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Overestimation of the *Legionella* spp. load in environmental samples by quantitative real-time PCR: pretreatment with propidium monoazide as a tool for the assessment of an association between *Legionella* concentration and sanitary risk

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ABSTRACT

Quantitative polymerase chain reaction (qPCR) offers rapid, sensitive, and specific detection of *Legionella* in environmental water samples. In this study, qPCR and qPCR combined with propidium monoazide (PMA-qPCR) were both applied to hot-water system samples and compared to traditional culture techniques. In addition, we evaluated the ability of PMA-qPCR to monitor the efficacy of different disinfection strategies. Comparison between the quantification obtained by culture and by qPCR or PMA-qPCR on environmental water samples confirms that the concentration of *Legionella* estimated by GU/L is generally higher than that estimated in CFU/L. Our results on 57 hot-water-system samples collected from 3 different sites show that: i) qPCR results were on average 178-fold higher than the culture results ($\Delta \log_{10} = 2.25$), ii) PMA-qPCR results were on average 27-fold higher than the culture results ($\Delta \log_{10} = 1.43$), iii) propidium monoazide–induced signal reduction in qPCR were nearly 10-fold ($\Delta \log_{10} = 0.95$), and that iv) different degrees of correlations between the 3 methods might be explained by different matrix properties, but also by different disinfection methods affecting cultivability of *Legionella*. In our study, we calculated the logarithmic differences between the results obtained by PMA-qPCR and those obtained by culture, and we suggested an algorithm for the interpretation of PMA-qPCR results for the routine monitoring of healthcare water systems using a commercial qPCR system (iQ-check real-time PCR kit; Bio-Rad, Marnes-la-Coquette, France).

1. Introduction

Contamination of hospital water with legionellae is a cause of nosocomial legionellosis, and rapid identification of the source of infection is essential to prevent further cases.

Culture is essential for identifying and typing *Legionella* strains. However, culturing *Legionella* requires long incubation times (up to 10 days), and additional time can be needed if additional confirmation procedures are applied. Moreover, various factors can influence method accuracy: differences in the membrane, such as pore size, batches, fragility, crinkling, and electrostatic interactions, and differences in the procedures for washing the organisms from the membrane, such as using a shaker/vortex, ultrasound, or finger and thumb scraping.

Quantitative polymerase chain reaction (qPCR) is an alternative tool that offers rapid, sensitive, and specific detection of *Legionella* bacteria in environmental water samples (Ballard et al., 2000; Behets et al., 2007; Dusserre et al., 2008; Joly et al., 2006; Wellinghausen et al., 2001; Yaradou et al., 2007). Currently, only culture methods (International Standards Organisation, 1998) and qPCR (Association Française de Normalisation, 2010; International Standards Organisation, 2012) are normalised. However, the major disadvantage of qPCR lies in its inability to distinguish between viable and membrane-compromised cells or extracellular DNA persisting in the environment (Josephson et al., 1993; Masters et al., 1994). The development of more rapid, culture-independent methods capable of discriminating between live and dead cells is of major interest for measuring the risk of *Legionella* infection and preventing legionellosis.

One of the most successful recent approaches used to detect viable cells utilised the technology based on sample treatment with the photoactivatable, cell membrane impermeable nucleic acid intercalating dyes, ethidium monoazide (EMA), or propidium monoazide (PMA), followed by light exposure prior to the extraction of DNA and amplification. Light activation of DNA-bound dye molecules results in irreversible DNA modification and subsequent inhibition of its amplification. The nucleic acid-binding dyes, EMA or PMA, used in combination with qPCR, are an attractive alternative for selectively detecting and

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enumerating viable bacteria (Chang et al., 2010; Delgado-Viscogliosi et al., 2009; Nocker et al., 2007).

This technique is not limited to use in bacteria but has also successfully been applied to fungi, protozoa, and viruses. Apart from sample-specific challenges, the 2 dyes both seem to have specific advantages and disadvantages. Whereas the greatest concern with EMA lies in its ability to penetrate bacterial cells with intact membranes causing underestimation of live cell population, the greatest concern with PMA is the generation of false-positive signals, due to incomplete signal suppression. Several comparative studies confirm that PMA outperforms EMA in selective removal of dead cells when combined with real-time PCR (Cawthorn and Witthuhn, 2008; Nocker et al., 2006; Pan and Breidt, 2007).

For the successful application of qPCR, a few factors that can influence the outcome of the resulting data must be considered. These factors include, amongst others, the choice of the dye, its concentration, the contact mode (cells suspension or cells impinged on filters), the incubation conditions, the light source, the presence of a high number of dead cells, the presence of high levels of suspended solids or biomass in the analysed samples, the salt concentration in the reaction mix, the pH of the reaction mix, the length of the target gene, and the sequence of the target gene (Fittipaldi et al., 2012).

In a previous study (submitted), we compared the results obtained by conventional qPCR and culture methods on spiked samples inoculated with *Legionella pneumophila* serogroup 1 (ATCC 33152), and we analysed the potential for the PMA-qPCR method to selectively quantify viable *Legionella* cells.

In this study, we addressed the question of how efficiently signals would be suppressed by PMA in the presence of an environmental sample. For this purpose, the 2 methods (qPCR and PMA-qPCR) were both applied to hot-water system samples and compared to the culture technique.

In addition, we evaluated the ability of PMA-qPCR to monitor the efficacy of different disinfection strategies.

2. Methods

2.1. Sample collection

From April to June 2013, 57 hot water samples were collected from the in-building distribution systems of 3 healthcare facilities in the city of Turin. The healthcare facilities included in this study consisted of acute care hospitals that conducted environmental monitoring programmes for *Legionella* detection. These facilities were selected because they adopt different strategies for the control of *Legionella* contamination: facility A implemented continuous chlorination to ensure 2–10 ppm of free chlorine at the distal sites, facility B implemented continuous chlorination to ensure 1–2 ppm of free chlorine at the distal sites, and facility C implemented quarterly disinfection of the water supply by thermal shock. In facility C, we collected water samples 30 days after thermal shock.

Each sample was collected in a sterile, 3-litre plastic bottle and was divided into 3 equal parts for culture, qPCR, and PMA-qPCR. The physical and chemical parameters of the water distributed in the facilities are almost identical for all water samples and in compliance with legal requirements, except for chlorine and temperature that are facility dependent; we decided to measure only chlorine and temperature parameters.

2.2. Quantification by the culture method

Analyses for the detection and quantification of *Legionella* were carried out in accordance with the modified ISO 11731 method (Ditommaso et al., 2011) that recommended the use of different media for routine water tests in hospitals: BCYE (Buffered Charcol Yeast Extract with a-ketoglutarate, L-cysteine and ferric pyrophosphate) and MWY (modified Wadowsky and Yee medium supplemented with glycine, polymyxin B, vancomycin, anysomicin, bromthymol blue and bromcresol purple). BCYE gives a high recovery rate of positive samples, a much greater yield of *Legionella* spp. than MWY, and is necessary for the detection of non–*L. pneumophila* species, which grew poorly on selective media. Selective media (MWY) were necessary for the recovery of *Legionella* spp. when the nonselective medium (BCYE) was difficult to interpret because contaminating background flora.

The water samples were concentrated 100-fold by filtration through a 0.2-μm polycarbonate filter (Millipore, Billerica, MA, USA). The filter membrane was aseptically placed in one of the bottom corners inside the stomacher bag and rubbed with the finger and thumb of one hand for 1 min with 10 mL Page's solution (pH 6.8) to detach the bacteria. A 0.2-mL volume of the concentrated sample was spread on duplicate plates of BCYE agar and MWY agar (Oxoid, Wesel, Germany).

Under these experimental conditions, the detection limit (LOD) was 50 CFU/L.

2.3. Quantification by qPCR and PMA-qPCR

The second and third aliquots were filtered through a 0.45-μm polycarbonate filter (Millipore, Billerica, MA, USA) according to the manufacturer's instructions (Aquadien™; Bio-Rad, Marnes-la-Coquette, France).

The first filter was directly added to the lysis solution for DNA extraction, whilst the second was first overlaid with 500 μl of PMA (50 μM) in 90 mm Petri dishes and incubated in the dark for 10 min followed by a 10 min exposure to a 500 W light on ice at a distance of 20 cm from the light source. After irradiation, the filter was added to the lysis solution for DNA extraction. In order to eliminate the bacteria resuspension step (which could generate bacterial loss), the extraction of DNA was performed directly from membrane filters. The experimental conditions were optimised in our previous study (submitted paper).

Extracted genomic DNA was analysed for the presence of amplifiable sequences using qPCR.

Analysis was performed by the iQ-Check™ Quanti *Legionella* spp. kit according to the manufacturer's instructions (Bio-Rad, Marnes-la-Coquette, France). The iQ-Check™ Quanti *Legionella* spp. kit is NF VALIDATION certified (certificate numbers BRD07/15-12/15) and contains reagents to amplify and quantify a 100-bp fragment from the 5S rRNA gene of *Legionella* spp. This method allows the quantification of *Legionella* in water samples in less than 3 hours following the water sample filtration and DNA extraction steps. The LOD of this qPCR method is 5 GU per well; performing analysis in duplicate kits allows us to have a total method detection limit equal to 80 GU/L. The quantification limit was 10 GU/5 μL corresponding to 608 GU/L. The Amplification mix contains the internal control (IC), a linear plasmid, which is added to each PCR reaction and which should be amplified in all conditions. This control monitors the inhibitory effects that may take place in the reaction mix. The *Legionella* target and the IC are always amplified in the same PCR well.

3. Statistical analysis

All data generated by qPCR were analysed by the Opticon Monitor Analysis Software version 3.4 (Bio-Rad). The positive predictive value (PPV) and negative predictive value (NPV) of the qPCR and PMA-qPCR techniques were calculated. PPV corresponds to the ratio between the number of culture positive samples and the number of positive samples

Table 1
Comparison of the culture and qPCR results on 57 water samples from 3 hospital water supplies in the Piedmont region.

Samples		Number	%
qPCR(+)	Culture (+)	26	46
qPCR(−)	Culture (−)	19	33
qPCR(+)	Culture (−)	12	21
qPCR(−)	Culture (+)	0	0
Total		57	100

Observed concordance = 79%; Cohen κ = 0.591.

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