Molecular and Cellular Probes 29 (2015) 237–243

Contents lists available at ScienceDirect

Molecular and Cellular Probes

journal homepage: www.elsevier.com/locate/ymcpr

Viability-qPCR for detecting *Legionella*: Comparison of two assays based on different amplicon lengths

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ARTICLE INFO

Article history:
Received 20 February 2015
Received in revised form
20 May 2015
Accepted 22 May 2015
Available online 23 May 2015

Keywords:
Legionella spp.
PCR (polymerase chain reaction)
Propidium monoazide
Quantification
Environmental water samples

ABSTRACT

Two different real-time quantitative PCR (PMA-qPCR) assays were applied for quantification of *Legionella* spp. by targeting a long amplicon (approx 400 bp) of 16S rRNA gene and a short amplicon (approx. 100 bp) of 5S rRNA gene. Purified DNA extracts from pure cultures of *Legionella* spp. and from environmental water samples were quantified.

Application of the two assays to quantify *Legionella* in artificially contaminated water achieved that both assays were able to detect *Legionella* over a linear range of 10 to 10^5 cells ml^{-1}. A statistical analysis of the standard curves showed that both assays were linear with a good correlation coefficient ($R^2 = 0.99$) between the Ct and the copy number. Amplification with the reference assay was the most effective for detecting low copy numbers (1 bacterium per PCR mixture). Using selective quantification of viable *Legionella* by the PMA-qPCR method we obtained a greater inhibition of the amplification of the 400-bp 16S gene fragment ($\Delta\log_{10} = 3.74 \pm 0.39$ $\log_{10}$ GU ml^{-1}). A complete inhibition of the PCR signal was obtained when heat-killed cells in a concentration below 1×10^5 cells ml^{-1} were pretreated with PMA. Analysing short amplicon sizes led to only 2.08 log reductions in the *Legionella* dead-cell signal.

When we tested environmental water samples, the two qPCR assays were in good agreement according to the kappa index (0.741). Applying qPCR combined with PMA treatment, we also obtained a good agreement (kappa index 0.615). The comparison of quantitative results shows that both assays yielded the same quantification sensitivity (mean log = 4.59 vs mean log = 4.31).

1. Introduction

The most commonly used method for the environmental surveillance of *Legionella* spp. is the standard culture technique, by which *Legionella* spp. organisms can be isolated and their number in environmental samples can be estimated. Quantitative polymerase chain reaction (qPCR) is an alternative tool that [1] enables rapid, sensitive, and specific detection of *Legionella* bacteria in environmental water samples and in clinical samples [1–8]. One of the most successful recent approaches used to detect viable cells utilised qPCR 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 and amplification of DNA [9–13]. The rapid sample extraction and analysis methodology by PMA-qPCR or EMA-qPCR has been shown to be effective for the detection and quantification of live bacterial cells in water samples. The photoreactive dye forms an irreversible cross-linkage with DNA when exposed to visible light and prevents PCR amplification. However, some studies have found that the PMA-qPCR method was not fully effective at removing the signal from dead cells: Kralik et al. [14] reported that a decrease in the PCR signal of no more than 2 logs could be obtained using membrane-permeable *Mycobacterium avium paratuberculosis* cells. Pan and Breidt [15] also showed that PMA-qPCR did not always eliminate the signal of heat-killed *Listeria monocytogenes*. Similar results showing the incomplete suppression of the dead-cell signal have also been reported using EMA-qPCR [16–18]. In addition, the use of membrane-based viability assays with inactivation mechanisms that do not affect the cell membrane has often been questioned [19,20]. In many environments, bacteria can be killed by processes [e.g., ultraviolet (UV) light] that do not directly cause membrane damage but instead cause damage to the genetic material. PMA treatment, for instance, was not successful at differentiating between live and UV-killed *Escherichia coli* O157:H7 [19].

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http://dx.doi.org/10.1016/j.mcp.2015.05.011
0890-8508/

The amplicon length affects qPCR: longer DNA sequences correlate with a higher probability that the DNA polymerase will encounter modifications in the stretch of DNA that is targeted by the primers, resulting in an increased suppression of signals from membrane-compromised cells [21–26]. After applying PMA to heat-killed *Legionella pneumophila*, there was a significant difference in the number of dead cells measured when qPCR was based on the amplification of 16S rRNA (454 bp) or 5S rRNA (108 bp) [9].

In our previous study, both qPCR and qPCR combined with propidium monoazide (PMA-qPCR) were applied to artificial samples and to hot-water system samples and compared with traditional culture techniques [27]. The analysis was performed by iQ-Check™ Quanti *Legionella spp*. (Bio-Rad, Marnes-la-Coquette, France), which amplify and quantify a fragment of approximately 100 bp from the 5S rRNA gene of *Legionella* spp. Applying qPCR combined with PMA treatment, we obtained a reduction of 98.5% ($\Delta Log\ mean = 1.83$) of the qPCR signal from dead cells. We observed a dissimilarity in the ability of PMA to suppress the PCR signal in samples with different amounts of bacteria: the effective elimination of detecting signals by PMA depended on the concentration of genomic unit (GU), and increasing cell amounts resulted in higher reduction values.

To investigate the effects of the amplicon length on the specific detection of viable *Legionella* cells in water samples, we studied the selective quantification of viable *Legionella* cells by the PMA-qPCR method, using either a fragment of 400 bp (from the 16S rRNA gene) or a fragment of 100 bp (from the 5S rRNA gene) as a target.

2. Materials and methods

2.1. Sample preparation, DNA extraction and PMA treatment

In the present study, we analysed the frozen extracted DNA (artificial samples and environmental samples) prepared in our previous study [27,28] as specified below.

2.1.1. Samples with live Legionella cells

Briefly, *L. pneumophila* serogroup 1 (ATCC 33152) was inoculated into AYE with 0.0025% ferric pyrophosphate (Sigma) and 0.04% L-cysteine (Oxoid) and incubated with shaking at 170 r.p.m. until the culture broth reached an OD 600 of 0.5. The bacterial suspension was prepared in Page's saline and adjusted to a concentration of 1×10^6 cells ml^{-1} which was confirmed by plate counting on BCYEα agar. For qPCR quantification, the cell cultures were serial diluted to obtain suspensions that contained 1×10^5, 1×10^4, 1×10^3, 1×10^2 and 1×10^1 cells ml^{-1}. Ten samples were prepared for each contamination level in order to evaluate the repeatability of the technique. The DNA extraction were performed following the Aquadien™ Bio-Rad protocol for the lysis, concentration and purification steps (without sample filtration).

2.1.2. Samples with heat-killed Legionella cells

A bacterial suspension was prepared in Page's saline and adjusted to a concentration of 10^8 cells ml^{-1} as confirmed by plate counting. Seven serial dilutions of the bacterial suspension were prepared and each concentration was boiled for 15 min. The efficacy of heat treatment was confirmed by plating the suspension on BCYEα medium. The DNA extraction were performed following the Aquadien™ Bio-Rad protocol for the lysis, concentration and purification steps (without sample filtration).

2.1.3. Environmental samples

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.

For each sample, 2 L of water was collected into sterile bottles containing sodium thiosulfate (20 mg/L) to neutralize any residual chlorine. The water samples were tested in less than 24 h.

2.2. PMA treatment

The ability of qPCR-PMA to detect dead cells was tested either on different amounts of heat killed *L. pneumophila* cells and on environmental water sample. For each sample two fractions were obtained and filtered through a 0.4 μm porosity polycarbonate. The first filter was directly added to the lysis solution for DNA extraction, while 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 following the Aquadien™ Bio-Rad protocol. DNA extracts were kept at −20 °C until use.

2.3. Quantification by real-time PCR

2.3.1. Quantification by qPCR: 16S rRNA gene (long amplicon)

The analyses were performed by a kit custom "Quantification of *Legionella* (all species) 400" based on TaqMan chemistry. Genesig, PrimerDesign Ltd (Southampton, United Kingdom) was the source of the design and gene detection kit. It contains reagents to amplify and quantify a 376 bp fragment of the 16S ribosomal RNA gene by the primers 5′-GGGAGGAGGGTTGATAGGTTA-3′ and 5′-ATCGTTTACAGCGTGGACTAC-3′ in less than 2 h following the water sample filtration and DNA extraction steps. A standard curve was constructed using a stock solution of *Legionella* genomic DNA (contained in the kit) titrated at 2×10^5 DNA copies μl^{-1}. Six serial dilutions of this *Legionella* DNA were performed, ranging from 2 to 2×10^5 genome copies μl^{-1}. Alternatively, a single dilution can be used as the positive control when a full quantitative analysis of the samples is not required. Genesig also supplied an exogenous plasmidic DNA, used as an internal control (IC), and a separate primer-probe mix. This control monitors the inhibitory effects that may take place in the reaction mix. The *Legionella* target and the IC were always amplified in the same PCR well.

The limit of detection (LOD) of this qPCR method is 10 genomic units (GU) per well, corresponding to 160 GU L^{-1}. The limit of quantification (LOQ) of the whole method was 320 GU L^{-1} considering a Z value of 32 (i.e., the fraction of analysed sample). A genomic unit includes 3 copies of the 16S rRNA gene.

This assay (alternative) was tested at same times with the Bio-Rad assay (reference) on artificial samples and environmental samples.

2.3.2. Quantification by qPCR: 5S rRNA gene (short amplicon)

The analyses were performed by "iQ-Check™ Quanti *Legionella spp*.", according to the manufacturer's instructions (Bio-Rad). The iQ-Check™ Quanti *Legionella spp*. kit is NF VALIDATION certified (certificate numbers BRD07/15-12/15), and it contains reagents to amplify and quantify a fragment of approximately 100 bp from the 5S rRNA gene (primer sequences are not provided by Bio-Rad) of *Legionella* spp. by molecular beacon probes. Using this method, the number of *Legionella* bacteria can be quantified in less than 3 h following the water sample filtration and DNA extraction steps. The LOD of this qPCR method is 5 GU per well, corresponding to 80 GU L^{-1}. The theoretical LOQ of the whole method (sample