BASELINE

SELECTION AND IMPROVING OF FIT-FOR-PURPOSE SAMPLING PROCEDURES FOR SPECIFIC FOODS AND RISKS

# PROJECT DELIVERABLE

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SEVENTH FRAMEWORK PROGRAMME

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## SUMMARY

In this deliverable a complete description of the design and optimization of quantitative detection methods for *Listeria monocytogenes* and *Salmonella enterica* using EMA-qPCR is described. The methodology described has sown an exceptional performance with excellent analytical sensitivity (down to 10 cfu/g of RTE lettuce) and with quantification capacities (linearity and PCR efficiency close to the ideal - $R^2 > 0.998$ - and PCR efficiency close to 100%).

# INTRODUCTION

The determination of bacterial viability is a key issue for the application of food risk management, and thus a rational approach to detect only viable bacterial cells by using molecular-based methods is necessary. However, PCR-based methods detect DNA which survives cell death. For this purpose the use of mRNA as template for amplification can be a promising solution (Klein and Juneja, 1997), though this requires removing any trace of bacterial DNA in the reaction in order to avoid false-positive results in viability assays (Cook, 2003).

## *NASBA*

Nucleic Acid Sequence Based Amplification (NASBA) technique is a promising diagnostic tool for the analysis of viable microorganisms, since it is based on amplification of RNA rather than DNA. This technique is a sensitive transcription-based amplification system specifically designed for the continuous amplification of RNA in a single mixture at isothermal conditions (Compton, 1991; Deiman et al., 2002). Thus, this technique has the potential for detection of viable cells through selective amplification of messenger RNA, even in a background of genomic DNA, which PCR does not possess. NASBA employs a battery of three enzymes (AMV reverse transcriptase, RNase H, and T7 RNA polymerase) to amplify sequences from an original single-stranded RNA template, leading to a main amplification product of single stranded RNA. The reaction also includes two oligonucleotide primers, complementary to the RNA region of interest. One of the primers also contains a promoter sequence that is recognized by T7 RNA polymerase at the 5’-end. The reaction is performed at a single temperature, normally 41ºC for 1 to 2 h in a self-sustained manner. At this temperature, the genomic DNA from the target remains double-stranded and does not become a substrate for amplification. The principal characteristics of NASBA are summarized in Table 1.

**Table 1.** Characteristics of NASBA[a].

A single step isothermal amplification reaction at 41ºC.

Especially suited for RNA analytes because of the integration of RT into the amplification process.

The single-stranded RNA product is an ideal target for detection by various methods including real-time detection using molecular beacons.

The fidelity of NASBA is comparable to other amplification processes that use DNA polymerases lacking the 3' exonuclease activity.

The use of a single temperature eliminates the need for special thermocycling equipment.

Efficient ongoing process results in exponential kinetics caused by production of multiple RNA copies by transcription from a given cDNA product.

Unlike amplification processes such as PCR, in which the initial primer level limits the maximum yield of product, the amount of RNA obtained in NASBA exceeds the level of primers by at least one order of magnitude.

NASBA RNA product can be sequenced directly with a dideoxy method using RT and a labelled oligonucleotide primer.

The intermediate cDNA product can be made double-stranded, ligated into plasmids, and cloned.

Three enzymes are required to be active at the same reaction conditions.

Low temperature can increase the non-specific interactions of the primers. However, these interactions are minimized by the inclusion of DMSO.

A single melting step is required to allow the annealing of the primers to the target.

The NASBA enzymes are not thermoestable and thus can only be added after the melting temperature.

The length of the target sequence to be amplified efficiently is limited to approximately 100 to 250 nucleotides.

[a] Source: Modified from Deiman, B., Van Aarle, P., and Sillekens, P. (2002).

The application of NASBA for detection of foodborne pathogens is at around the same stage as PCR was a decade or so ago, with a few methods being sporadically published in the scientific press (Table 2) (Cook et al., 2003; Rodríguez-Lázaro et al., 2006). Hence, considerable further development is required before NASBA can follow in PCR's footsteps to realize its potential for routine use. However, since NASBA can equal the rapidity and accuracy of PCR and has the additional potential advantage of unambiguous detection of viable pathogens, NASBA is a very promising diagnostic tool for food and clinical microbiology, and could become a reference in future decades.

**Table 2.** NASBA Methods for Detection of Pathogenic Microorganisms in Food[a].

| Target | Food | Reference |
|---|---|---|
| *Bacillus* spp. | Milk | Gore et al., 2003 |
| *Campylobacter jejuni, C. coli, C. lari* | Poultry products | Uyttendaele et al., 1994 |
| *C. jejuni* | Poultry products | Uyttendaele et al., 1995a |
| *C. jejuni* | Poultry products | Uyttendaele et al., 1995b |
| *C. jejuni* | Poultry products | Uyttendaele et al., 1997 |
| *C. jejuni* | Poultry products, meat products, dairy products | Uyttendaele et al., 1999 |
| Hepatitis A virus | Blueberries and lettuce | Jean et al., 2001 |
| Hepatitis A virus and norovirus | Deli sliced turkey and lettuce | Jean et al., 2004 |
| *Listeria monocytogenes* | Poultry products, meat products, seafood, vegetables, dairy products | Uyttendaele et al., 1995c |
| *L. monocytogenes* | Egg products, dairy products | Blais et al., 1997 |
| *M. paratuberculosis* | Milk, water | Rodríguez-Lázaro et al., 2004e |
| *Salmonella enteritidis* | Liquid egg | Cook et al., 2002 |
| *Salmonella enteritidis* | Fresh meats, poultry, fish, ready-to-eat salads and bakery products | D'Souza et al., 2003 |

[a] Source: Adapted from Rodríguez-Lázaro et al., 2006.

### *EMA and PMA*

An PCR-based approach has recently been devised to distinguish viable bacterial cells is the staining of cells with a blocking agent such as ethidium monoazide bromide (EMA) prior to DNA extraction and PCR to inhibit the amplification of DNA from dead cells (Nogva et al., 2003; Rudi et al., 2005a). This strategy combines the use of viability (live-dead)-discriminating dye with the speed, specificity, and selectivity of amplification-based techniques such as real-time PCR. The principle is that these dyes do not penetrate the cell walls of viable cells, but will penetrate those of dead cells. They can intercalate in DNA and prevent amplification, and thus amplification signals will only be obtained from viable cells that the dye could not penetrate. EMA is a phenanthridinium nucleic acid-intercalating agent (Waring, 1965), and photolysis of EMA with visible light produces a nitrene that can form stable covalent links to DNA (Coffman et al., 1982; Hixon et al., 1975). The unbound EMA, remaining free in solution, is simultaneously photolysed and converted to hydroxylamine, and is no longer capable of covalent attachment to DNA (DeTraglia et al., 1978). Thus, the application of EMA prior to bacterial DNA extraction can lead to selective removal of DNA from dead cells. This approach has already been tested with different foodborne pathogens such as *Escherichia coli* 0157:H7 (Guy et al., 2006; Nocker et al., 2006; Nogva et al., 2003), *Salmonella* (Guy et al., 2006; Nocker et al., 2006; Nogva et al., 2003), *Listeria monocytogenes* (Guy et al., 2006; Nocker et al., 2006; Nogva et al., 2003; Rudi et al., 2005a,b), *Campylobacter* (Rudi et al., 2005a), and *Vibrio vulnificus* (Wang and Levin, 2005). However, it has been reported that EMA can also penetrate the membrane of viable bacterial cells and covalently cross-linked with the DNA during photolysis, resulting in loss of a percentage of the genomic DNA of viable cells and PCR inhibition (Nocker and Camper, 2006; Rueckert et al., 2005). This drawback can be overcome using a similar staining strategy with a more selective molecule such as

propidium monoazide (PMA). PMA is a modification of propidium iodide that does not penetrate the membrane of viable cells, but is efficiently taken up by permeabilised cells (Nocker et al., 2006). Promising though this approach appears, it still contains a potential for ambiguity in that it is not completely assured that there are no circumstances in which dye is taken up by viable cells. In such circumstances, the potential for overlooking the presence of a pathogen in a food sample exists, and much further work is necessary before the dye approach can be confidently taken up in actual food analysis.

## OBJECTIVES

Within the BASELINE'S **WP5 "Selection and optimisation of sampling plan for the different risk target in plant products"**, the task WP5.4 "Assess the need of new or adapted analysis methods for sampling and analysis of the risk factors" was devoted to evaluate novel strategies for analytical methodologies for detecting the main risk in plant products. This task involved 6 partners and the task coordinator was Dr. Dario De Medici (ISS).

**Table 3.** Partners involved in Task 5.4.

| Number | Name | Short name | Country |
|---|---|---|---|
| 2 | Veterinaerinstituttet - National Veterinary Institute | NVI | Norway |
| 3 | Centro Nacional de Tecnología y Seguridad Alimentaria | CNTA | Spain |
| 6 | Hungarian Food Safety Office | HFSO | Hungary |
| 7 Task leader | Istituto Superiore di Sanità | ISS | Italy |
| 8 | Instituto Tecnológico Agrario de Castilla y León | ITACyL | Spain |
| 13 | University of Navarra | UN | Spain |

The Task 5.4 started in Month 18 and ended in Month 38. The main activities of this task were focused on the evaluation of currently used methods regarding specificity, quantitative uncertainty, as well as the risk of false positive and negative results. The latter is particularly relevant for analysis methods of emerging pathogens where there is limited experience regarding the interference between food matrix and pathogenic agent. In addition within this task, the subtask 5.4.1 "*Development of methods for human pathogens detection in plant products*" was focused in the need for quantitative, more specific methods and methods allowing lower detection levels as the PO values in early process stages should be very low for human pathogens that may grow during processing. In particular within this task two different strategies were followed: a harmonized method to use Real Time PCR for detection of HAV and noroviruses (D.5.2) and molecular methods to detect *Listeria monocytogenes* and *Salmonella enterica* using EMA-Real Time PCR will be also developed (D5.6).

Therefore, the **main objective** of the Deliverable 5.6 was to develop a feasible protocol for detect quantitatively *Listeria monocytogenes* and *Salmonella enterica* using EMA-PCR in plant products. This deliverable had to be ready for Month 38.

# DEVELOPMENT OF THE METHOD FOR *LISTERIA MONOCYTOGENES*

## *Optimisation of parameters*

Although the protocol was aimed to be developed using EMA, after the literature revision prior to start the work (see introduction), a promising blocking dye was identified (PMA), and both were evaluated in parallel.

The first step in the preparation of the methodology using blocking dyes coupled to real-time PCR was to optimise the different variables that could affect the performance of the method. In general, a pre-treatment using blocking dyes prior to DNA extraction compromises several steps:

1. Dilute the dye (EMA or PMA) in nuclease-free distilled water to create a stock solution and stored at −20 °C in the dark.

2. Add an appropriate volume of dye stock (EMA or PMA) solution into 0.5 mL of cell suspension to obtain the desired working concentration.

3. After dye (EMA or PMA) addition, thoroughly mix the sample and incubate in the dark at room temperature with occasional mixing to allow the dye to penetrate the dead cells and bind to the DNA.

4. Photoactivate the samples using the lamp Led-Active Blue photoactivation system.

5. Centrifuge the sample at 5000 g for 5 min and discard the supernatant.

6. Add 1000 µl of PBS and resuspend the pellet.

7. Repeat step 5.

8. Follow with the DNA isolation procedure.

The steps that can be optimised are in bold (steps 2, 3 and 5). Based in a literature search of times for incubation and photoactivation used previously in addition to preliminary experiments, the times for incubation (5 minutes) and photoactivation (15 minutes) were selected. Finally, dye concentration for EMA and PMA was optimised. For evaluation of concentration of blocking dyes, three different concentrations were selected for each dye **EMA: 12.5, 25 and 50 µM and PMA: 25, 50 and µM).** The experiments were performed in triplicate. Therefore, the activity was as follows:

Prepare an overnight culture of *Listeria monocytogenes* (strain ITACyL 1010) and adjust the cell concentration to $10^5$ cfu/mL.
Divide the prepared culture suspension in two tubes, and one will be heat-treated (100ºC for 10 minutes; dead cells) and the other will not be heat-treated (live cells).
Prepare the dye (EMA or PMA) in nuclease-free distilled water to create a stock solution and stored at −20 °C in the dark.

4. Add an appropriate volume of dye stock (EMA or PMA) solution into 0.5ml of cell suspension to obtain the desired working concentration (EMA: 12.5, 25 and 50 µM and PMA: 25, 50 and µM).


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5. After dye (EMA or PMA) addition, thoroughly mix the sample and incubate in the dark for 5 minutes at room temperature with occasional mixing to allow the dye to penetrate the dead cells and bind to the DNA.

6. Photoactivate the samples for 15 minutes using the lamp Led-Active Blue photoactivation system.

7. Centrifuge the sample at 5000 g for 5 min and discard the supernatant.

8. Add 1000 µl of PBS and resuspend the pellet.

9. Repeat step 5.

10. Follow with the DNA isolation procedure (QIAGEN column).

11. Perform real-time PCR using the protocol for quantitative detection of *Listeria monocytogenes* (Rodríguez-Lázaro et al., 2004).

In general EMA showed better performance than PMA, and the concentration with a better performance was 12.5 µM**.**

**Table 4.** Results of optimisation of blocking dye concentration.

| | EMA (µM) | | | PMA (µM) | | |
|---|---|---|---|---|---|---|
| | 12.5 | 25 | 50 | 25 | 50 | 100 |
| $C_T$ (mean ± ES) | 28.73 ± 0.09 | 30.50 ± 0.15 | 32.17 ±0.33 | 28.78 ± 0.34 | 29.33 ± 0.35 | 29.88 ± 0.23 |

Consequently, EMA was selected as blocking dye for subsequent experiments, and the concentration was 12.5 µM.

## *Evaluation of performance criteria*

**Aft**er the optimisation of the blocking dye concentration, the subsequent step was to establish the quantification capacity (limit of quantification) and analytical sensitivity (limit of detection) using pure cultures of *Listeria monocytogenes*.

For this, ten-fold dilutions of an overnight culture of *Listeria monocytogenes* were prepared (from $10^6$ to 1 cfu/mL), and 1 mL of each dilution was subjected to EMA treatment (see below) prior to a bacterial DNA extraction and real-time PCR. In parallel, 1 mL was subjected to a bacterial DNA extraction and real-time PCR without previous EMA treatment. Finally 1 mL of the dilutions containing $10^5$ and $10^3$ cfu were heat treated at 100ºC for 10 minutes and subjected to EMA treatment (see below) prior to a bacterial DNA extraction and real-time PCR. Three independent experiments were performed with an ample range of dilution series of *L. monocytogenes* equivalent to approximately to $1{\times}10^6$, $1{\times}10^5$, $1{\times}10^4$, $1{\times}10^3$, 100, 10 and 1 cfu per mL (i.e. $5{\times}10^4$, $5{\times}10^3$, $5{\times}10^2$, 50, 5, 0.5, and 0.05 genome equivalents per reaction).

The protocol was as follows:

Prepare the dye (EMA or PMA) in nuclease-free distilled water to create a stock solution and stored at −20 °C in the dark.
Add an appropriate volume of EMA stock solution into 1 mL of cell suspension to obtain the desired working concentration (EMA 12.5 µM).
After EMA addition, thoroughly mix the sample and incubate in the dark for 5 minutes at room temperature with occasional mixing to allow the dye to penetrate the dead cells and bind to the DNA.
Photoactivate the samples for 15 minutes using the lamp Led-Active Blue photoactivation system.
Centrifuge the sample at 5000 g for 5 min and discard the supernatant.
Add 1000 µl of PBS and resuspend the pellet.
Repeat step 5.
Follow with the DNA isolation procedure (QIAGEN column).
Perform real-time PCR using the protocol for quantitative detection of *Listeria monocytogenes* (Rodríguez-Lázaro et al., 2004).

Table 5 shows the mean $C_T$ values of a total of 9 PCR replicates from three independent experiments. Positive amplifications in all the replicates were obtained when the concentration of the target in the PCR was 5 genome equivalents or more and 1 genomic equivalent was detected with a 11% probability. Consequenlty, the probability of detection with a probability of 95% or higher was down to **$10^2$ cfu/mL** (equivalent to 5 theoretical genome equivalent per reaction), but the detection was possible down to **$10^1$ cfu/mL** (equivalent to 0.5 theoretical genome equivalent per reaction) in 11.11% of the samples (i.e. 1/9), and only in one of the three independent experiments. Therefore, the theoretical limit of detection (i.e. $10^2$ cfu/mL; 2nd column) was coincident to the experimental limit of detection (i.e. $10^2$ cfu/mL; 3rd).

**Table 5.** Results of the EMA-qPCR using pure cultures of Listeria monocytogenes[a].

| Initial inoculum | Inoculum | EMA-qPCR | qPCR |
|---|---|---|---|

| (cfu/mL) | (cfu/PCR)[b] | Signal ratio[c] | $C_T$ value [d] | Signal ratio | $C_T$ value [d] |
|---|---|---|---|---|---|
| $10^6$ | $5 \times 10^4$ | 9/9 | 24.22 ± 0.31 | 9/9 | 21.55 ± 0.21 |
| $10^5$ | $5 \times 10^3$ | 9/9 | 27.78 ± 0.22 | 9/9 | 25.04 ± 0.25 |
| $10^5$ dead | | 0/9 | n.a.[e] | 9/9 | 25.30 ± 0.22 |
| $10^4$ | $5 \times 10^2$ | 9/9 | 31.09 ± 0.18 | 9/9 | 28.98 ± 0.47 |
| $10^3$ | $5 \times 10^1$ | 9/9 | 34.22 ± 0.15 | 9/9 | 32.50 ± 0.22 |
| $10^3$ dead | | 0/9 | n.a. | 9/9 | 32.70 ± 0.26 |
| $10^2$ | $5 \times 10^0$ | 9/9 | 37.89 ± 0.30 | 9/9 | 35.76 ± 0.71 |
| $10^1$ | $5 \times 10^{-1}$ | 1/9 | n.a. | 1/9 | n.a. |
| $10^0$ | $5 \times 10^{-2}$ | 0/9 | n.a. | 0/9 | n.a. |

[a] Results of three independent experiments, with three PCR replicates used in each. The overall efficiency was 1.027, and the linearity ($R^2$) was 0.999

[b] Estimated number of L. monocytogenes genome equivalents in each PCR run, assuming 100% DNA extraction efficiency (each reaction contained 5 µL of a DNA preparation of 100 µL extracted from mL of pure culture).

[c] Positive results out of 9 reactions.

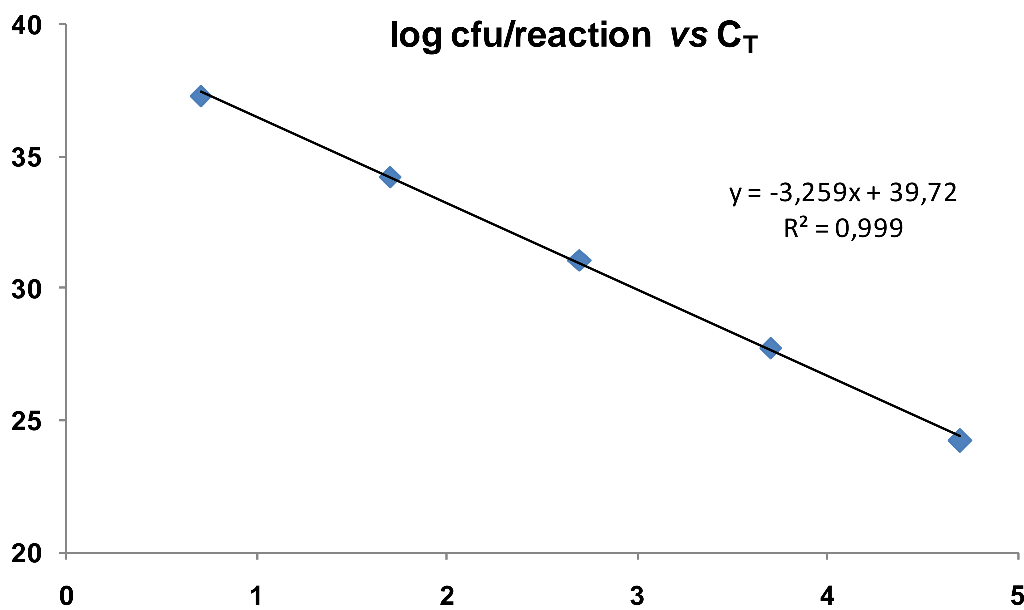
[d] Cycle number at which fluorescence intensity equals a fixed threshold. Mean value ± standard error of the mean. The experimental results were statistically significant ($P<0.05$), taking into account unavoidable error associated with serial dilutions.

[e] Not applicable.

The main parameters determining the quantification capacity of a qPCR assay are the linearity, the PCR efficiency (*E*), the quantification range and the limit of quantification (Rodríguez-Lázaro et al. 2007). Linearity is the ability of the method to generate results proportional to the amount of analyte present in the sample and is represented by the regression coefficient ($R^2$ value). PCR efficiency is the capacity of the PCR to duplicate the amplicon molecules in each cycle and is calculated from the slope of the linear regression curve (s) from the equation $E = 10^{1/s} - 1$. The EMA-qPCR linear regression curves were constructed by plotting the mean $C_T$ values obtained for each dilution against the logarithms of the number of genome equivalents per reaction (figure 1). The calculated $R^2$ value (0.999) was close to 1 showing a high linearity over a dynamic quantification range of at least 5 logs (from $5\times10^4$ to 5 genome equivalents or from $1\times10^6$ to 100 cfu/mL). The slope values (-3.259) correspond to E value of 1.027. It was very close to the theoretical value (-3.3219 and E = 1) showing an excellent efficiency. The limit of quantification (LOQ) was established as the lowest sample dilution in which the 99% confidence interval does not overlap with that of the next dilution. A statistical analysis (ANOVA and Tukey post-hoc method, $\alpha = 0.01$) determined the

absence of overlapping $C_T$ values and therefore confirmed reliable *L. monocytogenes* quantification was possible down to 5 genome equivalents of this bacterium per reaction (i.e. 100 cfu/mL).

**Figure 1.** Graphical represntation of log cfu vs $C_T$ values.




## *Quantitative detection of L. monocytogenes in RTE lettuce using EMA-qPCR*

The EMA-qPCR assay was used for the identification and quantification of *L. monocytogenes* in RTE lettuce. The samples were collected from a local market (Valladolid, Spain) and stored at 4ºC until used. They were analyzed by standard methods (ISO 11290-1:1996) to confirm the absence of *L. monocytogenes*. Serial ten-fold dilutions of three independent overnight cultures of three *L. monocytogenes* were used to inoculate the RTE lettuce samples in the artificial contamination challenge. All the artificial contamination assays were made in triplicate and included a non-contaminated sample (blank). In parallel, the serial dilutions were also plated on ALOA agar to estimate the colony forming unit numbers (CFUs) per sample (ISO 11290-2:1998) or the ISO 11290-1:1996 was used to determine the presence of *Listeria* when the enumeration technique was negative). Briefly, one millilitre of *L. monocytogenes* serial ten-fold dilutions containing approximately $1\times10^7$, $1\times10^6$, $1\times10^5$, $1\times10^4$, $1\times10^3$, $1\times10^2$, 10, and 5 *L. monocytogenes* per g were transferred onto the surface of RTE lettuce leaves (25 g) being inside of a Pulsifier bag. Two hundred and fifty mL of PBS were added to the Pulsifier bag, and were homogenised for 90 s using a Pulsifier (Microgen bioproducts, USA). Fifty mL of PBS were aseptically taken form the bag and transferred to a sterilised 50-mL centrifuge tube. The tube was centrifuged for 10 minutes at 4,000 × g. The supernatant was discarded and 1 mL of PBs was added to resuspend the pellet and the content was transferred into a clean 2-mL microcentrifuge tube. It was centrifuged for 5 minutes at 13,000 × g, the supernatant was discarded and the pellet was resupended using 1 mL of PBS. At this stage the EMA, DNA extraction and qPCR was performed as follows:

Add an appropriate volume of EMA stock solution into 1 mL of cell suspension to obtain the desired working concentration (EMA 12.5 µM).
After EMA addition, thoroughly mix the sample and incubate in the dark for 5 minutes at room temperature with occasional mixing to allow the dye to penetrate the dead cells and bind to the DNA.
Photoactivate the samples for 15 minutes using the lamp Led-Active Blue photoactivation system.
Centrifuge the sample at 5000 g for 5 min and discard the supernatant.
Add 1000 µl of PBS and resuspend the pellet.
Repeat step 5.
Follow with the DNA isolation procedure (QIAGEN column).
Perform real-time PCR using the protocol for quantitative detection of *Listeria monocytogenes* (Rodríguez-Lázaro et al., 2004).

Similarly to the previous experiments, aliquots with the same amount of *Listeria monocytogenes* were processed without EMA treatment, and heat-treated aliquots of $10^4$ and $10^3$ cfu/g of *Listeria monocytogenes* were also aasayed.

EMA-qPCR assay therefore yielded similar results in RTE lettuce in terms of absolute detection values (Table 6). It was possible to detect all the replicates containing as few as 10 CFUs per g of artificially contaminated RTE lettuce samples and to detect as few as 5 CFUs per g of artificially contaminated RTE lettuce samples with 33.33% probability (Table 6). Assuming 100% DNA isolation efficiency and taking into account that 100 µL of DNA was obtained from processing of 25 g of contaminated RTE salad, and that only 5 µL were used as template for the qPCR assay, the detection limit of 5 CFUs per g of RTE lettuce corresponds to approximately 1 genomic equivalents per reaction. These results are consistent with those obtained when pure cultures of *L. monocytogenes* was used (Table 5) and also similar to those published previously for other foodborne and animal pathogens (Rodríguez-Lázaro et al. 2004a; Rodríguez-Lázaro et al. 2004b; Rodríguez-Lázaro et al. 2004c; Rodríguez-Lázaro et al. 2005a; Rodríguez-Lázaro et al. 2005b; Rodríguez-Lázaro et al. 2005c; Rodríguez-Lázaro et al. 2006; López-Enríquez et al. 2007; Oravcová et al. 2009). In addition, the $R^2$ value (0.998) obtained showed a high linearity over a range of 6 logs (from approximately $2.5 \times 10^6$ to 25 genome equivalents per reaction) (Tables 6). Similarly, the PCR efficiency (0.9329) demonstrated that the performance of the EMA-PCR assay was also excellent (Figure 2).

**Table 6.** Quantification of Listeria monocytogenes in RTE lettuce using EMA-qPCR[a].

| Initial inoculum (cfu/g) | Inoculum (cfu/PCR)[b] | EMA-qPCR Signal ratio[c] | EMA-qPCR $C_T$ value [d] | qPCR Signal ratio[c] | qPCR $C_T$ value [d] | Relative accuracy |
|---|---|---|---|---|---|---|
| $10^7$ | $2.5 \times 10^6$ | 9/9 | 18.86 ± 0.16 | 9/9 | 18.62 ± 0.21 | 95.84 |
| $10^6$ | $2.5 \times 10^5$ | 9/9 | 22.10 ± 0.41 | 9/9 | 21.86 ± 0.34 | 113.31 |
| $10^5$ | $2.5 \times 10^4$ | 9/9 | 25.81 ± 0.13 | 9/9 | 25.45± 0.32 | 98.28 |

| | | | | | | |
|---|---|---|---|---|---|---|
| $10^4$ | $2.5 \times 10^3$ | 9/9 | 29.63 ± 0.26 | 9/9 | 29.01 ± 0.47 | 79.28 |
| $10^4$ dead | | 0/9 | n.a.[f] | 9/9 | 29.30 ± 0.22 | n.a. |
| $10^3$ | $2.5 \times 10^2$ | 9/9 | 32.50 ± 0.21 | 9/9 | 32.49 ± 0.39 | 119.60 |
| $10^3$ dead | | 0/9 | n.a. | 9/9 | 32.68 ± 0.29 | n.a. |
| $10^2$ | $2.5 \times 10^1$ | 9/9 | 36.32 ± 0.56 | 6/9 | 36.23 ± 0.33 | 96.48 |
| $10^1$ | 2.5 | 3/9 | n.a. | 1/9 | n.a. | n.a. |
| 5 | 1 | 0/9 | n.a. | 0/9 | n.a. | n.a. |

[a] *Results of three independent experiments, with three PCR replicates used in each. The overall efficiency was 0.9329, and the linearity ($R^2$) was 0.998*

[b] *Estimated number of L. monocytogenes genome equivalents in each PCR run, assuming 100% DNA extraction efficiency (each reaction contained 5 µL of a DNA preparation of 100 µL extracted from 25 g of RTE salad).*

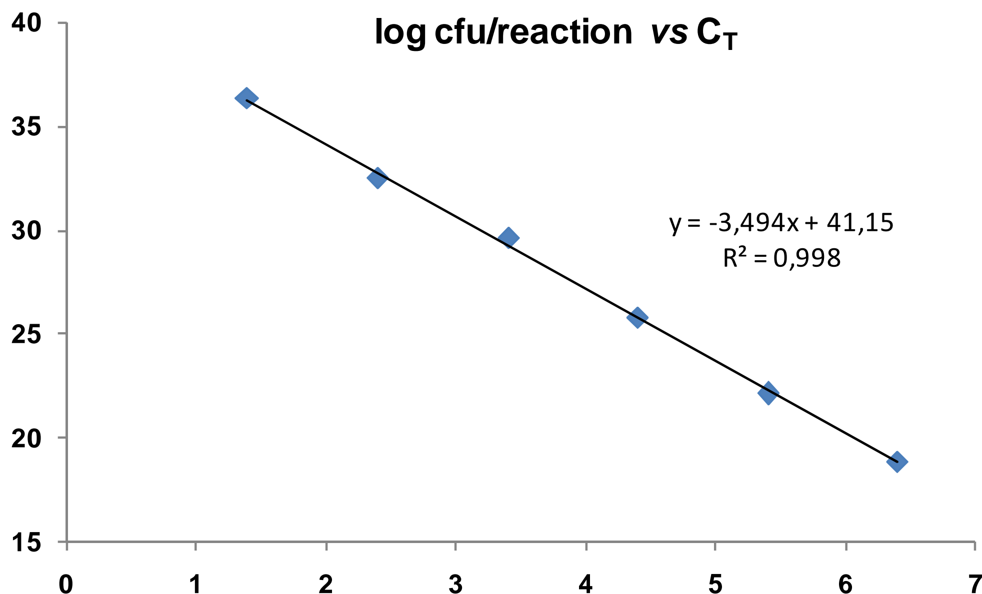
[c] *Positive results out of 9 reactions.*

[d] *Cycle number at which fluorescence intensity equals a fixed threshold. Mean value ± standard error of the mean. The experimental results were statistically significant (P<0.05), taking into account unavoidable error associated with serial dilutions.*

[e] *Degree of correspondence between the results obtained with the standard plating technique (L. monotyogenes cfu/g) and those obtained with the EMA-qPCR method (L. monotyogenes genome equivalents/g).*

[f] *Not applicable.*

**Figure 2.** Graphical representation of log cfu vs $C_T$ values.




The $C_T$ values obtained from the RTE lettuce samples artificially contaminated with *L. monocytogenes* were extrapolated to the corresponding standard regression curve, and the resulting theoretical numbers of *L. monocytogenes* were compared with those obtained with standard plating techniques (Table 6). Relative accuracy values ranged between 95.84% and 119.60%, indicating a high degree of correspondence between the quantitative results obtained by the reference method (number of *L. monocytogenes* CFUs/g as determined by standard plating) and the results obtained by the EMA-qPCR method (Table 6).

## Optimised protocol for detecting Listeria monocytogenes in RTE lettuce using EMA-QPCR

The complete protocol is as follows:

A. Preparation of RTE lettuce

Add 225 mL of PBS to 25 g of RTE lettuce sample.

Homogenise for 90 s in a Pulsifier (or similar homogeniser)

Transfer 50 mL of solution to a clean 50-mL centrifuge tube.

Centrifuge the sample at 4,000 × g for 10min and discard the supernatant.

Add 1000 µl of PBS and resuspend the pellet.

Transfer the content into a clean 2-mL microcentrifuge tube.

Centrifuged the solution for 5 minutes at 13,000 × g and discard the supernatant.



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Add 1000 µl of PBS and resuspend the pellet.

B. EMA treatment

Add an appropriate volume of EMA stock solution into 1 mL of sample suspension to obtain the working concentration (12.5 µM).

After EMA addition, thoroughly mix the sample and incubate in the dark for 5 minutes at room temperature with occasional mixing to allow the dye to penetrate the dead cells and bind to the DNA.

Photoactivate the samples for 15 minutes using the lamp Led-Active Blue photoactivation system.

Centrifuge the sample at 5,000 × g for 5 min and discard the supernatant.

Add 1000 µl of PBS and resuspend the pellet.

C. DNA extraction using columns

The protocol is based on the QIAGEN QIAMP mini kit (Hilden, Germany)

Transfer the content into a clean 1.5-mL microcentrifuge tube.

Centrifuge the sample at 13,000 × g for 5 min at 4ºC and discard the supernatant.

Suspend bacterial pellet in 180 µl of the appropriate enzyme solution (20 mg/mL lysozyme 20 mM Tris·HCl, pH 8.0; 2 mM EDTA; 1.2% Triton).

Incubate for at least 30 min at 37°C.

Add 20 µl proteinase K and 200 µl Buffer AL. Mix by vortexing.

Incubate at 56°C for 30 min and then for a further 15 min at 95°C.

Centrifuge for a few seconds.

Follow the "Protocol: DNA Purification from Tissues" from step 6

First add 4 µl RNase A (100 mg/mL), mix by pulse-vortexing for 15 s, and incubate for 2 min at room temperature.

Briefly centrifuge the 1.5 mL microcentrifuge tube to remove drops from inside the lid before adding 200 µl Buffer AL to the sample.

Mix again by pulse-vortexing for 15 s, and incubate at 70°C for 10 min.

Briefly centrifuge the 1.5 mL microcentrifuge tube to remove drops from inside the lid.

Add 200 µl Buffer AL to the sample, mix by pulse-vortexing for 15 s, and incubate at 70°C for 10 min.

Briefly centrifuge the 1.5 mL microcentrifuge tube to remove drops from inside the lid.

Add 200 µl ethanol (96–100%) to the sample, and mix by pulse-vortexing for 15 s.

After mixing, briefly centrifuge the 1.5 mL microcentrifuge tube to remove drops from inside the lid.

Carefully apply the mixture from step 6 (including the precipitate) to the QIAamp Mini spin column (in a 2 mL collection tube) without wetting the rim. Close the cap, and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 mL collection tube (provided), and discard the tube containing the filtrate.

Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW1 without wetting the rim. Close the cap, and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 mL collection tube (provided), and discard the collection tube containing the filtrate.

Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW2 without wetting the rim. Close the cap and centrifuge at full speed (20,000 x g; 14,000 rpm) for 3 min.

Place the QIAamp Mini spin column in a new 2 mL collection tube (not provided) and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min.

Place the QIAamp Mini spin column in a clean 1.5 mL microcentrifuge tube (not provided), and discard the collection tube containing the filtrate.

Carefully open the QIAamp Mini spin column and add 50 µl Buffer AE or distilled water.

Incubate at room temperature for 5 min, and then centrifuge at 6000 x g (8000 rpm) for 1 min.

Repeat step 35 and 36.

For long-term storage of DNA, eluting in Buffer AE and placing at –20°C is recommended

Yields of DNA will depend both on the amount and the type of tissue processed.

D. qPCR for Listeria monocytogenes

The qPCR protocol is that described by Rodríguez-Lázaro et al., 2004.

Prepare the mix including 0.10× more reaction for inaccuracies during pipetting. Prepare mix in a clean separate area as follows.

**Table 7.** QPCR mix (for one reaction).

| Reagent | Working concentration | Final concentration | Volume (µl) |
|---|---|---|---|
| Mix | 2× | 1× | 12.5 |
| Primer hlyF | 5 µM | 50 nM | 0.4 |
| Primer hlyR | 5 µM | 50 nM | 0.4 |
| Probe hlyQ | 5 µM | 100 nM | 0.8 |
| water | 2.5 µM | 50 nM | 5.90 |
| Total volume MIX | | | 20 |

| | |
|---|---|
| Sample | 5 |
| Final volume | 25 |

Once the mix has been prepared aliquot 20 µl into each well including the NTC. The total volume for one reaction after addition of target will be 25 µl (20 µl mix + 5 µl sample or standard).

Add samples (5 µl of the original sample) in a separate area.

Add 5 µl of the DNA standard of *L. monocytogenes* in duplicate.

Add 5 µl of nuclease-fee dd-water in the NTC wells.

Close wells with adhesive cover or caps.

Perform the QPCR in a real-time PCR platform, selecting the appropriate parameters (considering the total volume in each well, etc). Following activation of the UNG (2 min, 50°C) and activation of the Ampli*Taq* Gold for 10 min at 95°C, 45 cycles (15 s at 95°C and 1 min at 63°C) are performed.

Once the reaction is completed, store results and data as described in the user's manual of the equipment used.

The amount of DNA will be defined as the mean of the data obtained after correcting the dilution factor.

## DEVELOPMENT OF THE METHOD FOR *SALMONELLA ENTERICA*

### *Optimised protocol for detecting Listeria monocytogenes in RTE lettuce using EMA-QPCR*

As the performance of quantitative detection for *Listeria monocytogenes* using EMA-qPCR were not statistically significant (P<0.5) in comparison to that using qPCR and plating standard (ISO 11290-2:1998) assays, preliminary experiments were performed for *Salmonella enterica* using the same optimised conditions as for *L. monocytogenes* shown excellent performance (data not shown).

Therefore, the protocol is as follows:

A. Preparation of RTE lettuce

Add 225 mL of PBS to 25 g of RTE lettuce sample.

Homogenise for 90 s in a Pulsifier (or similar homogeniser)

Transfer 50 mL of solution ot a clean 50-mL centrifuge tube.

Centrifuge the sample at 4,000 × g for 10min and discard the supernatant.

Add 1000 µl of PBS and resuspend the pellet.

Transfer the content into a clean 2-mL microcentrifuge tube.

Centrifuged the solution for 5 minutes at 13,000 × g and discard the supernatant.

Add 1000 µl of PBS and resuspend the pellet.

B. EMA treatment

Add an appropriate volume of EMA stock solution into 1 mL of sample suspension to obtain the working concentration (12.5 µM).

After EMA addition, thoroughly mix the sample and incubate in the dark for 5 minutes at room temperature with occasional mixing to allow the dye to penetrate the dead cells and bind to the DNA.

Photoactivate the samples for 15 minutes using the lamp Led-Active Blue photoactivation system.

Centrifuge the sample at 5,000 × g for 5 min and discard the supernatant.

Add 1000 µl of PBS and resuspend the pellet.

C. DNA extraction using columns

The protocol is based on the QIAGEN QIAMP mini kit (Hilden, Germany)

Transfer the content into a clean 1.5-mL microcentrifuge tube.

Centrifuge the sample at 13,000 × g for 5 min at 4ºC and discard the supernatant.

Suspend bacterial pellet in 180 µl of the appropriate enzyme solution (20 mg/mL lysozyme 20 mM Tris·HCl, pH 8.0; 2 mM EDTA; 1.2% Triton).

Incubate for at least 30 min at 37°C.

Add 20 µl proteinase K and 200 µl Buffer AL. Mix by vortexing.

Incubate at 56°C for 30 min and then for a further 15 min at 95°C.

Centrifuge for a few seconds.

Follow the "Protocol: DNA Purification from Tissues" from step 6

First add 4 µl RNase A (100 mg/mL), mix by pulse-vortexing for 15 s, and incubate for 2 min at room temperature.

Briefly centrifuge the 1.5 mL microcentrifuge tube to remove drops from inside the lid before adding 200 µl Buffer AL to the sample.

Mix again by pulse-vortexing for 15 s, and incubate at 70°C for 10 min.

Briefly centrifuge the 1.5 mL microcentrifuge tube to remove drops from inside the lid.

Add 200 µl Buffer AL to the sample, mix by pulse-vortexing for 15 s, and incubate at 70°C for 10 min.

Briefly centrifuge the 1.5 mL microcentrifuge tube to remove drops from inside the lid.

Add 200 µl ethanol (96–100%) to the sample, and mix by pulse-vortexing for 15 s.

After mixing, briefly centrifuge the 1.5 mL microcentrifuge tube to remove drops from inside the lid.

Carefully apply the mixture from step 6 (including the precipitate) to the QIAamp Mini spin column (in a 2 mL collection tube) without wetting the rim. Close the cap, and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 mL collection tube (provided), and discard the tube containing the filtrate.

Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW1 without wetting the rim. Close the cap, and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 mL collection tube (provided), and discard the collection tube containing the filtrate.

Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW2 without wetting the rim. Close the cap and centrifuge at full speed (20,000 x g; 14,000 rpm) for 3 min.

Place the QIAamp Mini spin column in a new 2 mL collection tube (not provided) and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min.

Place the QIAamp Mini spin column in a clean 1.5 mL microcentrifuge tube (not provided), and discard the collection tube containing the filtrate.

Carefully open the QIAamp Mini spin column and add 50 µl Buffer AE or distilled water.

Incubate at room temperature for 5 min, and then centrifuge at 6000 x g (8000 rpm) for 1 min.

Repeat step 35 and 36.

For long-term storage of DNA, eluting in Buffer AE and placing at –20°C is recommended

Yields of DNA will depend both on the amount and the type of tissue processed.

D. qPCR for Listeria monocytogenes

The qPCR protocol is that described by Rodríguez-Lázaro et al., 2003.

Prepare the mix including 0.10× more reaction for inaccuracies during pipetting. Prepare mix in a clean separate area as follows.

**Table 8.** QPCR mix (for one reaction).

| Reagent | Working concentration | Final concentration | Volume (µl) |
|---|---|---|---|
| Mix | 2× | 1× | 12.5 |
| Primer invAF | 10 µM | 300 nM | 0.75 |
| Primer invAR | 10 µM | 300 nM | 0.75 |
| Probe invAQ | 5 µM | 100 nM | 0.8 |
| water | 2.5 µM | 50 nM | 5.20 |
| Total volume MIX | | | 20 |
| Sample | | | 5 |
| Final volume | | | 25 |

Once the mix has been prepared aliquot 20 µl into each well including the NTC. The total volume for one reaction after addition of target will be 25 µl (20 µl mix + 5 µl sample or standard).

Add samples (5 µl of the original sample) in a separate area.

Add 5 µl of the DNA standard of *Salmonella enterica* in duplicate.

Add 5 µl of nuclease-fee dd-water in the NTC wells.

Close wells with adhesive cover or caps.

Perform the QPCR in a real-time PCR platform, selecting the appropriate parameters (considering the total volume in each well, etc). Following activation of the UNG (2 min, 50°C) and activation of the Ampli*Taq* Gold for 10 min at 95°C, 45 cycles (15 s at 95°C and 1 min at 63°C) are performed.

Once the reaction is completed, store results and data as described in the user's manual of the equipment used.

The amount of DNA will be defined as the mean of the data obtained after correcting the dilution factor.

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