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Legionella spp. survival after different disinfection procedures: Comparison between conventional culture, qPCR and EMA–qPCR☆

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ABSTRACT

The development of rapid and sensitive methods for the detection and quantification of *Legionella* viable cells is essential for monitoring water quality and preventing legionellosis. The aim of this study was to verify the applicability of a quantitative PCR (qPCR) method used in combination with ethidium monoazide (EMA) to the quantification of *Legionella* spp. in samples collected from swimming pools, water recirculation systems and hot water systems in two fitness clubs. This molecular technique (EMA–qPCR) allows the amplification of target DNA from culturable and viable cells, but prevents the amplification of DNA from non-viable cells. The effectiveness of this new method able to detect alive legionellae was also compared with conventional qPCR and culture method. Our results confirm that EMA–qPCR allows to discriminate the non-viable cells from those viable and that it is particularly indicated for monitoring the effectiveness of thermal treatments for the *Legionella* contamination control in water environments, also providing information about the presence of Viable But Non-Culturable (VBNC) cells. Other Gram-negative bacteria typically associated with biofilm were identified in samples taken from swimming pools and balance tanks, suggesting that also the presence of biofilm should be monitored for a more general view of water contamination.

1. Introduction

Legionella spp. can cause legionellosis, a range of pneumonic and non-pneumonic disease; this Gram negative bacterium was identified for the first time after the 1976 Philadelphia outbreak during the American Legion convention where 29 people succumbed [1]. *Legionella* organisms are ubiquitous in aquatic environment and are able to multiply intracellularly in fresh water protozoa. These bacteria are found in many types of water sources and their growth is especially favoured in man-made warm water systems, including cooling towers and HVAC (Heating, Ventilation and Air-Conditioning) systems, hot tubs and spas [2,3]. Several cases of legionellosis have been associated with recreational water use like hot tubs, natural spa and aerosol-producing devices, like showers [4–7]. To date, outbreaks due to the use of swimming pools have not been reported, although *Legionella* spp. have been isolated from pool water and filter samples [8,9]. A wide variety of microorganisms can be found in swimming pools, and periodic controls for microbial parameters, including *Legionella* spp., are recommended by the World Health Organization [2]. *Legionella* risk assessment and the remedial actions eventually required are based on the results of the microbiological analysis performed in accordance with the ISO standard 11731 [10]. Culture is essential for identifying and typing the bacterial strains during outbreaks; however, culture-based methods call for prolonged incubation periods (up to 10 days), whereas the risk management strategy for *Legionella* spp. requires more rapid methods. Moreover, under certain conditions (i.e., low-nutrient environments, oxidative or osmotic stress, presence of biocides or toxic metal ions, etc.), some opportunistic pathogens, among which *Legionella pneumophila* and *Pseudomonas aeruginosa*, can enter a Viable But Non-Culturable (VBNC) state in which they are not detectable with standard culture methods but they are still alive and retain their virulence [11–13]. Thus, the presence of bacteria in VBNC state in man-made water systems may represent a public health hazard because they are potentially infectious. New investigation tools such as Polymerase Chain Reaction (PCR) [14,15] and especially quantitative PCR (qPCR) [16–19] should be used in combination with conventional culture methods to detect potential sources of infection and to monitor corrective actions. PCR techniques show several advantages including high sensitivity, accuracy and rapid evaluation of germ contamination; nevertheless, the main disadvantage of these molecular methods is that

Abbreviations: CFU, colony forming unit; VBNC, viable but non-culturable; EMA, ethidium monoazide bromide; GU, genomic unit; PCR, polymerase chain reaction; qPCR, quantitative PCR.

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don't distinguish between non-viable and viable cells, an important factor to take into account when the effectiveness of the corrective actions is evaluated [20,21]. The development of more rapid and sensitive methods for the detection and quantification of *Legionella* viable cells is essential for monitoring water quality and legionellosis prevention.

A new approach to detect viable cells by DNA-intercalating dyes (Ethidium MonoAzide Bromide, EMA or Propidium Monoazide, PMA) in combination with qPCR has been proposed over the last ten years [22]. This molecular technique allows the amplification of target DNA from culturable and viable cells, but prevents PCR amplification of DNA from non-viable cells (dead cells). EMA selectively binds DNA of cells with compromised membranes, whereas intact cell membrane represents a barrier for the dye. Once inside a non-viable cell, the azide group of the EMA allows the cross-linking to the DNA after exposure to strong visible light. The photolysis of EMA converts the azide group into a highly reactive nitrene radical which binds DNA. In this bound state, the DNA cannot be amplified by PCR, whereas DNA from viable cells do not bind EMA molecules and therefore can be amplified and quantified [23].

The aim of the present study was to verify the applicability of EMA–qPCR to the quantification of *Legionella* spp. in samples collected from swimming pools, water recirculation systems and hot water systems in two fitness clubs. In this study, EMA dye was first added to the water samples in a pre-treatment step. DNA was then extracted and further analysed by qPCR (EMA–qPCR). Furthermore, this new method able to detect alive legionellae was compared with conventional qPCR and culture method.

2. Materials and methods

2.1. Sampling

A total of 16 water samples were taken from several sites in two fitness clubs located in Rome. Eight samples were collected from four swimming pools and from the corresponding water recirculation from the balance tank before the purification treatment. The pool water was taken at a depth of 30 cm below the surface in sterile bottles; at the moment of sampling, the temperature, pH (direct reading pH meter, Orion 701 A, Cambridge, MA, USA) and free and total chlorine residual (Chlorine Test colorimetric DPD, Merck KGaA, Darmstadt, Germany) were annotated. Six water samples were taken from the showers of the hot water distribution system, while the remaining samples were represented by waters of supply. For each site, a four-litre water sample was collected into sterile bottles containing sodium thiosulfate (0.01 mg mL^{-1} final concentration) to neutralize any residual chlorine. All the samples were transported under refrigerated conditions into the laboratory and simultaneously analysed by culture method, qPCR and EMA–qPCR within 24–48 h of sampling. One-litre of each sample was analysed for *Legionella* spp. by culture method, two-litres were used for the bacterial quantification by qPCR and EMA–qPCR and the litre remaining was heat-treated in order to verify the effectiveness of EMA treatment on non-viable cells (see paragraph below). Water samples were also analysed by the membrane filter technique to determine the total heterotrophic counts at 36 °C (Plate Count Agar, Oxoid Ltd, Basingstoke, Hampshire, UK) according to the analytical reference methods [24]. Bacterial isolates were then subcultured and identified with a semi-automated system "GEN III Microstation System" (Biolog, Inc., CA).

2.2. Quantification by culture method

Isolation of *Legionella* from water samples was performed as specified in the ISO Standard 11731 [10]. One-litre samples of water were concentrated by filtration through 0.22 μm pore-diameter polycarbonate membrane (Isopore, Millipore). After filtration, membranes were placed into 10 mL of the original water sample and scraped to remove bacteria. A volume of 0.1 mL of concentrate was spread on a Petri dish containing α-BCYE (Buffered Charcol Yeast Extract with a-ketoglutarate, L-cysteine and ferric pyrophosphate) agar supplemented with vancomycin, polymyxin B, cycloheximide and glycine (GVPC medium) (Oxoid Ltd, Basingstoke, Hampshire, UK). For heat treatment, 1 mL of concentrate was treated at 50 ± 1 °C during 30 min. The inoculated plates were then incubated for 7–10 days at 36 ± 1 °C. Smooth colonies showing a greyish-white colour were chosen as suspected legionellae for subculture on a-BCYE agar and a-BCYE agar without L-cysteine. Isolated colonies that grew only on a-BCYE agar with L-cysteine were identified using a commercial agglutination test (Legionella Latex Test Oxoid, Ltd, Basingstoke, Hampshire, UK). The test allows a separate identification of *L. pneumophila* serogroup 1 and serogroups 2–14 and detection of seven *Legionella* (polyvalent) species (other than *L. pneumophila*) which have been implicated in human disease (*L. longbeachae*, *L. bozemanii* 1 and 2, *L. dumoffii*, *L. gormanii*, *L. jordanis*, *L. micdadei*, *L. anisa*). Results were expressed as number of Colony Forming Units per litre of water (CFU L^{-1}). The detection limit of culture method was 50 CFU L^{-1}.

2.3. EMA treatment

Water samples were EMA-treated prior to DNA extraction in order to prevent the PCR amplification of non-viable cells. Ethidium monoazide bromide (Sigma Chemical Co., St Louis, MO, USA) was diluted in sterile water to a concentration of 1 mg mL^{-1}. One-litre water sample was filtered and then the membrane was placed into a tube with 5 mL of PBS and scraped to remove bacteria, as described above. After the centrifugation at 8000 ×*g* for 10 min, pellet containing bacterial cells were resuspended in 1 mL of PBS. EMA was added to the concentrate sample in a transparent tube to a final concentration of 2.5 μg μL^{-1} as previously demonstrated [25]. EMA-treated cells were incubated in the dark for 10 min at room temperature (20 °C), then were placed on chipped ice and exposed to a halogen light (500 W) at a distance of 15 cm for 5 min. EMA-treated samples were washed by centrifugation (16,000 ×*g* for 10 min) with PBS and then pellets were resuspended in 30 μL of the corresponding residual supernatants before DNA extraction.

2.4. Effectiveness of EMA treatment

The EMA treatment used in this study allows amplification of target DNA of *Legionella* spp. from culturable and viable cells, but prevents the DNA amplification from non-viable cells when their concentration is approximately 10^5 CFU mL^{-1}. If the number of non-viable cells in the sample is greater, the amount of dye isn't enough to bind the DNA of all the non-viable cells *Legionella* spp. or other bacteria, therefore the DNA amplification can also occur from non-viable bacterial cells. In order to verify the effectiveness of EMA treatment on non-viable cells, an aliquot (one-litre) of each sample was heat-treated at 95 °C for 20 min to kill all bacterial cells. Death of the heat-treated *Legionella* cells was confirmed by spreading on the BCYE plate.

2.5. DNA extraction

DNA was extracted using the QIAamp DNA Mini kit (Qiagen, Hilden, Germany), according to manufacturer's instructions. DNA extracts were kept at −20 °C until use.

2.6. Quantification by qPCR

qPCR was performed with a LightCycler 1.2 instrument (Roche Diagnostics). PCR amplifications were carried out using the commercial kit "LightMix *Legionella* spp." (Roche Diagnostics) which amplifies a 386 bp fragment of 16S rRNA gene, according to the manufacturer's instructions. The threshold cycle (Ct) for each standard was plotted against the Log_{10} of the starting DNA quantity to generate a standard curve. The Ct value of each water sample was compared with those of the standards by means