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Viability qPCR, a new tool for *Legionella* risk management

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ABSTRACT

Background: Viability quantitative Polymerase Chain Reaction (v-qPCR) is a recent analytical approach for only detecting live microorganisms by DNA amplification-based methods This approach is based on the use of a reagent that irreversibly fixes dead cells DNA. In this study, we evaluate the utility of v-qPCR versus culture method for Legionellosis risk management.

Methods: The present study was performed using 116 real samples. Water samples were simultaneously analysed by culture, v-qPCR and qPCR methods. Results were compared by means of a non-parametric test.

Results: In 11.6% of samples using both methods (culture method and v-qPCR) results were positive, in 50.0% of samples both methods gave rise to negative results. As expected, equivalence between methods was not observed in all cases, as in 32.1% of samples positive results were obtained by v-qPCR and all of them gave rise to negative results by culture. Only in 6.3% of samples, with very low *Legionella* levels, was culture positive and v-qPCR negative. In 3.5% of samples, overgrowth of other bacteria did not allow performing the culture. When comparing both methods, significant differences between culture and v-qPCR were in the samples belonging to the cooling towers-evaporative condensers group. The v-qPCR method detected greater presence and obtained higher concentrations of Legionella spp. ($p < 0.001$). Otherwise, no significant differences between methods were found in the rest of the groups.

Conclusions: The v-qPCR method can be used as a quick tool to evaluate Legionellosis risk, especially in cooling towers-evaporative condensers, where this technique can detect higher levels than culture. The combined interpretation of PCR results along with the ratio of live cells is proposed as a tool for understanding the sample context and estimating the Legionellosis risk potential according to 4 levels of hierarchy.

1. Introduction

The genus *Legionella* comprises at least 61 species (tp://www.bacterio.net/legionella.html), 22 of them being associated to human disease (Winn, 2015). The most common pathogenic species is *L. pneumophila* serogroup 1 and is responsible for up to 80% of Legionellosis cases (Stout and Yu, 1997).

In nature, the genus *Legionella* can be considered ubiquitous in continental water environments and in man-made ecosystems; also can exist in appropriate environmental conditions that allow its proliferation. Respiratory infection by *Legionella pneumophila* (LP) is mainly attributed to inhalation of contaminated water aerosols produced by such systems and the aspiration of contaminated water has also been proposed as a possible mechanism of transmission (Yu, 1993).

Epidemiological data shows that Legionellosis outbreaks are related to cooling towers or hot water systems in big buildings as hotels and hospitals (Heymann, 2004). Nevertheless other man-made sources are related with infection, such as nebulizers (Woo et al., 1992), humidifiers (Endo and Ito, 2009), ornamental fountains (O'Loughlin et al., 2007), whirlpool spas (Benkel et al., 2000), water-birth baths (Franzin et al., 2004), ice-making machines (Yu and Stout, 2010), and domestic water blasters (Simmons et al., 2008). In addition, its presence is well known in dental units (Singh and Coogan, 2005) and also in rainwater on roads (Sakamoto et al., 2009). Therefore, virtually all man-made ecosystems containing water and with a capacity to disseminate may be related with Legionellosis risk.

As a general rule, for preventing *Legionella* transmission or minimizing water system colonization, total heterotrophic counts are only available as process indicators. Likewise, temperature and disinfectant level are key factors for control. From the practical management of water systems, a microbial indicator or model organisms correlated with the occurrence of *Legionella* do not exist. For this reason, in order

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to measure the risk of Legionellosis, it is necessary to perform direct analysis, by culture, which requires 10 days to confirm its presence or absence (ISO 11731, 1998ISO 11731, 1998).

Hence, much attention has been paid to alternative methods to culture such as quantitative PCR (qPCR) and immunomagnetic separation (IMS) (Albalat et al., 2014). In both cases, commercial solutions are now available with external validation that demonstrate their potential. However, the present challenge is to find a good correlation among the information generated by non-culture based methods and the current management criteria based on culture results (Díaz-Flores et al., 2015).

At this stage, the main weakness of qPCR methods is their lack of specificity for distinguishing between dead and live cells. The development of viability qPCR (v-qPCR) procedures that only detect DNA from live cells, with 'live' meaning with intact cell membrane, are changing the scenario (Delgado-Viscogliosi et al., 2009; Ditommaso et al., 2015).

By means of v-qPCR, today it is possible to detect the levels of live cells quickly, obtaining a direct measure of pathogen levels in a few hours. Although in *in vitro* experiments it is possible to obtain very good correlations between culture and v-qPCR methods, the practical experience dictates the use of additional strategies in order to control the bias for minimizing false positive results (Fittipaldi et al., 2011; Agustí et al., 2017).

According to a previously published approach (Fittipaldi et al., 2011), together with a newly optimized v-qPCR workflow (Agustí et al., 2017), herein we propose the use of absolute quantification results along with the ratio of live cells as tools for understanding the sample context and estimating Legionellosis risk.

In this work, various water samples from different sources have been simultaneously analysed by standard culture procedures and v-qPCR, with the aim of evaluating the potential of v-qPCR as a tool for managing the prevention and control of Legionellosis.

2. Methods

2.1. Materials and methods

2.1.1. Collection of water samples

During an 18-month period, a total of 116 water samples from different sampling points and buildings were analysed by Aconsa-Lab (Barcelona, Spain). Samples were categorized into 4 groups: 26 cooling towers and 12 evaporative condensers (n = 46 samples), 12 hot water systems (n = 19 samples), 12 cold water systems and 10 nebulizers (n = 29 samples), and others, 13 mainly fire-fighting water systems (tank reservoirs) and 9 swimming pools (n = 22 samples).

Throughout this study 22 cooling towers were sampled once, three were sampled twice, and one was sampled three times. Regarding the evaporative condensers nine were sampled once and three were sampled twice. All the other systems were sampled once although there was more than one sampling point in some of them.

Water samples of 1L were collected aseptically in sterile plastic containers containing sodium thiosulphate (10% w/v) and were transported at temperatures between 6 and 18 °C and shielded from the exposure to light. The transport preferably took less than 24 h but never more than 48 h. Samples were received by the laboratory and stored at 3–7 °C until needed, always within 24–48 h after sampling.

2.1.2. Quantification of Legionella using culture method

Culture-based assays for detecting and quantifying *Legionella* were conducted according to the reference culture method (ISO 11731, 1998ISO 11731, 1998) at an ISO 17025 accredited laboratory (cert. number: ENAC,962/LE1805). Briefly, 1 L of water was filtered (0.45 µm pore-size Nylon filter, Pall Corporation, NY, USA) and resuspended in 5 mL of Ringer 1/40 by vortexing for 10 min. Two 1 mL aliquots were heat-treated (50 °C for 30 min) or acid-treated (HCl-ClK buffer according to ISO 11731, pH 2.2 for 5 min). Aliquots of 100 µl of untreated, heat- and acid-treated specimens were plated onto selective agar for *Legionella*, Glycine-Vancomycin-Polymyxin and Cycloheximide agar (GVPC) (Reactivos para Diagnóstico, S.L., Barcelona, Spain). The plates were incubated at 36 ± 2 °C for 10 days and read from day 4 with a stereo microscope. Presumed *Legionella* colonies were subcultured on Buffered Charcoal Yeast Extract media (with cysteine) (BCYE) and Nutrient Agar (cysteine-free) media (Reactivos para Diagnóstico, S.L., Barcelona, Spain) and incubated at 36 ± 2 °C for 48–72 h. The presence of *Legionella* spp. was considered positive when there were growths in BCYE but not in nutrient agar. The *Legionella* colonies were counted and the result was given as colony-forming units per litre (cfu/L). The recovery rate for this procedure is usually 35–65%. Each sample batch contained a process negative control sample. The theoretical detection limit for culture was 50 cfu/L.

2.1.3. Quantification of viable Legionella using v-qPCR triple approach

A triple aliquot analysis approach was used with 500 µL from the concentrated water sample (Fittipaldi et al., 2011) in order to detect: (1) viable cells, (2) false positive results, and (3) total level of cells. The first aliquot (1) was directly treated with PEMAX™ monodoses (Std. Buffer) (GenIUL, Barcelona, Spain). Briefly, the microtubes were placed in a Dark Box system (GenIUL) for dark incubation at 37 °C for 20 min. Then, the sample was collected in a new polypropylene tube and was photo-activated at 100% for 15 min in a PhAST Blue system (GenIUL) (Agustí et al., 2017). The second aliquot (2) was exposed to heat treatment at 85 °C for 30 min in order to obtain a dead cell aliquot. After that, the bacterial suspension was treated with PEMAX™ monodoses (Std. Buffer), following the protocol described above. The third aliquot (3) was only concentrated by centrifugation (14,000*g* for 5 min) using a minicentrifuge (Minispin Plus-Eppendorf, Hamburg, Germany) and discarding the supernatant to obtain a pellet. Each sample batch contained a process negative control sample. The conversion factor x 28 between culture and PCR was selected according to Ditommaso et al. (2015).

2.1.4. DNA purification and PCR

In all cases, DNA was purified using the V-DNA reagent (GenIUL), according to the manufacturer's instructions. Following DNA purification, the samples were analysed by qPCR on the Mx3005P Real-Time PCR Platform (Agilent Technologies, Santa Clara, CA, USA). Amplifications and quantifications were made using the *Legionella* spp. qPCR detection kit (GenIUL). The PCR Kit meets the specification of ISO 12869:2012. Five microlitres of each extracted sample were used in the PCR reaction, following the cycling conditions: 15 min at 95 °C, 45 cycles of 15 s at 95 °C, 30 s at 55 °C, and 30 s at 72 °C followed by data acquisition at 82 °C during 20 s, and finally a melting temperature ramp from 65 °C to 95 °C at 0.1 °C/s.

Additionally, for each amplification round, a negative (RNase-Free water) and a positive control (Standard DNA supplied in the kit) were included. Besides reagents and enzyme for the detection of *Legionella* spp. target sequence, the PCR mix also contained an internal control to identify reaction inhibition. Likewise, external standard curves for quantification were also provided in the same commercial kit. The qPCR data were analysed using MxPro Analysis Software version 4.1 (Agilent Technologies). With the estimation of the total and viable *Legionella* genome units (GU) levels the percentage of live cells could be calculated (% live cells level = [(theoretical live cells GU − false positive cells GU)/total GU] × 100). The theoretical limit of detection of this qPCR method was 1200 GU/L.

2.2. Statistical analysis

In each sample group, results were categorized in 3 levels according to culture and v-qPCR results: no detection (detection limit < 50 cfu/L and < 1200 GU/L), positive detection of medium-low levels