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Optimization of viability qPCR for selective detection of membrane-intact *Legionella pneumophila*

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A B S T R A C T

Although a number of viability qPCR assays have been reported to selectively detect signals from membrane-intact *Legionella pneumophila*, the efficient suppression of amplification of DNA from dead membrane-compromised bacteria remains an ongoing challenge. This research aimed at establishing a new oligonucleotide combination that allows for a better exclusion of dead *Legionella pneumophila* on basis of the *mip* gene. Propidium monoazide (PMA) was chosen as viability dye. An oligonucleotide combination for the amplification of a 633 bp sequence was established with 100% specificity for different *Legionella pneumophila* strains compared with 17 other *Legionella* species tested. Apart from increasing amplicon length, the study aimed at optimizing dye incubation time and temperature. An incubation temperature of 45 °C for 10 min was found optimal. Dye treatment of heat-killed bacteria in the presence of EDTA improved signal suppression, whereas deoxycholate also affected signals from live intact bacteria. Suppression of signals from heat-treated bacteria was found to be approx. twice as efficient compared to a commercial kit, although the detection sensitivity is superior when targeting short amplicons. With a limit of detection of 10 genome copies per PCR well and a 6-log signal reduction of bacteria killed at 80 °C, the assay appears useful for applications where pathogen numbers are not limiting and where the priority is on the distinction between intact and damaged *Legionella pneumophila* for the evaluation of hygienic risk and of disinfection efficiency.

1. Introduction

Legionella pneumophila is the main causative agent of pneumonia and Legionnaires disease. Surveillance for *Legionella* is typically performed by cultivation with the disadvantage of a time demand of several days to develop colonies with a distinctive phenotype and subsequent necessity to confirmation of colonies on agar without cystein or other means (Hussong et al. 1987). Other limitations lie in the necessity to perform serotyping of suspected *Legionella* spp. colonies and the inability to detect viable but non-culturable (VBNC) cells. These drawbacks promote the application of cultivation-independent detection methods including quantitative polymerase chain reaction (qPCR; Behets et al. 2007; Ballard et al. 2000). With rapid analysis time, high sensitivity and unparalleled specificity, qPCR shows a couple of important advantages, however it is challenged with the necessity to differentiate between live and dead bacteria (Josephson et al. 1993; Masters et al. 1994). Traditional qPCR typically detects bacteria independent of their viability status due to the persistence of DNA after cell death (Young et al. 2007).

One approach to selectively detect live bacteria consists in the use of viability qPCR (v-qPCR; Nocker and Camper 2006; Rudi et al. 2005). The principle of viability PCR is based on the treatment of samples with a photoactive dye that selectively enters membrane-compromised cells and, once inside the cells, intercalates into their DNA (Nogva et al. 2003). Subsequent exposure with visible light leads to photo-activation and covalent binding of the dye to the DNA with the consequence that its qPCR amplification is suppressed (Soejima et al. 2007). One of the dyes currently used is propidium monoazide (PMA; Nocker et al. 2006) which has been used in a number of studies for detection of membrane-intact L. *pneumophila* (Yáñez et al. 2011; Li et al., 2015; Ditommaso et al. 2014). Although the use of PMA helps to reduce the signals of membrane-damaged bacteria, limitations in its capability to differentiate between dead and live bacteria have been reported (Scaturro et al. 2016). Hence a further minimization of false-positive signals from membrane-compromised cells remains a continuing challenge.

Several parameters to improve the efficiency of v-qPCR have been outlined in a a review by Fittipaldi et al. (2012) one of them being the amplification of longer sequences. The latter increases the probability of a dye binding event in the targeted region and subsequent stalling of the polymerase (Zhou et al. 2016; Soejima et al. 2011; Schnetzinger et al. 2013; Contreras et al. 2011; Banihashemi et al. 2012). The advantage of using longer amplicons in PMA-qPCR detection was nicely demonstrated for *Legionella* spp. by Ditommaso et al. (2014). Apart from increasing amplicon lengths, optimization of dye treatment conditions such as dye incubation time and temperature, repetitive light exposure cycles and use of membrane destabilizing deoxycholate in case of gram-negative bacteria have been successful for enhancing suppression of signals from membrane-damaged bacteria (Zhou et al.

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2016; Nkuipou-Kenfack et al. 2013; Fittipaldi et al., 2012). Another agent reported to modulate the permeability of gram-negative bacteria is ethylenediaminetetraacetate (EDTA) (Berney et al. 2007; Marvin et al. 1989). EDTA alters the outer cell envelope of gram-negative bacteria by chelating divalent cations that are responsible for stabilizing outer membrane lipopolysaccharides (LPS; Leive 1968). EDTA-treated bacteria have been reported to lose up to 50% of their LPS and minor amounts of proteins and phospholipids (Marvin et al. 1989). As a consequence, the presence of EDTA during dye treatment in v-qPCR can positively influence the uptake of the dye. The goal of this study was to make use of the advantage of amplicon length in viability PCR to develop a highly sensitive and specific v-qPCR assay for *L. pneumophila* that more efficiently discriminates between intact and damaged bacteria. In order to achieve this, a novel oligonucleotide combination was evaluated to amplify nearly the entire length of the *mip* gene of *L. pneumophila*. The potential benefit of the longer amplicon length was evaluated together with other sample treatment conditions to improve suppression of signals from membrane-damaged *Legionella pneumophila*. Eventually the improved assay conditions were tested in combination with different viability dyes.

2. Materials and methods

2.1. Oligonucleotide selection

A literature search was performed to identify oligonucleotides targeting the *mip* gene of *L. pneumophila*. A NCBI BLAST search was used as the first criterion for their specificity for *L. pneumophila* followed by a multiple sequence alignment of *mip* genes from a *L. pneumophila* strain and 17 non-pneumophila species with MafftWS. For better visualization of the alignment results, the MafftWS alignment was run on Jalview software (Waterhouse et al. 2009). Two primers were selected that embraced nearly the entire length of the gene and generate an amplicon of 633 bp (Table 1). A Taqman probe was designed using the same multiple sequence alignment.

2.2. Bacterial species and growth condition

Legionella species (Table 2) were streaked from cryo-stocks on buffered yeast charcoal extract (BYCE) agar (Xebios Diagnostics, Düsseldorf, Germany) and grown for approx. 3–4 days at 37 °C. A scoop of colonies was transferred into diluted yeast extract broth (YEB) medium (Karl Roth GmbH & Co KG, Karlsruhe, Germany) containing 0.4 g L-Cysteine and 0.25 g iron(III)pyrophosphate. The YEB broth was 5-fold diluted with sterile deionized water prior to inoculation. For initial experiments assessing assay specificity, all *Legionella* spp. were grown for 72 h on a rotary shaker set at 280 rpm at 30 °C. For all subsequent experiments performed with environmental cooling tower isolate *L. pneumophila* 711/17–2 (IWW, Mülheim, Germany), growth time in liquid cultures was reduced to 24 h to avoid accumulation of bacteria with compromised viability.

2.3. Sample preparation

The optical density (OD_{600}) after 24 h was adjusted to 0.3 by dilution with the growth medium equilibrated to 30 °C. Bacteria were harvested from 500 µL culture aliquots by centrifugation at 5000 *g* for 5 min followed by resuspension in 500 µL filtered (0.2 µm) mineral water (Evian, France). Heat treatment for generation of membrane-compromised cells was performed at 70 °C or at indicated other temperature for 30 min on a standard laboratory heat block (DITABIS AG, Germany) with bacterial suspensions in 2 mL microcentrifuge tubes to ensure efficient heat transfer.

Table 1
Primers and probe tested for specificity to *L. pneumophila*.

Oligonucleotide	Sequence 5′ – 3′	Reference
Wieland (forward)	TGGTGACTGCAGCTGTTATG	Wieland et al. 2002
Lpm2-R (reverse)	GGCCAATAGGTCCGCCAACG	Jaulhac et al. 1992
Lp-Probe1 (probe)	CTCATAGCGTCTTGCATGCCT	This study

2.4. Viability dye treatment

A stock solution of 2.5 mM PMA was prepared by dissolving the vial content (0.7 mg) of the QIAGEN Blu-V Viability Kit (QIAGEN GmbH; Hilden, Germany) in 550 µL sterile ultrapure water and stored at -20 °C. From this PMA stock, 2, 4 and 10 µL were added to 500 µL suspensions of L. *pneumophila* 711/17–2 to final concentrations of 10, 20 and 50 µM PMA, respectively. Following addition of PMA, samples were mixed (by inverting the tubes several times) and incubated in the dark at the indicated temperatures (20–50 °C) for 10–30 min with occasional mixing. When applying temperatures other than ambient room temperature (20 °C), incubation of PMA-containing samples was performed in a standard heat block covered with aluminum foil. PMA activation was achieved by light exposure of samples to blue LED light (Phast Blue, GenIUL; Spain) for 15 min with occasional mixing. Bacteria were harvested by centrifugation at 5000 *g* for 5 min followed by DNA extraction.

Similarly, samples were treated with the other viability dyes PMAxx (Biotium, Fremont, USA) and PEMAX (GenIUL, Barcelona, Spain) in the presence or absence of an enhancer solution (Biotium). As in the case of PMA, working solutions of 2.5 mM of these reagents were prepared with sterile ultrapure water. PEMAX combines the two photoreactive molecules ethidium monoazide (EMA) and PMA to distinguish between intact bacteria with active metabolism, intact bacteria with no metabolism and bacteria with damaged membranes (Codony et al. 2015). Whereas PMA distinguishes between intact and damaged cells, EMA can distinguish between active and inactive cells as the first can revert the uptake of EMA with the help of active efflux pumps.

2.5. Enhancement of PMA uptake by dead cells

A stock solution of 5% (*w/v*) deoxycholate (DOC; Sigma-Aldrich, Darmstadt, Germany) was prepared by dissolving 0.5 g of sodium DOC powder in 10 mL 0.2 µL filtered Evian water. Volumes of 1, 3, 10 and 30 µL from the 5% DOC stock were added to final volumes of 500 µL bacterial suspensions to achieve concentrations of 0.01, 0.03, 0.1, and 0.3% DOC, respectively. This was followed by addition of PMA (final concentration of 20 µM). A sample without DOC served as a control. In a similar manner, the effect of EDTA (Sigma-Aldrich, Darmstadt, Germany) was tested. Filtered Evian water was used to prepare EDTA solutions of 50, 100, 250 and 500 mM (undiluted) EDTA. Volumes of 10 µL of these solutions were added to 490 µL of bacterial suspensions to achieve final concentrations of 1, 2, 5 and 10 mM EDTA, respectively, before treatment with PMA. A sample without EDTA served as a control.

2.6. DNA extraction and quantification

Genomic DNA (gDNA) was extracted using the QIAGEN DNeasy® Blood and Tissue kit (QIAGEN GmbH; Hilden, Germany) according to the manufacturer's instructions for gram-negative bacteria with the following modifications: cell pellets were resuspended in 180 µL of animal tissue lysis (ATL) buffer followed by addition of 20 µL Proteinase K treatment and incubation at 56 °C for 10 min on a rotating heat block set at 180 rpm. Lysis (AL) buffer and ethanol (96%) were mixed in the ratio 1:1 prior to addition. Incubation in elution (EA) buffer was performed for 2 min at room temperature prior to centrifugation. Following extraction, DNA was quantified using the Qubit DNA quantification kit (Life Technologies, Eugene; Oregon, USA).

Quantification was done according to the manufacturer's instruction on a Qubit 3.0 fluorometer device (Life Technologies Holding Pte Ltd.; Malaysia). Isolated gDNA was processed directly or stored at -20 °C.

2.7. End-point DNA amplification for gel electrophoresis

For all end-point PCR reactions, 1 µL of 1 ng/µL template DNA was added to 19 µL PCR mixture containing 10 µL (2×) KAPA Fast Probe master mix (universal) (Kapa Biosystem Ltd., London, UK), 7.8 µL PCR grade water and 0.4 µL of 10 pmol/µL of each primer and probe. PCR was performed on a C1000 thermal cycler CFX96 Real-Time System (Bio-Rad Laboratories Inc., Munich, Germany). The cycling parameters were 3 min at 95 °C (initial activation), 45 cycles at 95 °C for 3 s (denaturation) and 63 °C for 1 min (annealing and extension). The amplification products were visualized on 2% agarose gels stained with SYBR Safe (Life Technologies, Eugene, USA) using a gel imaging system (Fast Gene B7G LED Transilluminator, FG-08, Nippon Genetics Co. Ltd., Tokyo, Japan).

2.8. Quantitative PCR

For all qPCR reactions, 2 µL of 1 ng/µL template DNA was added to 18 µL PCR mixture containing 10 µL (2×) KAPA Fast Probe master mix (universal) (Kapa Biosystem Ltd., London UK), 7.4 µL PCR grade water and 0.2 µL of 10 pmol/µL of each primer and probe in a total volume of 20 µL. The thermal cycler and the temperature cycling parameters were the same as for the end-point PCR. Quantification cycle (Cq) values were automatically calculated by the C1000 thermal cycler CFX96™ Real-Time System with the threshold set to 200 relative fluorescent units (RFU).

2.9. Standard curves, amplification efficiencies, limit of detection (LoD) and limit of quantification (LoQ)

For the preparation of standard curves, gDNA of *L. pneumophila* 711/17–2 was serially diluted in DNA elution buffer (EB) from the QIAGEN extraction kit. Standard curves were done in a way that a defined number of genome copies were added as template to each well of a 96 well plate used for qPCR assuming 3.7 fg for one genome copy. Standard curves were generated by plotting Cq values against DNA concentrations. Corresponding amplification efficiencies were automatically calculated by the software.

For determining the LoD, ten identical replicates of qPCR reactions containing 1, 2, 5, 10, 20 and 50 genome copies were run. The LoD was calculated as the lowest concentration that could be detected with 95% confidence (Armbruster and Pry 2008). The LoQ was determined as the first level of the calibration range and the smallest number of GU that can be quantified. Non-template controls showed no amplification within 45 cycles and were thus clearly distinguishable.

2.10. Flow cytometry

Flow cytometry (FCM) was used for cell counting and to study the membrane integrity of *L. pneumophila* 711/17–2. Samples (200 µL) of bacterial suspensions (OD_{600} = 1) diluted 2000-fold in filtered (0.1 µm) mineral water were stained with either 100 x SYBR Green I (SG) or a mixture of SG and propidium iodide (PI) as described in Nocker et al., (2017). Measurements were performed using an Accuri® C6 flow cytometer (BD Bioscience, Oxford, UK). Sample volumes of 50 µL were analyzed with a medium flow rate with the trigger set to FL-1 and a threshold of 2000 RFUs.

2.11. Statistical analysis

Statistical analyses were conducted using GraphPad Prism 5.0 (GraphPad Software, California, USA). Mann-Whitney statistics was used to compare differences in performance between the new assay and the BioRad kit in terms of signal reduction and log reduction of genomic unit. The Kruskal-Wallis-test was used to evaluate differences in signal reductions between different treatments followed by Dunn's Multiple Comparison Test as a post hoc-test to assess differences between pairs of treatments. The *P*-value for all statistical evaluations was set at < 0.05.

Table 2
List of *Legionella* species used in this study. Isolates were derived from real cooling tower water samples.

Legionella species	Strain
Legionella pneumophila	
L. pneumophila	DSM 7513
L. pneumophila	Isolate 711/17–1 serogroup 2
L. pneumophila	Isolate 711/17–2 serogroup 1
L. pneumophila	Isolate 712/17 serogroup 1
L. pneumophila	Isolate 693–4 serogroup 2
L. pneumophila	Isolate 689 serogroup 2
L. pneumophila	Isolate 705
Legionella spp.	
L. gratiana	ATCC 49413
L. anisa	ATCC35292
L. longbeachae	ATCC 33462
L. norlandica	CDC 2163
L. rubrilucens	ATCC 35304
L. oakridgensis	Isolate W09–391-2
L. jordanis	ATCC 33623
L. parisiensis	ATCC 35299
L. brunensis	ATCC 43878
L. tucsonensis	ATCC 49180
L. feelei	ATCC 35072
L. erythra	ATCC 35303
L. londiniensis	ATCC 49180
L. worsleiensis	ATCC 49508
L. micdadei	DSM 16640
L. dumoffii	ATCC 33279
L. bozemanii	DSM 16523

3. Results

3.1. Selection of oligonucleotide combination specific for L. pneumophila

Oligonucleotides (primers and probe, Table 1) were tested for specificity for *L. pneumophila* using 18 different *Legionella* species including L. *pneumophila* (Table 2) and end-point PCR (45 cycles with 1 ng template). Amplification products were visualized on agarose gels (Fig. 1). An amplification product of 633 bp was exclusively obtained with genomic DNA from L. *pneumophila* DSM 7513 corroborating the specificity of the chosen oligonucleotides for *L. pneumophila*. The oligonucleotides were further tested for inclusivity using 1 ng genomic DNA of seven different L. *pneumophila* strains (Table 2) using a qPCR approach. The results suggested that DNA from all *L. pneumophila* strains could be successfully amplified with minor differences in the Cq values (data not shown). *L. pneumophila* strain 711/17–2 (an environmental cooling tower isolate) was chosen for further experiments. The amplification efficiency of the oligonucleotide combination derived from the standard curve with a slope of −3.55 and R^2 of 0.99 was calculated to be 91.29% which falls within the recommended range of 90–105%.

In the following experiments, dye incubation temperature, time and co-incubation with membrane destabilizing agents were investigated for optimal discrimination between live and dead *L. pneumophila* cells by PMA-qPCR. Pure culture suspensions of *L. pneumophila 711/17–2* containing 1×10^9 cells/mL were used. Heat treatment was done at 70 °C for 30 min. The heat treatment temperature resulted in > 85% of bacteria showing loss of membrane integrity as revealed by flow cytometry (supplementary Fig. 1S).

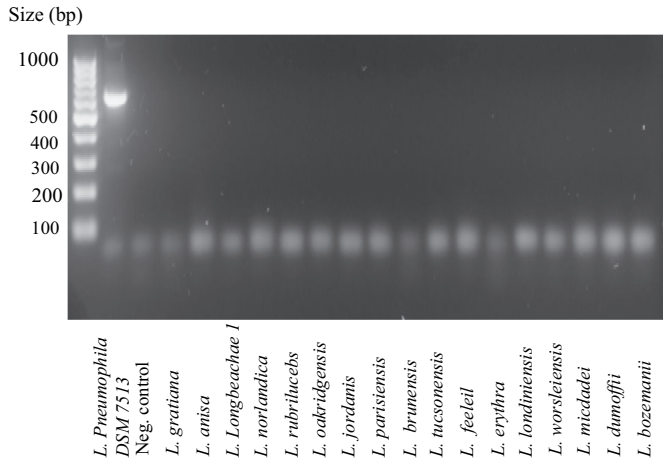


Fig. 1. Specificity of selected primer combinations for *L. pneumophila* as assessed by endpoint PCR with genomic DNA from 18 different *Legionella* spp. PCR products generated in 45 cycles were visualized on a 2% agarose gel together with a 100 bp ladder.

3.2. Effect of PMA incubation temperature and time on qPCR signal suppression

To assess the effect of dye incubation temperature and incubation time, both non-heated (intact) and heat-treated (70 °C, 30 min; dead) *L. pneumophila* were exposed to 10 µM PMA. PMA-treated samples were incubated at 20, 30, 37, 45 or 50 °C for 10, 20 or 30 min followed by extraction of genomic DNA and qPCR analysis (Table 3). For samples with intact bacteria, qPCR signal suppression was < 1 cycle (relative to a non-heated control without PMA) for all PMA incubation times and temperatures except at 50 °C, where signal suppression ranged from −1.40 cycles (10 min incubation) to −2.84 cycles (30 min incubation). Nevertheless the differences were not statistically significant. In case of heat-treated samples, the effect of elevated dye incubation temperature and time was more pronounced and was significantly different from their non-heated counterparts. Both longer incubation times and higher incubation temperatures resulted in increased signal suppressions from −8.50 cycles (20 °C, 10 min) to −17.71 cycles (50 °C, 30 min). Based on a moderate effect on live cells and a substantial effect on heat-killed cells, PMA incubation conditions of 45 °C for 10 min were considered adequately efficient for suppression of damaged cell signals while enabling rapid sample processing.

3.3. Effect of deoxycholate on PMA-qPCR signal suppression

Based on the reports of the use of sodium deoxycholate (DOC) to improve exclusion of dead cell signals while not affecting signals from live cells in case of other gram-negative bacteria, PMA treatment of *L. pneumophila 711/17–2* was performed in presence of increasing concentrations of DOC. Experiments were performed both with non-heated samples and heat-treated samples. Signals were related to intact samples without any treatment (DOC or PMA). Increasing concentrations of DOC resulted in increasing signal reductions both for non-heated and heated cells (Fig. 2). Although for any given DOC concentration, signal suppression of live samples was less than that of heat-killed samples, the differences were not significant. Hence, PMA treatment in presence of DOC was not considered advantageous and was not further pursued.

3.4. Effect of PMA concentration and comparison with commercial AFNOR ISO validated kit in regard to PMA treatment performance

The performance of the newly established assay for live-dead distinction was evaluated by comparing the results with that of the

Table 3
Effect of PMA incubation temperature and incubation time on qPCR signal suppression of non-heated ('live') and heat-treated ('dead'; 70 °C, 30 min) *L. pneumophila 711/17–2*. Samples were supplemented with PMA to a final concentration of 10 µM and incubated for 10, 20, or 30 min at either 20, 30, 37, 45, or 50 °C prior to light activation. Numbers represent signal reductions (ΔCq) calculated by subtracting the corresponding Cq values from the one of non-heated control without PMA treatment. Standard deviations from three independent repeats are shown in brackets. The extent of signal reduction is visualized by grey shading.

	Non-heated ("LIVE")					Heated ("DEAD")				
PMA incubation time	PMA incubation temperature					PMA incubation temperature				
	20°C	30°C	37°C	45°C	50°C	20°C	30°C	37°C	45°C	50°C
10 min	-0.05 (±0.68)	-0.15 (±0.22)	-0.46 (±0.23)	-0.67 (±0.41)	-1.40 (±0.8)	-8.50 (±1.03)	-9.33 (±1.23)	-11.48 (±2.83)	-13.61 (±1.07)	-14.67 (±0.49)
20 min	0.01 (±0.39)	-0.11 (±0.34)	-0.66 (±0.62)	-0.74 (±0.28)	-2.31 (±0.8)	-10.68 (±2.58)	-11.06 (±1.01)	-12.06 (±1.12)	-15.82 (±1.49)	-17.44 (±1.91)
30 min	-0.28 (±0.53)	0.17 (±0.43)	-0.38 (±0.29)	-0.88 (±0.2)	-2.84 (±0.85)	-10.70 (±1.87)	-12.11 (±1.96)	-12.66 (±2.84)	-15.90 (±1.68)	-17.71 (±1.28)

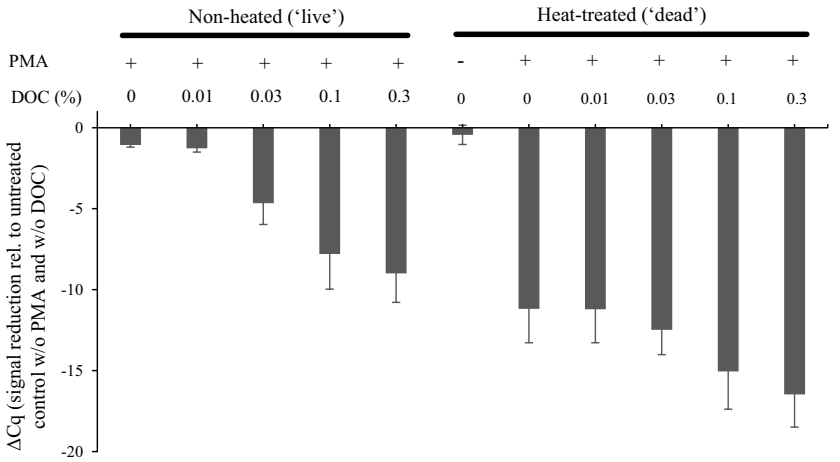
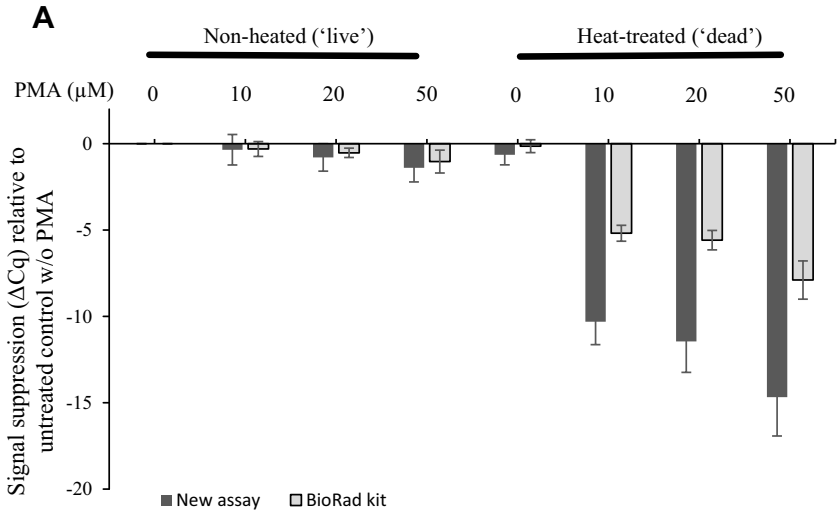


Fig. 2. Effect of deoxycholate (DOC) on PMA-qPCR signal suppression of both non-heated ('live') and heat-treated (70 °C, 30 min) *L. pneumophila* 711/17–2. Samples were exposed to 10 µM PMA (45 °C for 10 min) in the presence of 0, 0.01, 0.03, 0.1, or 0.3% DOC. Signal reductions (ΔCq) were calculated by subtracting the corresponding Cq values from the one of a non-heated control without PMA and without DOC. Error bars indicate standard deviations from three independent repeats.



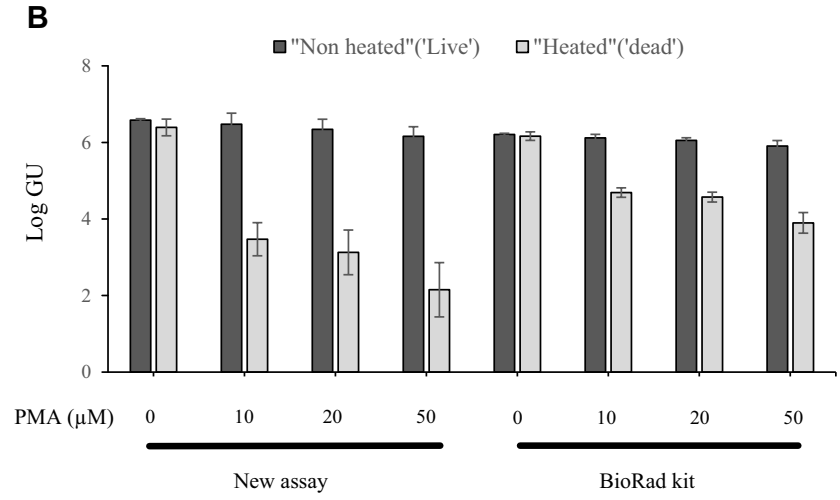


Fig. 3. Comparison of the performance of the new assay and the BioRad kit for live-dead distinction of *L. pneumophila*. Identical *L. pneumophila* 711/17–2 samples with either non-heated ('live') or heat-treated (70 °C, 30 min) bacteria were exposed to different PMA concentrations (10, 20 or 50 µM) at 45 °C for 10 min and analyzed by qPCR using the two assays. (A) Performance based on qPCR signal suppression (ΔCq) calculated by subtracting the corresponding Cq values from the one of a non-heated control without PMA treatment. (B) Performance based on log reduction of genomic units (GU). GU were calculated using the equations of the respective standard curves. Error bars represent standard deviations from three independent repeats.

commercial iQ-Check™ *L. pneumophila* kit (BioRad, USA) using identical samples treated with different PMA concentrations. For both approaches, the signal suppression increased with higher PMA concentrations (Fig. 3A). For intact bacteria, PMA concentrations of 10, 20 and 50 µM resulted in signal suppression values of −0.35, −0.79 and 1.4 cycles respectively for the new assay which was similar to that with BioRad kit of −0.31, −0.53 and 1.03 cycles, respectively. For heat-treated samples, on the other hand, the BioRad kit produced signal suppressions of 5.19, 5.59 and 7.89 unit with the different PMA concentrations, which was substantially < 10.31, 11.45 and 14.68, respectively, obtained with the new assay (Fig. 3A). As the two assays have different amplification efficiencies and sensitivities, the performances were also compared on the basis of log reduction of genomic units (Fig. 3B). Suppression of signals from heat-killed bacteria was

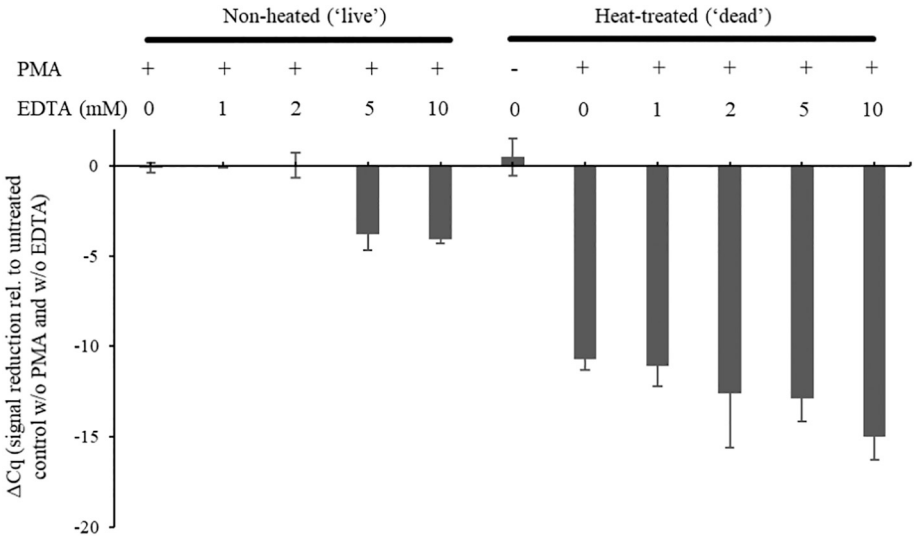


Fig. 4. Effect of EDTA on PMA-qPCR signal reduction of both non-heated ('live') and heat-treated (70 °C, 30 min) *L. pneumophila* 711/17–2. Samples were exposed to 20 µM PMA (45 °C for 10 min) in the presence of 0, 1, 2, 5 and 10 mM EDTA. Signal reduction (ΔCq) was calculated by subtracting the corresponding Cq values from the one of a non-heated control without PMA and without EDTA. Error bars indicate standard deviations of three independent repeats.

nearly twice as efficient when applying the new assay targeting longer DNA sequences. Differences were statistically significant.

In regard to the PMA concentrations, the following experiment was performed with 20 µM PMA due to its slightly higher efficiency to exclude dead cell signals compared to 10 µM. A PMA concentration of 50 µM was associated with a risk to suppress signals from intact bacteria in the pure cell suspensions (consistent with results from Scaturro et al. 2016), although such a concentration (or higher) might be necessary for treating environmental samples with a higher dye demand.

3.5. Effect of EDTA on PMA-qPCR signal suppression

The effect of EDTA in enhancing PMA effect on membrane-compromised *Legionella* was studied by exposing samples to increasing concentrations of EDTA (0–10 mM) followed by PMA treatment (20 µM final concentration and incubation at 45 °C for 10 min). Samples without EDTA served as controls and qPCR signals were related to non-heated control samples without treatment (neither with EDTA or PMA; Fig. 4). For non-heated samples with PMA treatment, EDTA did not have any effect on qPCR signal up to a concentration of 2 mM. From 5 mM, the signal suppression was as high as −3.78 and increased to −4.08 at 10 mM EDTA. For heat-treated bacteria, on the other hand, there was a more consistent increase and significant signal suppression with increasing EDTA concentration. Signals were not affect by EDTA alone in the absence of PMA (data not shown).

3.6. Comparison of performance of different viability dyes

The effects of different viability dyes (PMA, PMAxx and PEMAX) on non-heat treated and heat-treated *L. pneumophila* were compared at different concentrations, incubation temperatures and partly in absence or presence of a commercial enhancer solution (Fig. 5). Another test parameter included the number of light exposure cycles of 15 min each. As for previous experiments, the heat treatment (70 °C, 30 min) by itself did not lead to a qPCR signal reduction. Also, the commercial enhancer solution by itself did not affect the qPCR signals of live or heat-killed bacteria. An exposure to PMA at a final concentration of 25 µM resulted in a signal reduction of heat-killed cells of approx. 10.5 cycles in this experiment, when applying the optimized treatment conditions at 45 °C for 10 min. This was independent of whether the samples were light exposed once or twice suggesting that light was not the limiting factor. The incubation temperature was hereby more important than the PMA concentration as the incubation with 25 µM PMA at 45 °C for 10 min resulted in a substantially stronger signal reduction than an incubation with 50 µM PMA at room temperature with otherwise identical conditions. The presence of the enhancer in combination with PMA led to a strong increase in dead cell signal reduction, however at the cost of simultaneous suppression of live cell signals. The enhancer was therefore not seen beneficial in combination with PMA.

Sample treatment with PMAxx, in comparison, achieved signal reductions of approx. 5 and 7.5 cycles, when treating samples at room temperature with 25 or 50 µM dye concentration, respectively. The commercial enhancer solution, as in the case of PMA, led to stronger signal reductions both of live and dead bacteria, probably through enhanced dye penetration. As for PMA, the use of the enhancer solution was therefore not seen beneficial. Dye incubation of samples at 45 °C, on the other hand, was also advantageous in the case of PMAxx, although at the cost of a slight suppression of live cell signals.

When treating samples with PEMAX (a mixture of PMA and ethidium monoazide, EMA; Codony et al. 2015).), a recommended concentration of 50 µM was applied for 15 and 30 min at room temperature. The resulting dead cell signal reduction were approx. 7.7 and 10.1 cycles, respectively. The improved performance with an incubation time of 30 min was consistent with the recommendations by the manufacturer. An incubation temperature of 45 °C was avoided as active efflux of EMA was assumed to be temperature-dependent. Further research might be needed here.

3.7. Determination of LoD, LoQ and maximal exclusion efficiency of dead bacteria

The limit of detection (LoD) and the limit of quantification (LoQ) were determined to be 10 and 20 genomic units per PCR well using freshly extracted genomic DNA. Increasing numbers of freeze-thaw cycles reduced amplifiability probably due to strand breaks or other DNA damages. The extent of maximal dead cell signal exclusion was assessed by exposing suspensions of heat-killed *L. pneumophila* 711/17–2 to 20 µM PMA in the presence of 2 mM EDTA and performing the incubation at 45 °C for 10 min. When bacteria were exposed to 70 °C (30 min), signals from maximally 10^4 bacteria per mL could be suppressed (meaning no qPCR signal was obtained within 45 cycles, data not shown). When exposing bacteria to 80 °C for 30 min, signals from up to 10^7 bacteria per mL could be suppressed. This was consistent with flow cytometric data, where after 70 °C exposure and subsequent staining with SYBR Green I and propidium iodide approx. 12.5% of bacteria were still located in the gated region representing cells with intact cell membranes, whereas exposure to 80 °C led to the complete disappearance of intact cell signals (supplementary Fig. 1S).

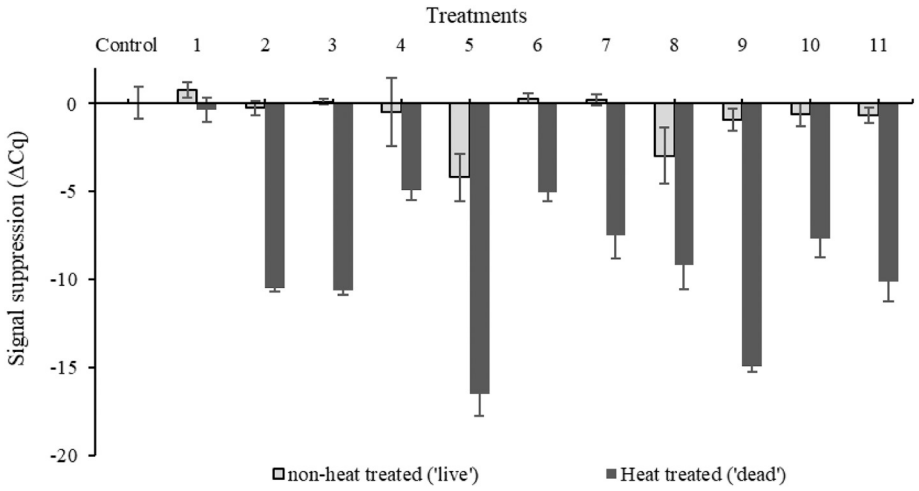


Fig. 5. Comparison of PMA, PEMAX, PMAxx and the effect of PMA enhancer on signal suppression of non-heated ('live') or heat-treated (70 °C, 30 min; 'dead') *L. pneumophila 711/17–2*. qPCR signal suppression (ΔCq) was calculated by subtracting the corresponding Cq values from the one of a non-heated control without PMA treatment. Concentrations of the corresponding dyes, the temperature applied for dye incubation (RT = room temperature), the time of dye exposure and the number of light exposures (15 min each) are indicated.

1	Enhancer\RT
2	PMA: 25 µM/45°C/10 min/1x light exposure
3	PMA: 25 µM/45°C/10 min/2x light exposure
4	PMA: 50 µM/RT/10 min/2x light exposure
5	PMA: 25 µM/enhancer/45°C/10 min/2x light
6	PMAxx: 25 µM/RT/10 min/2x light exposure
7	PMAxx: 50µM/RT/10 min/2x light exposure
8	PMAxx: 25 µM/enhancer/RT/10 min/2x light
9	PMAxx: 25 µM/45°C/10 min/1x light
10	PEMAX: 50 µM/RT/15 min/2x light exposure
11	PEMAX: 50 µM/RT/30 min/2x light exposure

4. Discussion

Given the importance of L. *pneumophila* as a human pathogen and the introduction of more stringent legislation to monitor concentrations of this pathogen in Germany (BMJV, 2017), a qPCR Taqman assay was developed with the aim to allow a better exclusion of signals from membrane-compromised bacteria. The assay was based on the gene encoding the macrophage infectivity potentiator (*mip*) gene which is considered a suitable target for the differentiation of L. *pneumophila* from non-pneumophila *Legionella* (Ratcliff et al. 1997) and has seen applications in a number of PCR assays for *L. pneumophila* (Wilson et al. 2003; Mahbubani et al. 1990; Lindsay et al. 1994; Bej et al. 1991). Both inclusivity and exclusivity of the qPCR assay in this study was 100% for *L. pneumophila* strains although the assessment was limited in regard to the number of tested strains and species and cannot guarantee the same performance for complex environmental samples. When working with L. *pneumophila* exposed to 70 °C for 30 min, signal reduction of this newly developed assay was found to be approx. double in comparison to an established commercial assay based on amplification of short gene sequences. It has to be pointed out, however, that the improved live-dead distinction came at the cost of a lower limit of detection being around 10 genome copies per PCR well. Amplicon length is known to be a critical determinant of amplification efficiency and thus has a direct influence on LoD (Debode et al. 2017). Whereas commercial assays thus have an advantage where highest sensitivity is desired, the long-amplicon assay presented here has a higher probability to achieve better signal exclusion of dead bacteria.

The majority of experiments was performed with *L. pneumophila* exposed to 70 °C for 30 min although flow cytometry with the applied staining conditions suggested that approx. 12.5% of bacteria had only suffered moderate/intermediate membrane damage. This temperature was chosen to leave room for assay improvement as temperatures ≥ 80 °C rendered nearly exclusively bacteria with heavy membrane damage. It is known that the extent of signal suppression in viability qPCR assays depends on the extent of membrane damage for heat-treated cells (Nocker et al. 2007; Contreras et al. 2011). More efficient dead cell signal exclusions can be obtained by applying stronger heat treatments, although extreme temperatures seem excessive when aiming at loss of viability instead of bacterial disintegration. Especially boiling can cause damage and cellular disintegration and thus appears little relevant for assay improvement. Such an extreme extent of damage is not common for disinfection scenarios where financial and environmental considerations often prohibit the application of such extreme disinfection strengths. On the other hand, the temperature of 55 °C that was applied for 10 min in the study by Scaturro et al. (2016) is not sufficient to cause dye uptake and thus to influence v-qPCR results in case of *Legionella.* When subjecting different *Legionella* species to such a mild heat treatment, the authors reported lower colony numbers, but no effect on PMA-qPCR results. Although the short amplicon BioRad assay was applied, probably the same result would have been obtained using the long amplicon assay described here as the heat conditions are too mild to inflict membrane damage with *Legionella*. The selective effect of 55 °C exposure on cultivation results (but not on v-qPCR) was interpreted by Scaturro et al. (2016) as a limitation of PMA-qPCR to differentiate between live and dead cells, although a different cultivation result might have been obtained in case of co-culture in the presence of free living amoeba. When applying *co*-culture conditions with *Acanthamoeba polyphaga*, *Legionella pneumophila* colonies were obtained even after exposing bacteria to 70 °C for 10 min (Allegra et al. 2008). No colonies were obtained in the absence of amoeba.

Raising the dye incubation temperature was found highly efficient for better exclusion of signals from membrane-damaged bacteria while not substantially affecting signals from intact cells. This probably owes itself to the temperature tolerance of mesophilic L. *pneumophila*. Also longer dye incubation times or the addition of EDTA proved beneficial for achieving greater signal suppression of heat-killed bacteria, while

the addition of DOC did not. DOC had been reported previously to improve dead cell signal exclusion in case of gram-negative bacteria *Vibrio vulnificus*, *Escherichia coli* and *Salmonella typhimurium* (Lee and Levin 2009; Nkuipou-Kenfack et al. 2013; Yang et al. 2011). Our research showed that this does not apply to all gram-negative bacteria and that DOC greatly affected signals of membrane-intact L. *pneumophila* preventing its use in combination with this bacterial species. The reason might lie in the fact that the route of infection of L. *pneumophila* is exclusively via inhalation and not through the digestive tract. In contrast with the other bacteria, *L. pneumophila* is not adapted to gall substances and appears susceptible. The benefit of co-incubation of a viability dye with DOC might therefore be limited to those gram-negative bacteria that are adapted to conditions in the intestines.

The presence of EDTA, on the other hand, was shown to be beneficial to selectively suppress signals of membrane-damaged L. *pneumophila*. EDTA acts by chelating membrane-associated divalent cations responsible for the tight cross linkage of outer membrane lipopolysaccharides in gram-negative bacteria, resulting in the release of a fraction of LPS and hence increasing the permeability of such treated bacteria (Leive 1968; Marvin et al. 1989). A differential effect of EDTA was observed in our study at a concentration of 2 mM EDTA (when applied in combination with PMA at 45 °C). Under these conditions PMA-induced signal suppression was enhanced in case of heat-treated L. *pneumophila*, whereas there was no measurable effect with non-heated ones. In other words, EDTA at this concentration and at 45 °C only affects outer membrane permeability if prior destabilization by heat has occurred. It should be noted that the EDTA effect on membrane permeability of fluorescent dyes is temperature-dependent. At room temperature, non-heated L. *pneumophila* can withstand an EDTA concentration of 5 mM without affecting PMA-qPCR results (relative to a control without any treatment; data not shown). The same result was obtained for 37 °C with flow cytometry where *L. pneumophila* was exposed to SYBR Green I and propidium iodide (3 μM) at 37 °C for 13 min (as in Nocker et al. 2017) in the presence or absence of 5 mM EDTA. The positions of the bacteria on the flow cytometric density plots were not affected by EDTA (data not shown). This fact was attributed to the lower staining temperature (37 °C) compared to the 45 °C applied for PMA treatment. Overall, EDTA concentration and incubation temperature have to be carefully adjusted in a species-specific manner to obtain a differential effect for intact and damaged bacteria. The differential effect disappears when either EDTA concentrations or incubation temperatures are chosen too high.

In conclusion, the dye incubation conditions presented here in combination with longer amplicon lengths and PMA treatment can lead to substantially better signal exclusion of membrane-compromised *L. pneumophila*. Although shorter amplicons tend to offer better sensitivity and therefore have an advantage for applications with very low pathogen numbers, the new assay seems beneficial when differentiation between intact and damaged cells is of primary interest and when pathogen numbers are not limiting (which is the case in many cooling tower water samples). The presented dye incubation parameters and qPCR approach might also be beneficial in combination with other viability dyes. A thorough validation of the presented assay and suggested dye incubation conditions on the basis of ISO 12869 still needs to be accomplished. Also further efforts will be necessary in applying improved assay conditions in combination with filtered samples. Described conditions are tailored for aqueous suspensions, but difficult to apply in a defined manner to filtered samples, where especially temperature control is hard to achieve.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mimet.2018.12.003.

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