection rather than an alternative.

qPCR enumeration

qPCR is an alternative method for rapid *Legionella* spp. enumeration from environmental samples (Joly et al., 2006). It simultaneously amplifies and quantifies a target DNA sequence (Templeton et al., 2003), giving the number of genome units (GU) per liter. An equivalence with the number of CFU has not been established and the results obtained are highly dependent upon the method used and the sample composition (Wellinghausen et al., 2001).

The rapid turn-around time and sensitivity of qPCR is advantageous when compared to traditional culture methods (Yaradou et al., 2007). The main limitation is a tendency to overestimate due to the amplification of non-viable or ''dead'' cells (Delgado-Viscogliosi et al., 2009). DNA within environmental samples can be very stable and may persist for extended lengths of time (Nocker et al., 2007). Josephson et al. (1993) demonstrated that in biofilm DNA from non-viable cells persisted from days to weeks depending on the microbial consortium present. Some studies have shown that pre-treatments with ethidium monoazide (EMA) and propium monoazide (PMA) prior to DNA extractions enable amplification of viable cells only (Chang et al., 2009; Chen & Chang 2010; Delgado-Viscogliosi et al., 2009; Qin et al., 2012). When exposed to light, EMA and PMA bind to DNA that is not protected by a cell membrane and prevents its amplification, and hence enumeration by qPCR. These methods have not been optimized for differing sample types and their reliability and accuracy of these results is still debated (Hein et al., 2006). Pisz et al. (2007) demonstrated that EMA was not effective in preventing the amplification of non-viable cells within biofilm samples, and suggested that the presence of extracellular polymeric substances could interfere with either the DNA binding or the photo-activation of EMA. Other studies have shown EMA to penetrate intact cells, with the extent of EMA-uptake-dependant on bacterial species and EMA concentration (Flekna et al., 2007; Kobayashi et al., 2009). Conversely, if the concentration of EMA or PMA is too low, insufficient free DNA will be bound resulting in further confounding unknowns (Fittipaldi et al., 2011).

Another difficultly with qPCR is the presence of environmental compounds inhibiting the qPCR reaction (Brooks et al., 2004). However, conducting 1:10 dilutions of DNA extracts has been shown to be effective at reducing inhibitors and enabling quantification of target DNA (Ballard et al., 2000). There are also a range of commercially available kits which contain components that may aid in the removal of qPCR inhibitors from DNA extracted from target samples (Wilson, 1997).

Comparison of current literature

Publications from the last 10 years which concurrently used culture and qPCR to detect *Legionella* spp. from environmental samples are collated in Table 1. Papers published in English and between the years 2003 and 2013 were included. Clinical and *in situ* experiments, including artificially spiked environmental samples were not included in this review.

Some studies which compared PCR (not qPCR) and culture are included in Table 1 if they provide a particular point of interest; however, for the basis of comparison the authors will only use the results obtained from the 28 studies which specifically enumerated *Legionella* using both culture and qPCR concurrently.

When the results of these studies are aggregated it becomes apparent that culture is more likely to underreport the presence of *Legionella* in water samples, with only 1/28 studies reporting higher detectable *Legionella* using culture and qPCR. In contrast, 25/28 studies reported higher detectable levels of *Legionella* using qPCR and one study reported equivalent results using both methods. On a sample per sample basis, samples analyzed concurrently by qPCR and culture were approximately 50% more likely to return a positive result by qPCR, with 2856/39 673 (72%) of all samples positive by qPCR and 1331/3967 (34%) of samples positive by culture.

The study by Levi et al. (2003), which reported higher detectable *Legionella* using culture compared to qPCR could be explained by the high limit of detection (800CFU/L) of the qPCR method used in this study. Four of the studies noted the presence of qPCR inhibitors (Behets et al., 2007; Brooks et al., 2004; Parthuisot et al., 2010; Yaradou et al., 2007), which either could not be removed or required additional sample processing to allow for qPCR detection. Whilst one study noted complete disparity between results, reporting five samples as positive by culture, five samples positive by qPCR, but no samples returning a positive result by both culture and qPCR (Hsu et al., 2009).

Conclusion

Legionella spp. continues to exist as a public health concern; an ongoing risk assessment focus and an obstacle for cooling tower operators and facility managers. The discrepancies between testing procedures highlights the requirement for adopting a standard method for *Legionella* spp. detection in environmental samples. This review identifies the numerous inconsistencies between culture and qPCR enumeration, with studies from the last decade reporting a 50% difference between methods. International consensus is required to develop a universality accepted testing protocol to ensure consistency of results for both research purposes and risk assessment and management legislation.

Declarations of interest

The authors report no declarations of interest.

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