Optimization of a viability-PCR method for the detection of *Listeria monocytogenes* in food samples

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ABSTRACT

Rapid detection of *Listeria* and other microbial pathogens in food is an essential part of quality control and it is critical for ensuring the safety of consumers. Culture-based methods for detecting foodborne pathogens are time-consuming, laborious and cannot detect viable but non-culturable microorganism, whereas viability PCR methodology provides quick results; it is able to detect viable but non-culturable cells, and allows for easier handling of large amount of samples. Although the most critical point to use viability PCR technique is achieving the complete exclusion of dead cells amplification signals, many improvements are being introduced to overcome this. In the present work, the yield of dead cell DNA neutralization was enhanced by incorporating two new sample treatment strategies: tube change combined with a double light treatment. This procedure was successfully tested using artificially contaminated food samples, showing improved neutralization of dead cell DNA.

Keywords: propidium monoazide, viability PCR, live-dead distinction, *Listeria monocytogenes*, foodborne pathogen detection

INTRODUCTION

Listeria monocytogenes is one of the most virulent foodborne pathogens. It is a Gram-positive and facultative anaerobic bacterium, which is capable of growing and replicating inside the host's cells. This bacterium causes listeriosis, the third-leading cause of death among foodborne bacterial pathogens, with fatality rates exceeding even *Salmonella* and *Clostridium botulinum* [19].

Listeria is commonly found in dairy products, meat, poultry, and seafood. Foods that are ready to eat, require refrigeration, and if are stored for an extended period of time have a higher likelihood of contamination than other products. *L. monocytogenes* has the ability to adapt to a wide range of conditions and survives in stressful environments, such as nutrient starvation, low refrigeration temperatures and osmotic and oxidative stress [2, 12]. To prevent *Listeria* infection and guarantee consumer safety, the control of this bacterium is required. Cell culture is the commonly performed method for detection and isolation of *L. monocytogenes* according to the U.S. Food and Drug Administration (FDA) specifications [7].

The culture approach is a time consuming methodology. Standard methods for detection of *L. monocytogenes* by culture at levels lower than 100 CFU g^{-1} require at least 48h. As a first step, a primary selective enrichment (24h) is needed, followed by plate isolation (24h) and in some cases (depending on each analytical standard) by a biochemical confirmation step. Although the isolation with differential media can be done with the primary enrichment, according to the International Organization Standardisation (ISO) method (UNE-EN ISO 11290-1:2017) [21], it is also necessary an additional secondary enrichment in order to ensure a complete cell growth. Moreover, this method presents other limitations such as the inability to detect viable but non-culturable (VBNC) cells [26] and detection difficulties as result of the possible presence of other *Listeria* species that usually outgrow and mask *L. monocytogenes* detection.

Listeria direct enumeration by plating on selective and differential agar plates, can only be done in samples in which high levels of *Listeria* cells are suspected to be present (>100 CFU g^{-1}). On the other hand, the quality criterion from some CE rules (CE 1441/2007) specifies the absence of the pathogen in 25 gr in certain foods. Under this scenario, it is highly likely that in the majority of the cases previous culture enrichment be necessary before any detection method can be applied.

The development of more rapid and sensitive methods for the detection and quantification of viable *L. monocytogenes* cells is essential for monitoring food quality and listeriosis prevention [14], but also for extending commercial life of short-term food products. In order to cover these needs, new methods using PCR techniques have been developed [3, 15]. PCR techniques allow quick results, the detection of VBNC *Listeria* cells, easier handling of large samples and, moreover, fit perfectly with the current workflows, such as being applicable after the primary enrichment. In this case the *L. monocytogenes* absence can be confirmed in 24h. However, PCR detects DNA in both live and dead cells and, as a consequence of this, it typically overestimates the quantity of infectious *Listeria* cells. Among other molecular strategies, viability PCR (v-PCR) and RT-PCR methods have been proposed as alternatives to culture-dependent procedure to discriminate between dead and viable cells [6, 24].

The v-PCR is based in the use of photo reactive DNA-intercalating agents, like ethidium monoazide (EMA), propidium monoazide (PMA) and PEMAX™, which can only penetrate membrane-damaged cells and neutralize DNA by the means of light treatment. The application of this sample treatment in conjunction with PCR, allows for the detection of viable bacteria. V-PCR methodology has been assessed to detect viable *L. monocytogenes* by several authors [5, 8, 17, 18] but with drawbacks, the problem of false-positive results being the most important among them. This issue hinders results interpretation, especially in the analysis of complex samples [10].

The DNA neutralization from dead cells, without affecting live cells is the common challenge when a new v-PCR procedure is developed. Different researchers have followed diverse strategies for improving the procedures and overcome this bias. Up to now, most of the attention is focused on dye selection, incubation time, dye photoactivation exposure, reaction buffer composition, and PCR amplicon size [9]. In addition, the impact of the microtube on the v-qPCR analytical bias has been recently demonstrated [1].

In this work, a new scheme which combines a tube change procedure with a double light treatment was explored as a strategy for obtaining an optimum *L. monocytogenes* qualitative real-time v-PCR method. Aiming at developing an improved methodology to exclusively detect live *Listeria* cells in different food matrices, while avoiding time-consuming tasks and limitations of culture based methods. The performance of this novel approach was evaluated using artificially contaminated food samples.

MATERIAL AND METHODS

Bacterial inoculums

L. monocytogenes (CECT 4032) was incubated at 37ºC for 24 hours on PCA medium (Liofilchem, VWR, Barcelona, Spain). Bacterial cells were harvested from the agar plates and diluted in sterile phosphate-buffered saline (PBS 1X, pH 7.4) to obtain a working bacterial suspension. The cell density was adjusted to an OD600 of 0.30, corresponding to 5.0×10^{8} cells ml^{-1}.

To obtain dead cells stocks, working bacterial suspension was heated at 85ºC for 35 min using a standard laboratory heat block (Termomixer comfort, Eppendorf, Hamburg, Germany) and stored at -20ºC until use. Dead cells were plated on PCA medium in order to ensure the non-viability of the cells.

Optimization of the viability dye treatment

To assess the effect of photoactivation conditions and the impact of microtubes changes on v-qPCR, three different approaches were evaluated with live and dead cells stocks (5.0×10^{7} CFU $sample^{-1}$):

Treatment 1, Single light treatment without change of tube.

Treatment 2, Single light treatment and double change of tube.

Treatment 3, Double light treatment and double change of tube.

Two independents experiments were carried out, and in each case treatments were evaluated by duplicate.

Viability dye treatment

PMA dye (GenIUL, Barcelona, Spain) was resuspended in PCR grade water (VWR) to obtain stock dye solution of 2 mM. It was dispensed into dark tubes and stored at −20°C until needed.

500 µl of sample aliquots were centrifuged at 14,100×g for 5 min, and the cell pellets were resuspended in PBS at a final volume of 500 µl. PMA stock solution was added to the samples, to obtain a final dye concentration of 50 µM. Samples were incubated in the dark at 24ºC for 30 min to allow dye penetration into dead cells with damaged membranes. After incubation in the dark, the samples with Treatment 2 and 3 were totally transferred to a new tube. Samples with Treatment 1 were kept in the same tube. All samples were then exposed to light treatment, single (15 min of light) or double (15 min of light +10 min of darkness +15 min of light) using PhAST Blue system (GenIUL) at 100% intensity. After photoactivation, samples with Treatment 2 and 3 were transferred again to a new tube. Samples with

Treatment 1 were kept in the same tube. The samples were subsequently centrifuged at 14,100×g for 5 min and the supernatant was discarded. In addition, 500 µl of control samples untreated with PMA dye were concentrated by centrifugation and the supernatant was discarded.

DNA purification and qualitative real-time PCR assay

DNA was extracted using the v-DNA reagent (GenIUL), according to the manufacturer's instructions. Briefly, the cell pellets were resuspended in 200 µl of v-DNA reagent (GenIUL) and were vortexed at 3,200 rpm for 5 min using multiplate shaker (Biosan, Riga, Latvia). Then, the cells were incubated at 80ºC for 10 min at 1,200 rpm using a heat block (Termomixer comfort, Eppendorf). Then, 600 µl of v-DNA buffer (GenIUL) were added and samples were vortexed again at 3,200 rpm for 2 min. Thereafter, they were centrifuged at 7,500×g for 2 min and 100 µl of supernatant were transferred to a new tube. Following DNA purification, the samples were analysed by qualitative real-time PCR.

For *L. monocytogenes* detection, a PCR procedure previously described by D'Agostino et al. [4] was adapted. Reactions were performed in the PikoReal^{TM} real-time PCR system (Thermo Fisher Scientific, Massachusetts, USA) with the following real-time PCR cycling conditions optimized previously (data not shown): 12 min at 95°C, 45 cycles of 10 sec at 95°C, 20 sec at 60°C, and 20 sec at 72ºC followed by data acquisition, and finally a melting temperature (Tm) ramp from 65°C to 95°C at 0.2°C s^{-1}. All reactions were performed at a final volume of 20 µl and contained 4 µl of 5x HOT FIREPol® Evagreen® qPCR Supermix (Solis BioDyne, Tartu, Estonia), 5 µl of DNA template and 0.25 µM of primers. The primers according to D'Agostino et al. [4] amplified a 274 bp fragment of the prfA gene. Negative control, water PCR grade (VWR), and positive control, DNA from *L. monocytogenes*, were included in each qualitative real-time PCR assay. PCR inhibitions were evaluated by melting curve analysis and Tm value.

Food Samples Handling

A total of 26 commercial processed foods were purchased from local markets. The samples were not tested for *Listeria* before artificial speaking. For each food, 10 g were mixed with 90 ml of Half Fraser broth (Reactivos para diagnóstico, Barcelona, Spain) into a sample filter bag (IUL S.A., Barcelona, Spain). This amount was chosen taking into account the ratio (1:9) specified in the ISO regulation (UNE-EN ISO 11290-1 (2017) [21]. Then, live and heat-treated *L. monocytogenes* cells at 1.0-5.0×10^1 and 1.0-5.0×10^7 CFU g^{-1} respectively, were spiked into food suspensions and homogenized for 30 s in a paddle

blender homogenizer (Masticator, IUL). 100 ml of Half Fraser broth inoculated with bacteria was used as control. Artificially contaminated samples were incubated at 30°C for 24 h.

Listeria detection in processed food samples by culture

Presence and CFU levels of *L. monocytogenes* in spiked food samples were measured by plate culture. For that, 1 ml sample aliquots were taken after 24 h of enrichment, and were serially logarithmic diluted in PBS, spread-plated on Ottaviani Agosti *Listeria* agar (Reactivos para diagnóstico), and incubated at 37ºC for 24 h.

Listeria detection in processed food samples by qualitative real-time v-PCR

Sample aliquots of 1 ml taken at time 0 h and 24 h were centrifuged at 800×g for 2 min, separating the food sample debris pellet. 100 µl of supernatant were transferred in a new microtube, centrifuged at 14,100×g for 5 min, and the cell pellets were resuspended in 200 µl of PBS and analysed by qualitative real-time v-PCR as it is depicted in the viability dye treatment section. In this case, the treatment 3 before mentioned was used since it was determined as the optimum one.

Statistical analysis

Mean values and standard deviations were calculated on the basis of two independent experiments, each performed in duplicate. Microsoft Office Excel 2010 (Microsoft Corporation, Redmond, WA, USA) was used for statistical analysis. Shapiro–Wilk test was conducted to check for the normality distribution of data, in each treatment group, considering a normality distribution at p-value>0.05. One-way analysis of variance (ANOVA) was used to examine differences between the treatments. Tukey's HSD test was used to identify significant differences between treatment groups. Differences and correlations were considered statistically significant at p-value<0.05.

RESULTS

Optimization of qualitative real-time v-PCR cell sample treatment

The effects of the different approaches evaluated are depicted in Figure 1. As expected, PMA treatment induced a great ΔCt value (dead with PMA−dead without PMA) in dead cells suspensions with a minimum impact on live cells suspension. The results of ΔCt were: Treatment 1, 14.3 with a 95% confidence interval (CI) of (16.0–12.6), Treatment 2, 15.9 with a 95% CI of (17.6–14.2) and Treatment 3, 18.0 with a 95% CI of (18.5–17.5). Statistical significant differences in dead cells were observed among

the three tested treatments ($p < 0.05$). Between Treatment 1, the conventional approach, and Treatment 3, the herein proposed new protocol, the results show a significant difference of 3.7 Ct, being the biggest difference among the treatments evaluated. Based on these results, Treatment 3 was selected for the analysis using artificially contaminated food samples.

Listeria monocytogenes detection in artificially contaminated food samples

26 food samples artificially contaminated with live and dead *L. monocytogenes* cells were analysed. Enrichment broth at time 0h and 24h were analysed by qualitative real-time PCR and v-PCR. At 24h, CFU levels were also evaluated by culture. The results obtained were summarized in the Table 1. At time 0h and before enrichment culture at 30ºC, the Ct values of samples tested only by qualitative real-time PCR (PCR), relating to the amount of total cells spiked in samples, showed mean Ct values of 28.4 ± 0.7. Moreover, these samples treated with PMA (v-PCR samples) showed negative fluorescent signal detection (Ct signal higher than 40) in 21 samples (80.8 %) as well as in the control sample. Other 4 samples (15.4 %) showed Ct signal higher than the qualitative real-time PCR detection limit (Ct = 36.2). Only in one food, the reduction was not complete as expected, with a low observed signal, Ct 35.4, very close to the detection limit. This result is probably related to the food matrix complexity (powdered milk).

After 24 hours of enrichment, the mean Ct values of all samples without and with PMA treatment were 18.5 ± 1.1 and 19.2 ± 0.9, respectively, indicating the cell growth of live cells in the Half Fraser broth with food sample. These results show that, at this point, the presence of a high amount of dead cells does not interfere negatively in the real-time v-PCR results.

The culture-based results showed the same results for all samples and were coherent with qualitative real-time v-PCR results. Furthermore, in two of all samples tested, marinated salmon and ground pork, *Listeria* cell growth was not observed at 24h for both analytical methods used, culture and qualitative real-time v-PCR, strengthening the suitability of v-PCR methodology.

DISCUSSION

Nowadays, there is a growing demand for rapid, sensitive, and accurate methods to detect foodborne pathogens such as *L. monocytogenes*. Development of rapid PCR methods that detect only viable bacteria, such as v-PCR methodology, is required in microbiological quality control in the food industry in order to shorten culture method workflow. To this end, different authors have applied v-PCR technique for accurate detection of viable *L. monocytogenes* cells in foods showing its suitability in the discrimination

between live and dead *L. monocytogenes* cells [5, 8, 16, 17, 18]. However, in such approaches the elimination of false positive results in samples with high amounts of dead cells (1.0×10^{6}-1.0×10^{8} CFU sample^{-1}) was not achieved. That suggests that further optimization of the used v-PCR procedures for *Listeria* detection is needed.

The first evidence of the ability of total Ct signal suppression on dead *Listeria* cells by v-qPCR in raw shrimp was reported by Zhang et al. 2015 [25]. These authors optimized a PMA TaqMan-based multiplex real-time PCR tool for monitoring contamination of viable *V. parahaemolyticus* and *L. monocytogenes* in seafood assessing the suitability of v-qPCR in samples inoculated with different concentrations of viable or dead cells. In these studies the authors used high concentration of PMA, 100 μM, and evaluated the v-qPCR in sea foods right after cell inoculation, without pre-enrichment step in a selective broth medium.

Working in this direction, in this study, two strategies for improving qualitative real-time v-PCR for *L. monocytogenes* were assessed, aiming at false positive results suppression. The obtained results indicate that the optimal qualitative real-time v-PCR protocol, which allows maximum Ct signal suppression for dead *L. monocytogenes* cells, comprised a sample treatment with 50 μM of PMA dye at 24ºC for 30 min followed by double photo-activation for 15 min light, 10 min dark, and 15 min light. Moreover, the changing of tube throughout the procedure allowed for achieving the best results in qualitative real-time v-PCR for *Listeria*. Previously, we have successfully evaluated this tube change procedure in *Salmonella* [1] and *Legionella* cells [11]. Thus, demonstrating the microtube impact on the incomplete exclusion of dead cells amplification signals in v-qPCR. In that study, the suitability of tube change to remove the extracellular DNA retained in microtube walls and thereby avoiding false positive results was also evidenced. Taking to account these improvements, the change of tube procedure was introduced and assessed in these *Listeria* studies (Treatment 2). We found that the results obtained were better than without the tube change (Treatment 1), obtaining similar results improvements as pointed out by Agustí et al. 2016 [1]. However, in these experiments total Ct signal reduction from dead cells was not achieved. Considering this and in order to increase the procedure efficiency, the double treatment with PMA dye was introduced. This double dye incubation approach has been already tested by different authors [13, 20]. In the mentioned studies the PMA treatment step was performed twice. Briefly, samples were double treated with PMA and in each PMA treatment the dye was exposed to light. On the other hand, Pan and Breidt 2007 [17] tested this procedure in *L. monocytogenes* cells, reporting better results when samples were treated two or three times with PMA at 50 μM, but without complete dead cells Ct signal reduction.

We tested this double dye methodology in our studies introducing a modification in double treated PMA methodology described by these authors by using a double light treatment without further addition of dye during the procedure. Moreover, this double photoactivation step was combined with tube change approach (Treatment 3). The results showed for first time the suitability of double light treatment in conjunction with a double change of tube in the improvement of qualitative real-time v-PCR results in *L. monocytogenes* cells detection.

Additionally, we assessed the enhanced qualitative real-time v-PCR methodology relevance for routine practice in food control. For that, 26 different food samples were spiked with different concentrations of live cells and high amounts of dead cells ($1.0\text{-}5.0\times10^{7}$ CFU g^{-1}) and the suitability of the developed technique was evaluated before and after 24 hr of enrichment step. The qualitative real-time v-PCR results in enrichment broth at time 0, showed the limits of this technology for direct analysis in food samples at very high levels of dead cells. The current detection limit of the qualitative real-time PCR used in this work was internally established using *Listeria* DNA standard curve at 36.2 Ct (data not shown), which corresponds to a range of 4-10 target copies per reaction according to a Poisson distribution as stated in UNE-EN ISO 7218:2007 [22]. However, under the context of a method validation the real detection limit of PCR assay for the detection of foodborne pathogen must to be estimated according to ISO method (UNE-EN ISO 16140-2016) [23]. Although levels lower than this, as 1-4 target per reaction, will not show signal in 50% of cases or less than this, the theoretical detection limit can be estimated around 39.5 Ct. Considering that, qualitative real-time PCR and v-PCR results greater than 40 also should be considered as negative. Likely, some signal beyond this range is the result of a residual unspecific amplification accumulated during 45 amplification cycles. In Table 1 and according to this criterion, at time 0h, 5 out of 26 samples (19.2%) still have positive signal. On the other hand, the current procedure is able to neutralize in 80.8% of cases with high levels of dead cells in artificially contaminated food samples. In future studies higher dye concentrations should be evaluated to observe if better results can be achieved with this technique.

Nevertheless, as in most cases for food pathogen the rules specify *L. monocytogenes* absence, therefore it does not make sense a direct analysis by PCR because up to now it is impossible a complete analysis of total bacterial DNA in a 25 gr of food sample by PCR. For this reason, it does not make sense to focus all the efforts in evaluating the maximum neutralization capability for this application at time 0. For *L. monocytogenes* it is a key to ensure that after the enrichment step, all PCR signals are not influenced by

high levels of dead cells background. And based in the results obtained after the enrichment step, we can observe that, at that point and using the developed qualitative real-time v-PCR procedure, the presence of a high amount of dead cells does not interfere negatively in the qualitative real-time v-PCR results.

Formally the herein developed method has not been assessed with contamination levels close to the detection limit and/or on naturally contaminated samples, reason for this technical evaluation cannot be extrapolated directly to real samples Nevertheless, since it has been developed following a conventional enrichment step, in order to increase the cell population at levels it can be detected by PCR and culture, the obtained results suggest that the depicted sample treatment approach is suitable for *Listeria* detection in food samples by v-PCR. However, more validation tests using naturally contaminated food samples should be performed to strengthen the suitability of this method in routine control.

Results obtained in our study highlight that the use of qualitative real-time v-PCR methodology allows the exclusive detection of live *L. monocytogenes* cells in spiked food samples, minimizing the effect of false positive results. The new protocol herein proposed, in which the improvements introduced, did not add a longer times, showed good efficiencies and was able to minimize false positive results even working with high concentrations of dead cells.

CONFLICT OF INTEREST

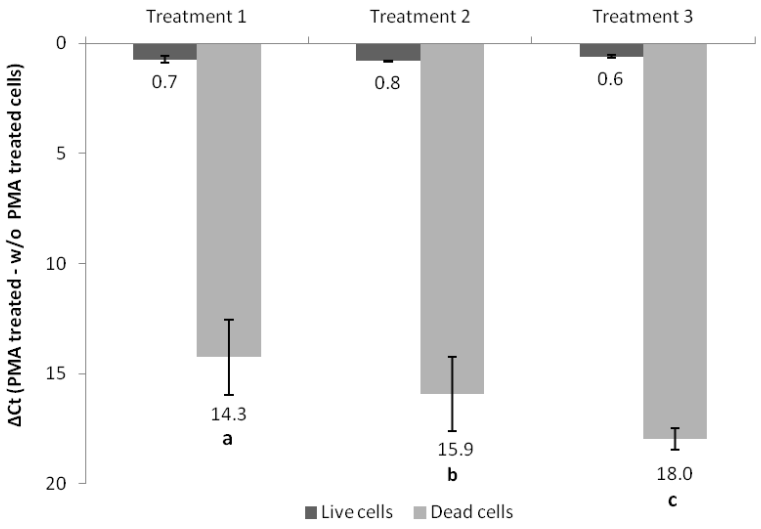
The authors declare that they have no conflict of interest

TABLES AND FIGURES

Table 1. Results of the analysis of different foods spiked with live ($1.0\text{-}5.0\times10^{1}$ CFU g^{-1}) and dead ($1.0\text{-}5.0\times10^{7}$ CFU g^{-1}) *L. monocytogenes* cells by qualitative real-time PCR (PCR) and v-PCR (Ct values) and culture based method (culture confirmation), before (0h) and after (24h) enrichment culture at 30ºC. Samples for v-PCR were treated with 50 µM PMA. n.s., no fluorescent signal detection. >40, real-time PCR signal after 40 cycles. +/-, positive/negative plate counting results.

Time/ Treatment	0h		24h		
	PCR	v-PCR	PCR	v-PCR	Culture confirmation
Control	26.2	>40	16.1	17.0	+
Meat	25.5	>40	24.4	25.9	+
Salad	25.1	39.0	17.4	17.7	+
Powdered milk	31.6	35.4	17.4	18.6	+
Ham	28.3	n.s	20.4	20.6	+
Mortadella	28.6	38.3	18.0	18.9	+
Fresh cheese	29.1	>40	19.5	21.2	+
Vegetable cream	28.1	36.9	17.3	18.4	+
Lentils	27.6	37.2	17.9	18.9	+
Marinated salmon	30.7	>40	27.6	>40	-
Chicken noodle soup	29.0	>40	17.5	18.2	+
Chicken	28.9	>40	17.9	19.5	+
Champignon mushroom	28.2	>40	18.1	19.4	+
Vegetable couscous	29.9	>40	17.6	18.9	+
Green peas	24.8	>40	21.4	21.8	+
German sausage	25.2	>40	16.6	18.0	+
Rice	26.2	>40	16.2	17.9	+
Green cream	28.5	>40	20.1	21.5	+
Turkey ham	29.3	>40	16.6	18.2	+
Vegetal lasagne	27.4	>40	16.6	17.9	+
Rice with vegetables and chicken	28.8	>40	15.3	15.8	+
Spaghetti with tomato sauce	29.0	n.s	15.4	16.0	+
Chickpea	31.4	n.s	21.3	23.6	+
Green bean	30.0	>40	15.9	17.1	+
Turkey breast	31.9	n.s	18.6	18.7	+
Ground pork	29.2	n.s	>40	>40	-
Mozzarella cheese	28.6	n.s	19.0	19.1	+

Figure 1. Mean of ΔCt (Ct dye-treated dead cells − Ct non-treated dead cells) using three different v-PCR treatments. Treatment 1, single light treatment without change of tube. Treatment 2, single light treatment and double change of tube. Treatment 3, double light treatment and double change of tube. Error bars indicate the confidence interval from two independent assays. Statistically significant differences ($p < 0.05$) between treatments are indicated by different subscript letters.



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