Assessment of Viability of Legionella Pneumophila in Environmental Samples: On Filter Application of Ethidium Monoazide Bromide

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Short Report

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Abstract

Purpose: Culture method, Real-Time PCR (qPCR) and Ethidium Monoazide Bromide (EMA) qPCR have been compared in order to detect *Legionella pneumophila* (Lp) in water samples, to identify a method able to speed up the procedures, detect the “viable but not cultivable” bacteria (VBNC) and exclude dead bacteria using a commercial kit for extraction and amplification and modifying the protocol.

Methods: Using these three methods, 34 environmental water samples and a series of samples artificially spiked with alive, dead and VBNC Lp ATCC 33152 were analysed. ISO 11731-2-2004 culture method was applied, whereas a commercial kit was selected for both qPCR and EMA qPCR pretreatment.

Results: only 35% (12/34) of the environmental samples were positive in both culture and qPCR methods. With regard to EMA qPCR, results showed the absence of dye toxicity on viable and VBNC strains and an incomplete effectiveness on the dead ones. In both viable and VBNC strains a decrease of bacterial DNA amplification was recorded as a function of sample dilution but not of EMA concentration.

Conclusions: Discrepancies between culture method and EMA-qPCR were observed and could be due to different causes as membrane-dye interactions, presence of interfering compounds and the relatively low sensitivity of the kit used.

Significance and Impact of the Study:

In presence of one or more suspected cases of nosocomial legionellosis, the application of a rapid molecular method able to identify only the viable and VBNC Lp would be useful in order to quickly identify the source of infection and to intervene with sanitation treatments. However, because in our experience EMA pretreatment on filter membrane has not given the expected results, it would be necessary to proceed with other experiments and different dyes.

Key Points

- Need for a rapid method able to identify both viable and VBNC *Legionella pneumophila*
- Modification of the protocol and EMA pretreatment directly on filter
- Results with discrepancies, no toxic effects even with increasing dye concentration

Background

Legionella is a Gram-negative, aerobic obligate bacterium, whose habitats are represented by both natural and artificial aquatic environments, in a free-living form, as parasites of protozoa and within biofilms (Bianchi et al. 2016). Colonization by *Legionella* occurs more easily if the artificial water network presents stagnation, biofilm, corrosion, limestone and temperatures between 25°C and 42°C.

Legionella, and particularly the species *L.pneumophila* (*Lp*), is the causative agent of Legionellosis, a generic term used to describe both pneumonic and non-pneumonic forms. *Legionella* is commonly transmitted by inhalation of contaminated aerosols, originating from air conditioning, cooling towers, hot and cold water systems, dental unit, humidifiers and whirlpool spas, but also by aspiration of contaminated water (Tesauro et al. 2018).

Legionellosis varies from a mild febrile illness (Pontiac fever) to a serious and sometimes fatal form of pneumonia (Legionnaires' disease) with an approximate mortality rate of 10-30% (Italian guidelines). Elderly, immunocompromised, transplanted and onco-hematological patients are particularly at risk (Fields et al. 2002).

Some authors (Dusserre et al. 2008, Ducret et al. 2014, Li et al. 2014, Mansi et al. 2014, Casini et al. 2018) reports that *Lp*, if submitted to prolonged stress, such as continuous disinfection treatments, can enter in a state of low metabolic activity that makes it "viable but not cultivable" (VBNC). VBNC bacteria are not able to replicate in culture but regain virulence when environmental conditions return to be optimal, representing a risk for human health.

Laboratory methods currently used for both research and quantification of *Lp* in water are culture method and Real-Time PCR, both with strengths and limitations.

Culture method allows to detect and isolate all *Legionella* species and permits to correlate clinical strains with the environmental ones. Using this method, however, the results, expressed in Colony Forming Units (CFU), can be reported after long time, usually 11 days after the beginning of the analysis. In addition, an underestimation of *Legionella* species may occur because culture method detects only living bacteria and can be influenced by the presence of interfering bacterial flora (Leoni et al. 2001, Collins et al. 2015, Kirschner et al. 2016, Collins et al. 2017, De Giglio et al. 2020). Otherwise, qPCR strengths regard *i*) the ability to amplify and simultaneously quantify the DNA of the species of *Legionella*, expressing the results in Genomic Units (GU), *ii*) the rapid response times from the beginning of the analysis (hours instead of days) and *iii*) the capacity to also detect VBNC bacteria (Fittipaldi et al. 2011, Whiley and Taylor 2016, Boss et al. 2018). On the other hand, this technique does not have ISO validation and, as a substantial limit, it also detects the DNA of dead bacteria. Furthermore, at the moment, it is not possible to convert in a univocal way GU into CFU (Joly et al. 2006, Shih et al. 2006, Ditommaso et al. 2014, Collins et al. 2015, Collins et al. 2017, Reyneke et al. 2017, Whiley and Taylor 2016, Boss et al. 2018), as required by national and international guidelines.

To overcome this problem, some authors (Chang et al. 2009, Chang et al. 2010, Chen and Chang 2010, Qin et al. 2012, Mansi et al. 2014, Inoue et al. 2015a and 2015b, Reyneke et al. 2017) highlighted the possibility of adding to qPCR a pretreatment on the bacterial cells with Ethidium Monoazide Bromide (EMA), a fluorescent dye capable of crossing damaged cell membrane of dead bacteria and covalently binding to their DNA. Thereafter, exposing the bacterial colonies to a light of 500W, the dye is activated, with consequent induction of irreversible damage to the nucleic acids which cannot be amplified in a subsequent amplification.

Combination of qPCR and EMA pretreatment would therefore allow to amplify only the DNA of viable and VBNC bacteria, but also in this case there are limits. EMA, being toxic, could penetrate living cells and damage them, causing an underestimation of living bacteria (Reyneke *et al.* 2017, Boss *et al.* 2018). Furthermore, in case of high concentration of dead bacteria ($>10^5$ CFU/ml), the amount of EMA may not be enough to inactivate their DNA, with risk of having false positives (Fittipaldi *et al.* 2011, Qin *et al.* 2012). Subsequently, it would be necessary to optimize EMA concentration according to chlorine concentration possibly present in examined water samples. Finally, it is important to consider the rather significant cost of the method.

Aims of this work were *i)* to compare the culture and the qPCR methods on the same water samples and *ii)* to applicate and develop the EMA qPCR, using a commercial kit and modifying its protocol, in order to identify a rapid method able to speed up the procedures, detect the viable and “viable but not cultivable” bacteria (VBNC) and exclude dead bacteria, especially in situations where discriminating is particularly relevant, as in presence of clusters or outbreaks of Legionellosis.

Methods

Study design has included several phases: 1) Applicate both culture and molecular analysis on the same water sample. Thirty-four water samples of 2 liters were collected using sterile bottles containing Sodium Thiosulfate (LP italiana SpA, Milan); refrigerated samples were transported to the laboratory where they were analyzed both by culture method and qPCR, within 24 hours from the sampling. The samples were collected from Healthcare Facilities distributed in Lombardy, Piedmont and Liguria (North Italy). Some of these water systems were treated in continuous with disinfectants as chlorine dioxide and monochloramine. 2) Applicate and develop EMA qPCR method. Water samples artificially spiked with viable, dead and VBNC *Lp* ATCC 33152 in serial dilutions were tested. Initially, 3 different concentrations of the dye (1,25-2,5-5mg/ml) were used, and higher concentrations (2,5-10-20 mg/ml) were subsequently tested. Dead cells were obtained by heat treating, in thermostat, at 95°C for 15 minutes (Qin *et al.* 2012, Ditommaso *et al.* 2015, Slimani *et al.* 2012) while VBNC cells were treated at 59°C for 30 minutes (Slimani *et al.* 2012).

Culture method in environmental samples

ISO 11731-2-2004 "Water quality- detection and enumeration of *Legionella*" Part 2: "Direct membrane filtration method for waters with low bacterial counts" was followed to test viable cells through culture method. Briefly, after filtration, the cellulose acetate membrane (0,22µm) was placed directly on GVPN Agar Base medium and *Petri* dishes were incubated in thermostat with CO₂ at 2.5%, at 37°C for 10 days. Subsequently, suspicious colonies were tested for identification of genus both on BCYE Agar and CYE Agar (ThermoFisher Scientific, Oxoid, Rodano, Italy) and only those BYCE positive were verified through latex agglutination tests (*Legionella* Latex Test, ThermoFisher Scientific, Oxoid, Rodano, Italy) to identify species and serogroups.

DNA extraction

The AquaScreen® FastExtract, validated according to ISO/TS 12869: 2012, was used to extract DNA from water samples, following the instructions provided by the manufacturer. Water samples were filtered through the polycarbonate membranes (size pores of 0.45µm), provided by the kit, and any bacteria present was subjected to lysis directly onto the membrane, as required by the supplier's protocol. Then, we proceeded with binding, removal of contaminants and elution of DNA.

Real-Time PCR

Bacterial DNA was amplified by qPCR using the AquaScreen® *Legionella pneumophila* kit, validated according to AFNOR T90-471 and ISO/TS 12869: 2012, following the instructions provided by the manufacturer and using the StepOnePlus Thermocycler (Applied Biosystems, Foster City, California, USA)

Target of amplification is the *Lp* mip gene, which encodes for a protein implicated in the bacterium virulence mechanisms. The related primers are: Forward Legmip_f: 5'-GGG (AG)ATT(ACG)T TTATGA AGA TGA (AG)A(CT) TGG; Reverse Legmip_r: 5'-TC(AG) TT(ATCG) GG(ATG) CC(ATG) AT(ATCG)GG(ATCG) CC(ATG) CC.

A Reaction Mix, provided by the kit, containing an internal control, was used for the amplification of the extracted DNA.

For each group of samples subjected to amplification, a negative control (PCR grade water), a positive control (provided by the kit) and a series of 6 standard dilutions of *Lp* were also analyzed.

Standard dilutions (PCR Quantification Standard), made from a stock solution of 10⁸ UG provided by the kit, were necessary for the construction of a standard reference curve and the quantification of the amplified DNA (UG).

For the amplification reaction, 1 cycle was performed at 95°C for 5 minutes, followed by 45 cycles so composed: "denaturation" (95°C for 30 seconds), "annealing" (55°C for 30 seconds) and "elongation and data collection" (60°C for 45 seconds).

EMA treatment

Each artificially spiked sample was filtered using membranes supplied by the AquaScreen FastExtract (Fx) extraction kit and EMA treatment was carried out directly on membranes, to integrate dye treatment with the kit protocol.

Two different experimental tests were carried out: one with EMA at concentrations of 1,25-2,5-5 mg/ml and a second with EMA at concentrations of 2.5-10-20 mg/ml (Mansi *et al.* 2014, Quin *et al.* 2012, Chang

et al. 2009, Chen *et al.* 2010, Delgado *et al.* 2009). Each membrane was placed in a Petri dish and covered with 500ml of EMA at different concentrations. Petri dishes were incubated in dark in a thermostat at 21°C for 10 minutes, then placed in a tray with ice and exposed for 5 minutes to light (500W) at 15/20 cm from the light source. Finally, each membrane was taken and placed in a new Petri dish for bacterial DNA extraction and amplification procedures, as provided by kit instructions.

Data interpretation

Values of Threshold cycle (Ct) indicate presence or absence of *Lp* and the amplification reaction is to be considered positive with Ct <40 and negative with Ct ≥ 40.

The GU determined refer to the total number of bacterial cells, alive and cultivable, alive but not cultivable and death. It should be noted that the kit has a "Limit of Detection" (LOD) of 20 GU and a "Limit of Quantification" (LOQ) of 50 GU.

Results

Data regarding the comparison between culture method and qPCR, carried out on 34 environmental samples and collected in different 13 Healthcare facilities in North Italy are synthetically reported in Table 1. There is correspondence between samples positive and negative with both methods in 12/34 (35%) and 10/34 (29%) cases, respectively. In the other cases there were discrepancies between the two methods. In 9/12 water samples chlorine dioxide concentration was reported, with mean values of 0.32 mg/L and a range of 0.18-0.58 mg/L. *Lp* was present in the water network with variable concentrations ranging from “not detected” to 3.3Log and 50% of the isolated strains belonged to *Lp* serogroup 1. GUs range from 2.48Log to 5Log, only in rare circumstances high values of CFU/L correspond to high values of GU.

Table 1

comparison of culture method and qPCR on environmental samples. Pos= positive Neg= negative

	CFU/L pos	CFU/L neg
qPCR pos	12/34 (35%)	8/34 (24%)
qPCR neg	4/34 (12%)	10/34 (29%)

With regard to EMA qPCR, repeated experiments at low dye concentrations (EMA 1.25-2.5-5 mg /ml) gave differing and controversial results (not reported in this paper) and, therefore higher concentrations of dye (2.5-10-20 mg/ml) were verified. For this purpose, diluted samples (1st dilution= 10⁵ UFC/ml; 3rd dilution= 10³ UFC/ml; 4th dilution= 10² UFC/ml) of viable, VBNC and dead *Lp* ATCC 33152, prepared from the same stock solution, were analyzed using EMA qPCR and repeated for three times.

As shown in Figure 1, we observed a decrease of DNA amplification as a function of sample dilution, both in viable and VBNC strains at all three dye concentrations, almost overlapping. EMA inhibited the amplification of DNA of dead bacterial cells only at the concentration of 2.5µg/ml (4th dilution) and of 10µg/ml (3rd and 4th dilution); in the other cases, qPCR detected genetic material. Confirmation that these bacteria were died was given by culture method, which always gave results equal to 0 CFU/L.

Discussion And Conclusion

Legionnaires' disease represents an emerging problem for public health, especially if it occurs in healthcare facilities, causing not only isolated cases but also clusters or outbreaks. Hence, environmental surveillance in health facilities plays an important role in preventing these infections, in order to establish usual or extraordinary treatment interventions to reduce and control the risk of acquiring the infection. Currently, with the aim to detect and quantify the contamination degree in water networks, culture method is considered the gold standard, but qPCR is still possible on water samples, even without international validation.

Many studies confirmed qPCR as a fast and sensitive method to detect and amplify the *mip* gene of *Lp*, and useful to highlight also VBNC bacteria. Nevertheless, the problem of DNA detection from dead bacteria remains, along with other economic and methodological limitations. Other studies highlight the possibility of overcoming the problem of overestimating the presence of dead bacterial cells, using Ethidium bromide monoazide (EMA) to bind to DNA of dead cells and prevent its amplification by PCR (EMA-qPCR) (Reyneke *et al.* 2017).

Culture method, qPCR and EMA-qPCR, with a variation to the protocol applying the dye directly on the filter, were compared in the environmental water samples and artificially spiked ones with serial dilutions of alive, dead and VBNC *Legionella pneumophila* ATCC 33152.

The comparison between culture method and qPCR gave overlapping results in most of the cases, showing that the AquaScreen® FastExtract kit and AquaScreen® *Legionella pneumophila* kit, respectively for extraction and amplification, have acceptable and satisfactory characteristics. We can hypothesize that discrepancies regarding samples with positive cultures and negative qPCR are due to presence of ferruginous deposits or other substances, such as residues of disinfection treatments, that may play a role, interfering with DNA extraction and blocking DNA amplification. We cannot exclude that there was also a problem regarding the analytical sensitivity of the q-PCR (20UG/L), being not able to

detect very low charges of the microorganism, a condition that often occurs in structures with continuous treatment with biocide. In this case, the likelihood of acquiring the infection is very small, as demonstrated by the absence of cases of Legionnaire's disease among the patients.

Otherwise, in the case of samples with negative cultures and qPCR positive, we can hypothesize that the presence of dead or VBNC bacterial cells has been highlighted, with the amplification of bacterial DNA. Thereby, we consider essential to try to solve or contain this possibility.

Unfortunately, our experiments with EMA-qPCR were not satisfactory at low concentrations of dye (1.25-2.5-5 µg/ml) and even at higher concentrations of 2.5-10-20 µg/ml. Despite toxicity data to bacteria reported in literature, in our series of tests the dye has not proven to be toxic on live bacteria or even on VBNC ones, observing amplifications at all dilutions. At the same time, it has not been shown to be effective in inhibiting the amplification of the DNA of dead bacterial cells.

Our idea to associate qPCR to the use of dye directly on the filter to evidence VBNC cells and exclude the dead ones derived from the necessity to best represent the real contamination from *Lp* in the water network in a short time, as emerged also in other studies. Boss *et al.* (2018) transferred starved bacteria into a fresh nutritional medium that stimulates them, resulting in a boosted rRNA synthesis, unlike dead cells, followed by RT PCR. Ezenarro *et al.* (2020) presented a combination of sample concentration, immunoassay detection, and measurement by chronoamperometry. A nitrocellulose microfiltration membrane is used as support for both the water sample concentration and the *Legionella* immunodetection. Samhan *et al.* (2017) reported a rapid isothermal amplification method for *Lp* 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), *Lp* could be detected in 1e2 h at ~1 cfu/100 ml of tap water. Pàrraga *et al.* (2018) developed a membrane filter method to capture and immunodetect *Lp* in water samples. This membrane filter is used to retain the bacteria using a nitrocellulose disc inside a home-made cartridge. Subsequently the immunodetection of the bacteria retained in the nitrocellulose (blocking, antibody incubation, washings and developing) was performed.

We assumed the same idea to use and optimize the filtration on membrane to the following phase of selection of dead cells from viable and VBNC ones in our experiments. We choose not to carry out the treatment on pellets but directly on membranes in order to use the commercial qPCR kit and to speed up the duration of the analyses. Unfortunately, from our results, we can hypothesize that interactions have occurred between filter and the dye, making its effect less effective on bacterial cells.

In conclusion, while cultural method remains the Gold standard for the research of *Lp* in waters, it is necessary to develop alternative methods that allow to shorten the analyses time and therefore to be able to intervene more quickly in case of positivity. At the same time, there is still a need to have a laboratory method that can detect VBNC bacteria and exclude dead ones. For this purpose, the qPCR could be conducted in parallel with culture method, even better when combined with a pre-treatment that allows to

exclude dead bacteria. We therefore plan to continue our experience, modifying conditions of isolation, reagents, or dye.

Declarations

The data analyzed during the current study are available from the corresponding author.

Ethics approval and consent to participate

not applicable.

Consent for publication

not applicable.

competing interests

The authors declare that they have no competing interests.

funding

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Author's contribution

MT and MC conceived and designed research. MC, AG conducted experiments. ET contributed new reagents or analytical tools. AG, SS and MT analyzed data. MC, AG, MG and MT wrote the manuscript. All authors read and approved the manuscript.

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Figures

Figure 1

comparison among viable, dead and VBNC *Lp* ATCC 33152 dilutions expressed in log UG/1L for both qPCR and EMA-qPCR. EMA concentrations 2,5 µg/ml, 10 µg/ml and 20 µg/ml.

Supplementary Files

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