Detection and Enumeration of *Listeria monocytogenes* in Fresh Cut Vegetables Using MPN-Real-Time PCR

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Abstract

***Listeria monocytogenes* is a gram positive, rod shaped, pathogenic bacterium, causative agent of a severe infection generally known as listeriosis. Packaging and storage conditions of fresh cut vegetables may favour the growth of this psychrotrophic pathogen leading to potential health threat. Detection and enumeration of *L. monocytogenes* in concentrations up to 10^3 CFU/g, usually implies use of the most-probable-number technique (MPN) which may take up to seven days for verified identification of the pathogen. We developed a fast and reliable protocol combining MPN with a Real-Time quantitative PCR (qPCR) approach. Samples of fresh cut salads (25 g) purchased at local shops were spiked with 1 to 10^5 CFU/g of *L. monocytogenes*. Samples were homogenized, and triplicate series of tubes containing 10^{-5} to 10 g of food were incubated in Fraser broth at 30°C for 48 h for standard MPN analysis. After incubation, broth samples were taken from each tube and DNA was extracted. DNA from enrichment tubes was used as template in a qPCR assay targeting a 64 bp *hlyA* gene sequence of *L. monocytogenes*. Results of this assay were than compared with those of standard MPN analysis and a complete accordance was observed. Furthermore, we tested an enrichment free approach using the same qPCR assay. Samples were prepared as described for MPN-qPCR while DNA extraction was performed prior to enrichment of inoculated salads. This approach allowed us to identify *L. monocytogenes* in samples spiked with 10-10^5 CFU/g. The whole process, including DNA extraction, required less than four hours, thus providing a fast and reliable tool for detection of *L. monocytogenes* in fresh cut vegetables.**

INTRODUCTION

Listeria monocytogenes is a rod shaped, non spore-forming, Gram positive facultative anaerobic bacterium (Seeliger and Jones, 1986). *L. monocytogenes* is the causative agent of listeriosis, a severe, potentially fatal infection. During the early stages, human listeriosis often displays non-specific flu-like symptoms and gastroenteritis. However, if listeriosis is not medically treated, it can develop into septicaemia, meningitis, encephalitis, abortion and, in about 30% of cases, death (Liu, 2006). A wide range of foods have been associated to outbreaks of listeriosis including several ready-to-eat (RTE) foods and fresh-cut products (Gillespie et al., 2010; Little et al., 2010; Gaul et al., 2013). *L. monocytogenes* is a pathogen of particular concern for safety of fresh cut products as it grows across a broad pH range (4.3-9.8) and temperatures around 4°C (Ghandi and Chikindas, 2007). Moreover, it can grow in aerobic modified atmospheres also with competitive microorganisms (Wimpfheimer et al., 1990). It is commonly accepted that the minimal infectious dose for *L. monocytogenes* is at least 100 CFU per gram of food (Nørrung, 2000). However, depending on both the food considered and food safety legislations, accepted concentrations of *L. monocytogenes* range from 0 to

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100 CFU/g (Yang et al., 2007; Commission Regulation (EC) No. 2073/2005). Enumeration of *L. monocytogenes* in food matrices is usually carried out by the Most Probable Number (MPN) method which is laborious and time-consuming (5-10 days), as it requires preparation of replicated dilution series in selective enrichment broth, followed by plating on selective agar and tests for species identification (Berrada et al., 2006; Churchill et al., 2006). The development of fast and reliable detection and quantification methods for *L. monocytogenes* is important for verifying food safety and prevention of listeriosis. During the past decade a number of PCR-based methods, targeting mostly virulence factors and 16srRNA genes, have been proposed for detection and/or quantification of *L. monocytogenes* in foods (Pan and Breidt, 2007; O'Grady et al., 2008; Rantsiou et al., 2008). The aim of this study was to evaluate a combination of MPN and qPCR for quantification in fresh-cut vegetables along with development of a simple and fast protocol for detection and enumeration of *L. monocytogenes* from minimally processed vegetables relying on qPCR only.

MATERIALS AND METHODS

Food Samples

Minimally processed fresh cut vegetables (arugula, salad mixes) were randomly purchased at local shops in Foggia (Italy) and stored at 4°C.

Bacterial Strains, Media and Cultures

A total of 4 bacterial strains were used, including *Listeria monocytogenes* (CECT4031), *Listeria ivanovii* (IZSPB48), *Listeria innocua* (IZSPB45) and *Lactobacillus plantarum* (WCSF1). All strains were grown overnight in TSB broth (Oxoid, Hampshire, UK) at 37°C, without agitation.

Artificial Contamination of Samples

All tests were performed on artificially contaminated fresh cut vegetables. For both, *L. monocytogenes* and negative controls, 25 g samples of food were spiked with 10^0 to 10^5 cells/g. Bacterial cells were suspended in 1.0 ml TSB broth (Oxoid, Hampshire, UK).

DNA Isolation and Analysis

Three different DNA extraction protocols were evaluated, in order to select the most suitable one, on the basis of cost, time, yield, quality and personnel skills required.

1. DNeasy Blood and Tissue Kit (Qiagen, Milano, IT). The extraction was performed following the manufacturer's protocol for Gram-positive. Slight modifications were: adjustment to 40 min for lysozyme and proteinase K digestion, double elution of DNA with 100 and 50 µl aliquots of elution buffer.

2. Phenol-Chloroform Extraction. The organic extraction was performed according to standard method (Sambrook and Russel, 2001).

3. Power Soil DNA Isolation Kit (Mo-Bio, Carlsbad, CA, USA). Extraction was carried out following manufacturer's instruction. Mechanical lysis was performed using a Mini bead beater (Biospec products, Bartlesville OK, USA) for 30 s at half power.

The amount and purity of DNA were assessed by measuring absorbance at 260 nm (concentration) and the ratio between absorbance at 260 and 280 nm (purity) using a BioTek Eon spectrophotometer (BioTek, VT, USA). DNA purity was considered satisfactory when the A260/A280 ratio was >1.50. The degradation level of the extracted DNA was evaluated by electrophoresis in agarose gel (1% w/v). All DNA samples were stored at -20°C prior to use.

MPN Enumeration of *Listeria monocytogenes*

Inoculated vegetable (rocket salad) samples (25 g) were added to 225 ml of Fraser broth (Oxoid, Hampshire, UK) in sterile filter bags (BagFilter®, Interscience, Ile de

France, FR) and homogenized in a stomacher (Bag Mixer®, Interscience) for 2 min. Bags were incubated for 4 h at 37°C and Fraser selective supplement was successively added (Oxoid, SR0156E). Triplicate series of tubes were prepared to contain 10, 1, 0.1, $1\cdot10^{-2}$, $1\cdot10^{-3}$, $1\cdot10^{-4}$ and $1\cdot10^{-5}$ grams of food. Tubes were incubated for 44 h at 30°C. Aliquots (100 µl) of enrichment broth were taken from tubes containing presumptive *Listeria* spp. (dark colour) and streaked on plates containing Oxford agar (Oxoid, Hampshire, UK) and PALCAM agar (Oxoid, Hampshire, UK). Plates were incubated at 37°C for 48 h and five typical colonies were picked for purification on TSA+0.6% yeast extract plates (Oxoid, Hampshire, UK). Plates were incubated for 24 h at 37°C and Gram positive, catalase positive colonies were streaked on Blood agar (37°C, 24 h). Hemolytic colonies were identified as *Listeria monocytogenes* using API Listeria strips (Biomerieux, Marcy l'Etoile, FR). MPN was calculated using MPN Calculator Build 23 software (Mike Curiale).

QPCR Assays

All qPCR assays were performed on an AB 7300 Real Time PCR System (Life Technologies, Monza, ITA). Target genes, primers and probes employed are listed in Table 1. Four different assays were tested: 16S rRNA, *hlyA* (1), *hlyA* (2) and *iap*. All reactions were carried out using iTaq SYBR green Supermix with ROX (BIO-RAD) or l iTaq Universal probes Supermix (BIO-RAD) depending on the employed chemistry. Reagent concentration and amplification cycles are reported in the reference papers listed in Table 1.

RESULTS AND DISCUSSION

DNA Extraction

The first part of the present study was focused on the individuation of a suitable protocol for bacterial DNA extraction from fresh cut vegetables. Several parameters were considered. It was necessary to identify a procedure which could guarantee a good DNA yield combined with adequate purity and, especially, the absence of PCR inhibitors such as complex polysaccharides. Furthermore, we needed a simple, time efficient, possibly low cost procedure. Finally, possible manipulation of toxics had to be taken into account. Three different extraction procedures were evaluated, including two commercial kits (Qiagen Blood and Tissue, Mo-Bio Power Soil) and a Phenol-Chloroform extraction. DNA extraction was performed on artificially inoculated samples incubated at 37°C for 8 h using *Listeria monocytogenes* as a test organism (see Materials and methods section). Purity and concentration of DNA (quadruplicate samples) was evaluated with a BioTek Eon spectrophotometer (BioTek, Winooski, VT) as described in the previous section. All the extraction methods guaranteed adequate yield and purity (Table 2). However, phenol-chloroform method was discarded at this stage due to major drawbacks such as laboriousness, time required for extraction, necessity to store and manipulate significant amounts of toxic compounds. The eventual degradation of DNA extracted with Mo-Bio Power soil and Qiagen Blood and Tissue kits was evaluated by agarose gel electrophoresis. In both cases no significant degradation was observed (data not shown). Nonetheless Qiagen Blood and Tissue kit appears to be more suitable for our purposes because either assuring more than acceptable purity and yield of DNA, or less expensive and time consuming than Mo-Bio Power Soil Kit.

QPCR Assays

QPCR assay targeting *L. monocytogenes* 16S rRNA gene was initially chosen as it was assumed that presence of a multi-copy target sequence would enhance assay sensitivity. The assay was based on the use of SYBR green rather than fluorescent probes in order to reduce cost per reaction. Assay specificity was tested against DNA extracted from *L. monocytogenes*, *L. innocua*, *L. ivanovii* and *L. plantarum* (used as negative control strain). The assay showed the expected signal in the case of *L. monocytogenes* and

no signal in the case of the *Lactobacillus plantarum* strain. However, it lacked of specificity as a signal was also observed in the case of *L. innocua* and *L. ivanovii*. Due to this evidence, the assay was discarded and a SYBR green qPCR assay targeting *hlyA* gene (*hlyA*(1), see previous section) was tested. This test was expected to improve specificity as it was directed to detect a specific virulence related gene. However, even in this case, similar results were observed, with positive signal generated also with non-*monocytogenes* DNA (data not shown). Therefore, this assay was also discarded. Due to the unsatisfactory results obtained, we decided to perform further tests employing probe-based assays in order to identify an adequate specificity. Primers and probes targeting *iap* gene designed by Hein et al. (2001) were chosen for this assay. The assay was tested against the strains previously analyzed using the same protocols. In this case, the assay proved adequately specific, and only positive signal for *L. monocytogenes* strains were detected. Nonetheless, the assay was discarded as it was unsuitable for quantification purposes because of high serovar-related variability of results (data not shown). A second probe-based assay (*hlyA*(2), see previous section) was then tested. Primers and probes from Rodríguez-Lazaro et al. (2004) were adopted. The assay based on these oligonucleotides correctly identified *L. monocytogenes* in all of our tests while showing no signal in the case of non-*L. monocytogenes* strains. In Figure 1 the specificity of different qPCR assays are reported. Detection limits of the qPCR assay were successively investigated. For this purpose, duplicate serial dilutions of *L. monocytogenes* DNA were subjected to qPCR and a standard curve was constructed. The standard curve showed a linear relationship (spanning over 7 logs) between log input DNA and threshold cycle. The slope of the curve was -3.45 and the square regression coefficient was R^2=0.998. Detection limit was 0.28 picograms of DNA per reaction. These data led us to consider this qPCR assay suitable for development of MPN-qPCR and enrichment free enumeration protocols.

MPN-qPCR

MPN experiments were performed as illustrated in the Materials and Methods section. For DNA extraction, enrichment tube aliquots (1 ml) were taken from both positive and negative enrichment tubes. Then detection of *L. monocytogenes* using *hlyA*(2) qPCR protocol was performed. All of the tubes which tested positive after 48 h incubation in Fraser broth were eventually confirmed positive by conventional identification tests. In all of these samples *L. monocytogenes* was also detected using our qPCR protocol, while negative enrichment tubes gave negative results both in the qPCR assay and conventional methods. These findings illustrate complete accordance between our assay and MPN enumeration (Table 3). Our protocol can be employed to shorten MPN enumeration of *L. monocytogenes* from circa 5-10 days to 48 h with a faster and reliable confirmation of positive samples.

The development of a shorter MPN protocol for detection of *L. monocytogenes* may prove useful for assessing food safety due to its rapidity and sensitivity. This technique is also suitable for automation and high throughput applications, yet validation is necessary to adopt these molecular protocols in the food industry. Further development of the proposed qPCR based techniques includes use of propidium monoazide or similar compounds (i.e., etidium monoazide) in order to quantify viable cells exclusively.

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Tables

Table 1. QPCR assays, primers and probes.

Target gene	Forward primer (5'-3')	Reverse primer (5'-3')	Probe (5'FAM-TAMRA3')	Fragment size (bp)	Reference
16S rRNA*	CACGTGCTACAATGGATAG	AGAATAGTTTTATGGGATTAG	None	70	de Oliveira (2010)
hlyA(1)*	TGCAAGTCCTAAGACGCCA	CACTGCATCTCCGTGGTATACTAA	None	113	Nogva (2000)
hlyA(2)$^{+}$	CATGGCACCACCAGCATCT	ATCCGCGTGTTTCTTTTCGA	CGCCTGCAAGTCCTAAGACGCCA	64	Rodriguez (2004)
Iap^{+}	CTAAAGCGGGAATCTCCCTT	CCATTGTCTTGCGCGTTAAT	CTTCTGGCGCACAATACGCTAGCACT	174	Hein (2001)

*SYBR green, $^{+}$TaqMan.

Table 2. DNA extraction methods comparison.

Extraction method	Cost per reaction (€)	Time (20 sample-run)	Yield (ng/µl)	Purity A_{260}/A_{280}	Manipulation of toxics
MoBio kit	7.00	2.2 h	75.13±8.46	>1.6	Not significant
Qiagen kit	5.00	1.3 h	64.85±4.89	>1.6	Not significant
Phen/chlor	0.50	3 h	87.17±6.74	>1.6	Significant

Table 3. MPN test results and concordance with qPCR.

Inoculum (CFU/g)	MPN (CFU/g)	% of tubes confirmed positive by qPCR
1	0.93(0.23-3.80)	100
10	4.3 (1-18)	100
100	43 (10-180)	100
1000	564 (155-1890)	100

Figures

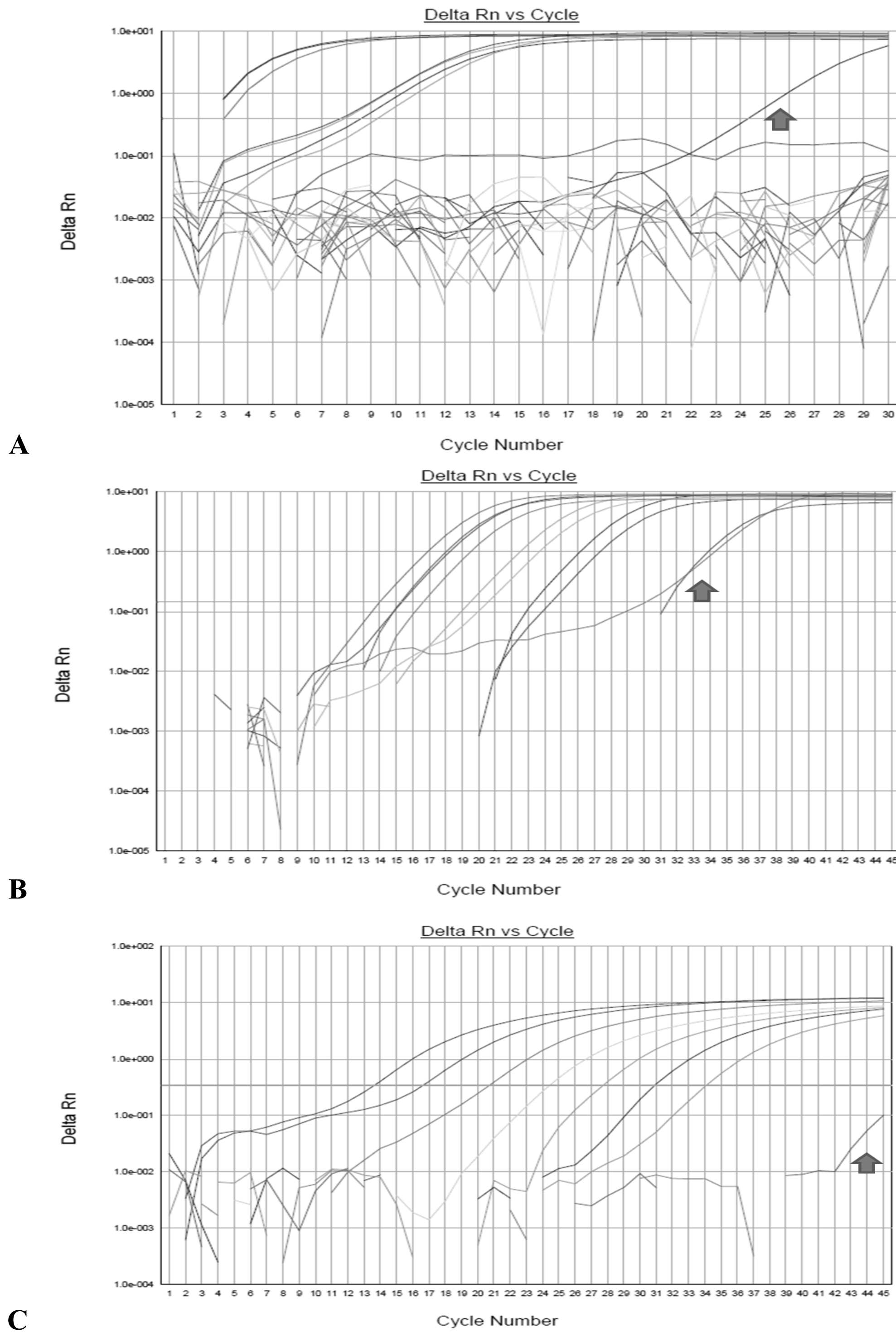

Fig. 1. QPCR amplification for *L. monocytogenes* CECT 4031, *Listeria ivanovii* (IZSPB48) and *Listeria innocua* (IZSPB45) by using as target gene 16S rRNA (A) and *hlyA* without (B) or with probe (C). Arrows indicate non-monocytogenes *Listeria* spp. strains.