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Evaluation of viability-qPCR detection system on viable and dead *Salmonella* serovar Enteritidis

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ABSTRACT

The propidium monoazide (PMA) coupled with PCR (viability PCR) is used in foodborne pathogen detection in order to detect only viable bacteria. Originally presented to fully remove the signal of dead bacteria, the limits of the viability PCR rapidly came out in the literature. In this study, the use of PMA in a viability-qPCR (v-qPCR) was assessed on viable and dead cells of *Salmonella enterica* subsp. *enterica* serovar Enteritidis. The PMA treatment protocol was modified (dark incubation duration, concentration of PMA) to evaluate if a complete negative signal of dead *Salmonella* was possible. However, none of these modifications was found to improve the removal of the remaining qPCR signal observed in the presence of dead bacteria. The present research also underlines that PMA may unexpectedly decrease the qPCR signal observed on living *S.* Enteritidis at low concentration. Finally, the use of *S.* Enteritidis cells killed by processes altering or not the cell-wall/membrane gives us a clue to answering the question about the non-total extinction of the signal of dead cells sample in the v-qPCR assay. Indeed, the data strongly indicate that the remaining qPCR signal observed in non-culturable cells does not only depend on the cell-wall/membrane integrity of the bacteria. According to these results, the authors suggest that for a rapid and reliable foodborne bacteria detection system, an enrichment followed by a qPCR analysis should be preferred to a v-qPCR.

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1. Introduction

Foodborne pathogens are an important concern as illustrated for Europe in 2012, where 91,034 human cases have been reported, resulting in 61 deaths (EFSA and ECDC, 2014). To be able to prevent the occurrence of such outbreaks, foodstuffs are monitored according to the Regulation EC 2073/2005 in which microbiological criteria are given for each food category (Commission of EU, 2005). In this regulation, the reference methods used to search for the presence of foodborne pathogens are mostly culture-based (e.g. ISO, 1996a, 1996b, 2001, 2002, 2006a, 2006b). These conventional methods are efficient and detect only viable bacteria but are time-consuming and labour-intensive. Hence, they are not suited in case of outbreaks where a rapid answer is necessary.

Molecular methods are progressively recognised as valuable alternatives since they are fast, sensitive and specific. However, polymerase chain reaction (PCR) or real-time PCR (qPCR) amplify DNA from both dead and viable bacteria as DNA remains stable after the death of bacteria (Li et al., 2013; Masters et al., 1994; Wolffs et al., 2005). Two techniques can potentially be used to detect only viable bacteria. The first one is based on the detection of mRNA by the use of reverse-transcriptase (RT) (q)PCR (Gonzalez-Escalona et al., 2009; McIngvale et al., 2002; Yaron and Matthews, 2002). However, this detection technique requires expression of the targeted gene(s), which can vary under conditions of stress. Furthermore, RNA is very sensitive to degradation in complex matrices such as food. Overall the use of RT-(q)PCR technology is more adapted for gene expression studies than as a detection system for foodborne pathogens (for a recent review, see (Postollec et al., 2011)). The second available technique relates to the viability PCR (v-PCR) or viability qPCR (v-qPCR) that is based on DNA detection of cells with intact cell/wall membranes. In v-(q)PCR, before (q)PCR amplification, a viability discrimination step is performed. In this step two known molecules can be used: ethidium monoazide (EMA) and propidium monoazide (PMA), derivatives from ethidium bromide and propidium iodide, respectively. EMA

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and PMA intercalate DNA every 4–5 nucleotides (Waring, 1965). They are positively charged molecules thus they are excluded by intact, negatively charged, bacterial cell-walls but can enter bacteria with damaged cell-wall/membranes (Nocker et al., 2006). EMA was firstly reported to be useful to quantify viable bacteria by v-PCR (Nocker and Camper, 2006; Rudi et al., 2005) but can penetrate and be toxic for viable bacteria (Nocker et al., 2006; Pan and Breidt, 2007). Afterwards, PMA was used as a non-toxic alternative (Nocker et al., 2006; Pan and Breidt, 2007) able of covalent cross-linkage with DNA under light exposure (Coffman et al., 1982). The precise mode of action of PMA remains unclear: the DNA charge changes (Nocker and Camper, 2006) and/or the DNA is cleaved (Soejima et al., 2007), leading to reduced ability to extract or amplify DNA, respectively. Since PMA is not able to penetrate bacteria with intact cell-wall/membranes, only the DNA from bacteria with compromised cell-wall/membranes is bound and is thus not amplified by (q)PCR (Nocker et al., 2006; Shapiro, 2003).

The definition of "bacterial viability" is still a subject of controversy. The most usual one is: when a sample is plated out on an appropriate solid medium, dead bacteria are unable to produce colony forming unit (CFU) whereas a viable bacteria is able to form CFU (Trevors, 2012). In this definition, the cell wall/membrane integrity of the different states is not taken into account. Another approach, based on culturability, metabolic activity and membrane integrity has been described (Nocker and Camper, 2009). In this model, four different states are postulated. The "living" bacteria are defined as culturable, metabolically active and with an intact cell-wall/membrane. The "viable but non-culturable" (VBNC) bacteria are defined as metabolically active, with an intact cell-wall/membrane but non-culturable. The "ghost" bacteria have an intact cell-wall/membrane but are metabolically inactive and non-culturable. Finally the "membrane compromised" cells have a compromised cell-wall/membrane, are non-culturable and have no metabolic activity.

The use of PMA was recently coupled with PCR (Banihashemi et al., 2012; Cawthorn and Witthuhn, 2008; Yang et al., 2012) or qPCR (Dinu and Bach, 2013; Elizaquivel et al., 2012a,2012b; Josefsen et al., 2010; Kim and Ko, 2012; Liang et al., 2011; Mamlouk et al., 2012; Singh et al., 2013; Soejima et al., 2012; van Frankenhuyzen et al., 2011) in order to detect only viable bacteria. PMA was originally reported to fully remove the signal of dead bacteria in v-PCR (Nocker et al., 2006) and v-qPCR analysis (Josefsen et al., 2010), but later several drawbacks of the technique were reported. Indeed, PMA treatment does not always lead to complete removal of the qPCR signal of dead bacteria (see review in (Fittipaldi et al., 2010)). In particular, recent studies have showed that PMA treatment does not fully remove the signal from dead bacteria if i) the amplicon size of the qPCR assay is short (Li and Chen, 2013; Luo et al., 2010; Martin et al., 2013; Schnetzinger et al., 2013), ii) the target bacteria is at high concentration (Elizaquivel et al., 2012c; Li and Chen, 2013; Pacholewicz et al., 2013; Zhu et al., 2012), iii) the concentration of Mg^{2+} in the PCR reaction is not adapted (Nocker et al., 2006), or iv) the fat content of food sample is high (Yang et al., 2011), and may also vary according to the "killing" treatment (Kobayashi et al., 2010; Liang and Keeley, 2012; Nocker et al., 2007; Yang et al., 2011).

In this study, optimisation of the PMA protocol (by variation of dark incubation and duration and PMA concentration) was first assessed to achieve full extinction of the dead bacteria qPCR signal. Afterwards, to evaluate the dynamic range of the v-qPCR assay, different amounts of living and isopropanol-killed *Salmonella* Enteritidis were tested. Finally, for a better understanding of the mode of action of PMA, cells of *S.* Enteritidis killed by different processes affecting or not the cell-wall/membrane integrity were simultaneously analysed with v-qPCR, culture-based and microscopic observation. The general applicability of v-qPCR for a *S.* Enteritidis detection system is also discussed.

2. Material and methods

2.1. Bacteria strain and growth conditions

Salmonella enterica Enteritidis (H,VI,6,32 from Belgian *Salmonella* NRC) was used as model bacteria for Gram-negative foodborne pathogens. One single typical colony was inoculated in 10 ml of sterile Brain Heart Infusion (BHI) broth, vortexed and incubated at 37 °C for about 16 h. Ten microliters of this overnight (ON) culture was inoculated into a new 10 ml sterile BHI broth and incubated without shaking for 3.5 to 4 h at 37 °C to get a culture at an exponential growth phase (OD 600 nm between 0.3 and 0.6).

2.2. Enumeration of S. Enteritidis

The initial concentration of *S.* Enteritidis was determined by performing a tenfold serial dilution, 100 μl of the dilutions −4 to −7 was plated on Nutrient Agar (NA) and incubated at 37 °C overnight. Plates with less than 300 CFU were retained for enumeration and calculation of the initial bacterial counts expressed as colony forming units/ml (CFU/ml).

2.3. Death control

The killing process used on viable *S.* Enteritidis was evaluated by enumerating the growing cells by streaking 100 μl of the treated sample on NA plate followed by 24 h of incubation at 37 °C.

2.4. PMA treatment

One hundred microliters of tested sample was transferred in a 1.5 ml Eppendorf tube. In a dark room (PMA is sensitive to light), 75 μM or 150 μM of PMA 20 mM (Biotium: BTIU40019) was added to each tube. Tubes were incubated in a dark room under rotating agitation for 5 or 60 min. At the dark incubation step, PMA penetrates the permeable cell-wall/membranes and gets access to the DNA of cell-wall/membrane compromised bacteria. Tubes were transferred to the PhAST blue lamp (GenIUL) to undergo the LED-light exposure for 15 min. The light treatment covalently binds PMA to DNA. Free PMA was removed by harvesting the bacteria 10 min at 6000 ×g. The pellet was re-suspended in 100 μl of DNAse/RNAse free water (Acros, Geel, Belgium).

2.5. Processes used to kill the S. Enteritidis cultures

The killing processes were chosen for their possible occurrence in food product (heat and freezing) or for the membrane status of the cell after treatment (isopropanol and kanamycin). All killing processes were applied to a culture at the exponential growth phase (between 10^6 and 10^8 CFU/ml).

- Isopropanol 70% was added to aliquots of *S.* Enteritidis culture and the bacterial suspension was incubated for 10 min. Isopropanol was removed by harvesting the *S.* Enteritidis 10 min at 6000 ×g prior to re-suspension in the initial volume of BHI.
- Heat: 400 or 500 μl of *S.* Enteritidis culture was heated for 15 min at 99 °C in a thermoblock (dry bath).
- Kanamycin: 5 ml of *S.* Enteritidis culture (sensitive to kanamycin) was supplemented with kanamycin at 1 mg/ml and incubated for 16–18 h at 37 °C.
- Freezing: 500 μl of *S.* Enteritidis culture was immersed into liquid nitrogen for 15 min and transfered to −20 °C for 16–18 h. Note that freezing is a stress process that leads to a portion of dead *S.* Enteritidis.

2.6. DNA extraction

DNA extraction was performed by heat-lysis for 15 min at 99 °C in a thermoblock. Cell debris was removed by centrifugation 10 min at 6000 ×*g*. The supernatant (containing the DNA) was collected in a new Eppendorf tube.

2.7. Real-time PCR assays

The qPCR assays were performed on an iCycler iQ™5 Real-TIME PCR Detection System (BioRad) with iCycler iQ™ PCR plates, 96 wells (BioRad) closed with the PCR Sealers Microseal B films (BioRad). The reaction was performed in a final volume of 25 μl containing 5 μl of template, 1X SYBR®Green PCR Mastermix (Diagenode), and 250 nM of each primer (*safC*-10 *Salmonella* spp. detection qPCR assay (72 bp) (Barbau-Piednoir et al., 2013)). The following thermal programme was applied: a single cycle of DNA polymerase activation for 10 min at 95 °C followed by 40 amplification cycles of 15 s at 95 °C (denaturing step) and 1 min at 60 °C (annealing-extension step). Subsequently, melting temperature analysis of the amplification products was performed by gradually increasing the temperature from 60 °C to 95 °C over 20 min (±0.6 °C/20 s). Positive control using gDNA of *S.* Enteritidis at 10^4 copies and negative control using DNAse and RNase free water (Acros, Geel, Belgium) were included in each qPCR reaction.

2.8. Data analysis

2.8.1. SYBR®Green qPCR assay

For the interpretation of SYBR®Green qPCR assays, two criteria were taken into consideration: the quantification cycle (Cq) value, and the melting temperature of the amplicon (Tm). The Cq-value represents the cycle at which the PCR amplification reaches the threshold level of the reaction (Bustin, 2000). To be considered as positive, a signal generated in SYBR®Green qPCR analysis should display an (exponential) amplification above the threshold level associated with the specific Tm-value of the amplicon.

2.8.2. PMA effect

To evaluate the effect of PMA treatment on a sample, the ΔCq was calculated. The ΔCq of a sample is the difference between the Cq-value obtained with PMA treated sample and the Cq-value obtained with not treated sample: $\Delta Cq = (Cq_{\text{sample w/ PMA}} - Cq_{\text{sample w/o PMA}})$.

2.8.3. Statistical analysis

The data were analysed with Splus 8.0 for Linux. A linear mixed model was set up with conditions as fixed factors and date of analysis and aliquot number as nested random factors. Predefined contrasts between conditions were calculated and their corresponding P-values were corrected for simultaneous hypothesis testing according to the Sidak correction. Differences with P values < 0.05 were considered statistically different.

2.9. Live/Dead® BacLight™ Bacterial Viability kit analysis

In order to discriminate by microscopy bacteria with compromised cell-wall/membranes (dead) from those with non-compromised cell-wall/membranes (viable) the LIVE/DEAD® BacLight™ Bacterial Viability kit (L7007 form Molecular probes) was used according to the manufacturer's recommendations. This kit provides a two-colour fluorescence assay: Syto®9 green-fluorescent nucleic acid stain (480/500 nm) and propidium iodine (PI) red-fluorescent nucleic acid stain (490/635 nm). Syto®9 stains all bacteria in a population whereas PI penetrates only cell-wall/membrane compromised bacteria and causes a reduction in SYTO®9 stain fluorescence when both are present. This kit displays an appropriate mixture of SYTO®9 and PI stains leading to green fluorescence with intact cell-wall/membranes (live) bacteria and red-fluorescence for bacteria with damaged cell-wall/membrane (dead).

A 100 μl aliquot of each tested sample was transferred into a sterile 1.5 ml Eppendorf and the pellet was harvested by centrifugation 10 min at 6000 × *g*. The pellet was re-suspended in 100 μl of physiologic water and stained with 0.3 μl of (v/v) mixture of components A and B (L7007 LIVE/DEAD® BacLight™ Bacterial Viability kit). Samples were mixed thoroughly and incubated at room temperature for 15 min in the dark. One-hundred microliter of agarose 2% in physiological water was added to each sample (final concentration 1% agarose). Seventy-five microliters was trapped between a slide and a 18 mm square cover slip. Confocal fluorescence microscopy (CFM) images were taken using an LSM710 Microscope (Zeiss) with a 40× oil immersion objective and 1× zoom. Fluorescent images of SYTO®9 and PI stained bacteria were acquired at 488 and 543 nm, respectively. The count of dead (red) and live (green) bacteria was performed using the Imaris Software.

3. Results and discussion

3.1. Optimisation of the PMA protocol

To obtain full extinction of the dead bacteria qPCR signal using v-qPCR, optimization of the PMA protocol was sought by varying the dark incubation duration and PMA concentration.

3.1.1. Effect of increased dark incubation time on qPCR signal reduction

To investigate whether duration of the PMA incubation in the dark has an effect on the extinction of the dead *S.* Enteritidis qPCR signal, 5 and 60 min dark incubation times were compared. Two concentrations of PMA, 0 and 75 μM, were used in both conditions, with *S.* Enteritidis suspensions at a concentration of ca. 8.7 log CFU/ml (4.7×10^8 and 6.4×10^8 CFU/ml for the two independent analyses). Samples were analysed in triplicate in each independent analysis (n = 6) and qPCR was performed in duplicate on each sample. The qPCR positive and negative controls gave the expected positive and negative results, respectively. For the dead samples, one of the most common killing processes, isopropanol 70%, was used to eliminate the bacteria. Death was checked on plate and around 37 CFU/ml (first repetition) and 0 CFU/ml (second repetition) of *S.* Enteritidis were recovered after isopropanol treatment.

As shown in Fig. 1A, no effect of PMA treatment was observed on living *S.* Enteritidis for both dark incubation times since their average signal reductions (ΔCq) were not significantly different. Also, as expected, PMA treatment induced a significant reduction of the qPCR signal (P = 0.0001) on isopropanol-killed *S.* Enteritidis. However, no significant difference was observed between 5 and 60 min of dark incubation (P = 0.85). The average Cq reduction was ca. 4 for both incubation periods. Therefore, increasing the incubation time from 5 to 60 min had no significant effect on the ΔCq of dead *S.* Enteritidis treated by PMA and thus cannot be used to improve the protocol.

3.1.2. Effect of PMA concentration on qPCR signal reduction

To investigate whether PMA concentration has an effect on the reduction of the qPCR signal associated with dead *S.* Enteritidis, 0, 75 and 150 μM PMA were compared (5 min dark incubation was used for all PMA concentrations). A dynamic range of *S.* Enteritidis culture of tenfold dilution from 7.9 to 3.9 log CFU/ml (7.6×10^7 CFU/ml to 7.6×10^3 CFU/ml) were used. Killing was performed by isopropanol 70%. The efficiency of the killing procedure was verified for each *S.* Enteritidis dilution by the absence of growth on nutrient agar. The Cq-value of the dead samples not treated with PMA (0 μM), treated with 75 and 150 μM was compared (Fig. 1B). The qPCR positive and negative controls gave the expected positive and negative results, respectively.

As expected, a higher Cq-value was observed with the PMA treatments (ΔCq = ca. 7) than with no PMA treatment (0 μM). Yet, for all

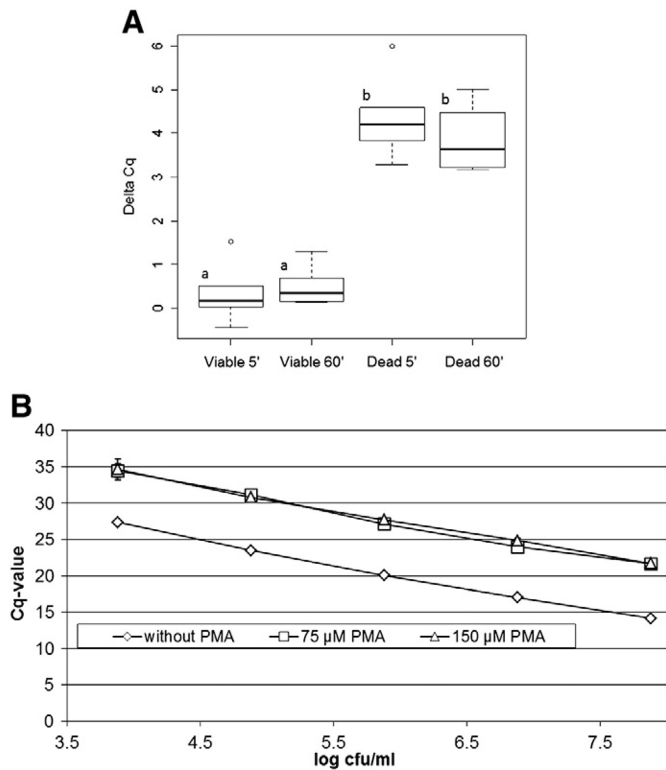

Fig. 1. Effect of dark incubation time and PMA concentration on viable and dead cultures of *S.* Enteritidis. A. Boxplot of different PMA incubation periods (5 and 60 min) on viable (n = 6) and dead (n = 6) bacteria at 8.7 log CFU/ml. Delta Cq: qPCR signal reduction between samples treated and not treated with PMA 75 μM, different letters denote values significantly different at $P \leq 0.05$. B. Effect of three PMA concentrations (0, 75 and 150 μM) on five different suspensions of dead cells: 7.9, 6.9, 5.9, 4.9 and 3.9 CFU/ml (n = 1).

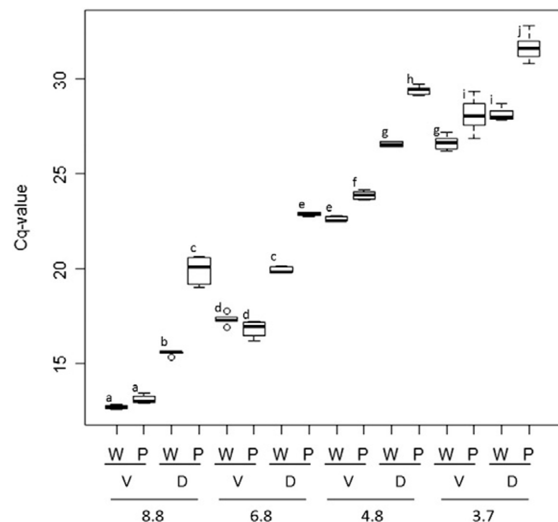

Fig. 2. Effect of PMA on the Cq-values of different concentrations of viable and dead *S.* Enteritidis (n = 3). W: without PMA, P: with PMA 75 μM, V:viable, D: dead. The numbers 8.8, 6.8, 4.8 and 3.7 indicate the cell concentration in log CFU/ml. Different letters denote values significantly different at $P \leq 0.05$.

the tested dilutions, the Cq-values obtained with 75 and 150 μM of PMA were not significantly different. Interestingly, these observations indicate that the increase in PMA concentration does not further reduce the qPCR signal of dead *S.* Enteritidis samples.

3.2. PMA effect on different S. Enteritidis concentrations

To assess whether the amount of bacteria in a sample may have an impact on the reduction of the v-qPCR signal of dead bacteria, four *S.* Enteritidis concentrations were used: 3.7, 4.8, 6.8 and 8.8 log CFU/ml. Each sample was tested in triplicate and qPCR was performed in duplicate on each replicate. The qPCR positive and negative controls gave the expected positive and negative results, respectively. Living and 70% isopropanol-killed *S.* Enteritidis were tested. The efficiency of the killing procedure was verified by the absence of colony on plate for all tested concentrations of *S.* Enteritidis. The Cq-values obtained with each type of sample were recorded (Fig. 2).

This experiment gave three distinct results. Firstly, the Cq-values obtained with living and dead samples not treated with PMA present an unexpected significant shift between 1 and 4 Cq for the four tested concentrations of cells. This illustrates that the death itself already induces a reduction of the qPCR signal which is probably due to the loss of DNA occurring at the harvesting cell step, from dead cells with highly damaged membrane/cell wall. Secondly, for dead *S.* Enteritidis, as expected, the Cq-values observed with PMA treated samples were significantly higher than with non PMA treated samples for all the studied concentrations (Fig. 2: 8.8D, 6.8D, 4.8D and 3.7D). The shift observed was significantly higher for 8.8 log CFU/ml with a ΔCq ca. 4.5 whereas it was the same for the 3 other concentrations with a ΔCq between 2.7 and 3.9. These results confirm the effect of PMA on dead sample but also demonstrate again that PMA is not able to completely remove the signal of dead cells at all studied concentrations. Thirdly, as previously shown, on living samples (8.8A and 6.8A) at high concentration (8.8 and 6.8 log CFU/ml), PMA treatment has no effect on the signal reduction since no significant differences were observed between Cq-values obtained on living cells (Fig. 2: 8.8A and 6.8A) treated or not with PMA. On the contrary, at lower concentrations (3.7 and 4.8 log CFU/ml), PMA has a significant effect on viable cells. Indeed, the Cq-values obtained with living samples treated with PMA are significantly higher, with a shift between 1 and 2.6 Cq, than the non treated living samples (Fig. 2: 4.8A and 3.7A). This reduction of the signal of living cells at low concentration may lead to underestimation of viable bacteria and, in the worst case scenario, may lead to false negative results with very low amount of viable bacteria. This reduction of viable cells signal was previously observed on *Legionella* spp. (Yanez et al., 2011) and on *Escherichia coli* (Liu and Mustapha, 2014).

3.3. Effect of different killing processes to the v-qPCR response

To better understand why PMA treatment does not completely remove the qPCR signal of dead *S.* Enteritidis (between 7.9 and 11 log CFU/ml), four distinct killing or stress processes (heat, isopropanol, antibiotic or freezing) were analysed using three different viability evaluation protocols: i) samples were plated on nutrient agar (conventional microbiological technique), ii) samples were analysed with v-qPCR (molecular technique) with 75 μM PMA and 60 min dark incubation, and iii) samples were dyed with the Live/Dead® BacLight™ Bacterial Viability kit and observed microscopically (microscopic technique). The four distinct killing or stress processes were chosen to act differently on the cell-wall/membrane integrity. Alcohols (such as isopropanol) dehydrate bacteria, disrupt cell-wall/membranes and cause coagulation of proteins. Heat denatures the proteins of the cell-wall and membrane, the phospholipids become more fluid, leading to disruption of the integrity of the cell-wall and membrane. Freezing forms ice crystals inside the bacteria that can rupture the cell-wall/membrane. Isopropanol, heat and freeze-killed samples should induce a high percentage of dead bacteria with disrupted cell-wall/membranes. Kanamycin has a different mode of action. By interacting with the 30S subunit of ribosomes, it induces mistranslation and indirectly inhibits protein synthesis (Misumi and Tanaka, 1980). Kanamycin-treated samples should therefore contain a high rate of dead bacteria with intact cell-wall/membrane (ghost cells: Nocker and Camper, 2009).

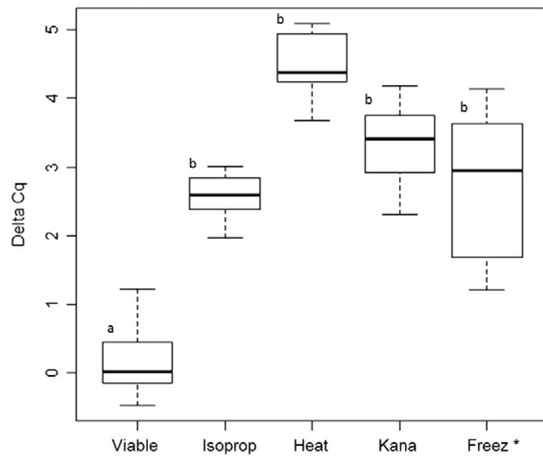

Fig. 3. Boxplot of the effect of PMA 75 μM on qPCR signal reduction of *S.* Enteritidis cells treated with isopropanol, heat, kanamycin or a freezing treatment (n = 9). *Not 100% dead; different letters denote values significantly different at P ≤ 0.05.

Compared to the control experiment using viable bacteria, the v-qPCR, displayed significant reduction of the signal (between 2.5 to 4.5 Cq) on bacteria samples (n = 9) killed by the four different processes (Fig. 3). Unexpectedly however, no significant difference in the Cq signal reduction was recorded between the different killing processes (Fig. 3). The qPCR positive and negative controls gave the expected positive and negative results respectively.

To further investigate these data in relation to cell-wall/membrane integrity, v-qPCR results were compared with plate counts and Live/Dead kit microscopic observations (Table 1). Plate count assesses the "culturability" of the bacteria whereas Live/Dead kit discriminates bacteria on the basis of their cell-wall/membrane integrity. The first interesting observation was that even if no culturable bacteria were observed, bacteria with intact cell-wall/membrane remained visible (e.g. isopropanol, kanamycin and heat treatments). Heat and isopropanol treated samples included bacteria presenting an intact cell-wall/membrane (5 and 13%) whereas no culturable bacteria were observed on plate (Table 1). For freezing, 2% of the cells had an intact membrane and between 0.5 and 1.7% were culturable. These intact cell-wall/membrane cells amongst non-culturable cells may explain the remaining qPCR signal of dead cells, as previously reported (Kralik et al., 2010; Lovdal et al., 2011; Pan and Breidt, 2007). Contrary to heat and isopropanol treatments and as expected, for kanamycin treated samples, a high percentage of bacteria with intact cell-wall/membranes (80%) was observed whereas none of the bacteria in the kanamycin treated sample were culturable. According to the PMA mode of action, this must have a drastic effect on the v-qPCR signal reduction: in such samples, no or very low extinction of the signal (ΔCq) should have been observed. However, this was not the case since the kanamycin treated samples ΔCq were not significantly different from the other killing processes (Table 1 and Fig. 3). These observations demonstrated that the v-qPCR results do not strictly correlate with the physical status of the bacterial cell-wall/membrane.

4. Conclusion

As the rapid detection of foodborne pathogens remains a challenge, the improvement of the currently available and internationally accepted methods is needed. The ISO/TS 13136 for the detection of Shiga toxin-producing *E. coli* (ISO, 2012) was the first reference method using qPCR instead of culture-based method as a screening system. This opened the way for internationally accepted qPCR detection systems in foodborne pathogen detection. However, there is still room to speed up the detection method by replacing the enrichment step which takes around 18 h. The use of PMA treatment instead of enrichment before qPCR analysis, called v-qPCR, was proposed as a potential alternative.

The aim of this study was first to optimise the v-qPCR protocol to obtain full extinction of the dead bacteria signal and secondly to study the results of v-qPCR on *S.* Enteritidis cells when different killing and stress conditions are applied.

The present study confirms previous observations where v-qPCR was shown as inefficient to completely remove qPCR signal of dead *Mycobacterium avium*, *Listeria innocua* and *Listeria monocytogenes* (Kralik et al., 2010; Lovdal et al., 2011; Pan and Breidt, 2007). Indeed, in this study, from 3.7 to 8.8 log CFU/ml of dead *S. enterica*, full extinction of the v-qPCR signal was never reached, only a reduction of the signal between 2 to 7 Cq was observed in the different experiments reported in this study. Neither longer dark incubation nor higher concentration of PMA resulted in a significant improvement of the signal reduction. Other studies describe possibilities of improvements of the v-qPCR by further disrupting of the cell-wall/membranes of dead *S. enterica*, *L. monocytogenes* and *E. coli* before PMA treatment (Nkuipou-Kenfack et al., 2013; Wang et al., 2014a, 2014b; Yang et al., 2011, 2014). In these papers, even if the reduction of the signal is better, a signal is still observed with dead bacteria. This may lead to overestimation of the presence of viable bacteria in a sample and a false positive signal with dead bacteria. This is not a major issue for detection purposes since detected bacteria should always be confirmed by isolation of the bacteria.

Our research also underlines that the PMA may reduce the signal of viable bacteria at low concentration and confirms other recent studies (Liu and Mustapha, 2014; Yanez et al., 2011). This represents a risk of false negative results if the reduced signal falls below the LOD of the qPCR assay. These false negatives are not acceptable in a foodborne pathogen detection system where zero tolerance is the rule, as is the case for *S.* Enteritidis (Commission of EU, 2005, 2013).

The last experiment performed on *S.* Enteritidis killed by processes altering or not the cell-wall/membrane gave some clues to the question about the non-total extinction of dead sample signal in v-qPCR assay. Indeed, a portion of the non-culturable bacteria (expected to be dead) has an intact membrane/cell-wall. Since PMA is able to penetrate only bacteria with compromised cell-walls/membranes, in v-qPCR assay, dead bacteria with intact membrane/cell-wall should give a signal similar to

Table 1
Comparison of different assays used to evaluate the percentage of dead *S.* Enteritidis in a sample.

Treatment	Microscopic observation					Plate count			v-PCR
	Alive cells (green)	Dead cells (red)	Total count	Percentage of cells with intact membrane	Percentage of membrane-compromised cells	CFU/ml	Percentage of "culturable" cells	Percentage of non "culturable" cells	ΔCq-PMA
No (living cells)	101	15	116	87	13	$8.1 \cdot 10^7$	Theoretically 100%	Theoretically 0%	0.9 ± 0.4
Isopropanol	38	252	290	13	87	<3	0	100	2.3 ± 0.2
Kanamycin	290	74	364	80	20	<3	0	100	3.1 ± 0.7
Heat	19	355	374	5	95	<3	0	100	4.5 ± 0.3
Freezing	17	904	921	2	98	$4.05 \cdot 10^5$ to $1.35 \cdot 10^6$*	0.5 to 1.7*	98.3 to 99.5*	2.5 ± 0.6

* None countable (bacterial lawn): approximated from other freezing experiments.

that of viable bacteria. However, the kanamycin-treated samples raised incoherence about the mode of action of the v-qPCR. Indeed, these types of inactivated samples displayed a high percentage of non-culturable bacteria with intact cell-wall/membrane although they unexpectedly gave one of the best ΔCq in v-qPCR. These results strikingly illustrate that the v-qPCR results are not only based on the bacterial cell-wall/ membrane integrity.

To conclude, according to the results of this study and of a recent review (Fittipaldi et al., 2012), for a qPCR foodborne pathogen detection system, it is not recommended to replace the enrichment step by a PMA treatment, in its current form. At this stage of current knowledge on foodborne pathogenic bacteria detection systems, the use of enrichment before qPCR analysis followed by confirmation of positive results with culture-based methods continues to remain as the most appropriate approach.

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