Food Microbiology 27 (2010) 19–23

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

Quantification of *Listeria monocytogenes* in minimally processed leafy vegetables using a combined method based on enrichment and 16S rRNA real-time PCR

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ARTICLE INFO

Article history:
Received 9 March 2009
Received in revised form
3 July 2009
Accepted 12 July 2009
Available online 17 July 2009

Keywords:
Listeria monocytogenes
Minimally processed vegetables
Real-time PCR
16S rRNA
Microbiological quality

ABSTRACT

Modern lifestyle markedly changed eating habits worldwide, with an increasing demand for ready-to-eat foods, such as minimally processed fruits and leafy greens. Packaging and storage conditions of those products may favor the growth of psychrotrophic bacteria, including the pathogen *Listeria monocytogenes*. In this work, minimally processed leafy vegetables samples ($n = 162$) from retail market from Ribeirão Preto, São Paulo, Brazil, were tested for the presence or absence of *Listeria* spp. by the immunoassay *Listeria* Rapid Test, Oxoid. Two *L. monocytogenes* positive and six artificially contaminated samples of minimally processed leafy vegetables were evaluated by the Most Probable Number (MPN) with detection by classical culture method and also culture method combined with real-time PCR (RTi-PCR) for 16S rRNA genes of *L. monocytogenes*. Positive MPN enrichment tubes were analyzed by RTi-PCR with primers specific for *L. monocytogenes* using the commercial preparation ABSOLUTE™ QPCR SYBR® Green Mix (ABgene, UK). Real-time PCR assay presented good exclusivity and inclusivity results and no statistical significant difference was found in comparison with the conventional culture method ($p < 0.05$). Moreover, RTi-PCR was fast and easy to perform, with MPN results obtained in ca. 48 h for RTi-PCR in comparison to 7 days for conventional method.

1. Introduction

All over the world, public health agencies are concerned with food safety assurance due to globalization of food markets, growing demand for minimally processed ready-to-eat (RTE) foods and increasing numbers of meals served outside home (Kennedy and Wall, 2007). RTE products are subjected to mild treatments and are often stored under conditions that may favor the growth of diverse spoilage and pathogenic microorganisms, such as *Listeria monocytogenes* (WHO/FAO, 2007). This psychrotrophic bacterium causes a disease with mild flu–like symptoms and rarely gastroenteritis in healthy adults, but it may manifest as severe infection in immunocompromised persons and during pregnancy, leading to abortion, stillbirth or perinatal morbidity (Abadias et al., 2008; Aureli et al., 2000; Berrada et al., 2006; Donnelly, 2001; Gasanov et al., 2005).

Vegetables are highly susceptible to microbiological contamination due to lack of good agricultural practices by use of untreated water, inappropriate organic fertilizers and fails during harvesting, handling and distribution (Francis et al., 1999). Minimal processing of leafy vegetables is intended to improve quality, microbiological safety and to extend shelf life, but it can also cause mechanical injuries to the tissues. The minimal processing of vegetables may cause loss of water and color changes during steps of selection, washing, peeling, cutting, sanitizing, rinsing, drying and packaging (Francis et al., 1999; Soares and Geraldine, 2007)

High populations of *L. monocytogenes* have been reported for some RTE products, including outbreak samples, but growth rate of *L. monocytogenes* in vegetables is generally lower compared to milk, dairy and processed meat products (Chen et al., 2006). It is also important to consider that minimally processed vegetables are often used as ingredients to prepare salads, which may have longer refrigerated shelf lives, with risk of increasing of *L. monocytogenes* population (Aguado et al., 2004).

The quantification of *L. monocytogenes* in foods is generally done by the classical Most Probably Number (MPN) method, which requires replicated dilution series of food in selective enrichment broth followed by plating on selective agar plates and various tests for species identification (Berrada et al., 2006; Churchill et al., 2006; De Martinis et al., 2007; Duvall et al., 2006).

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doi:10.1016/j.fm.2009.07.003

Rapid and sensitive methods for enumeration of *L. monocytogenes* are important for microbiological safety throughout the food production chain and in this last 10 years, a considerable number of detection methods using molecular tools have been proposed (Aguado et al., 2004; Amagliani et al., 2007; Aznar and Alarcon, 2002; Berrada et al., 2006; De Martinis et al., 2007; Duvall et al., 2006; Ingianni et al., 2001; Jung et al., 2003; Norton, 2002; O'Grady et al., 2008; Sommer and Kashi, 2003; Rosmanith et al., 2006).

Many papers have been published on the detection of *L. monocytogenes* by PCR, using primers targeted to virulence and non virulence factors, including hemolysin (*hly*), invasion associated protein (*iap*) and 16S rRNA genes (Berrada et al., 2006; Gasanov et al., 2005; Niederhauser et al., 1992; Thimothe et al., 2004; Sommer and Kashi, 2003; Wang et al., 2007). The PCR method based on 16S rRNA genes for detection of *L. monocytogenes* presents better sensitivity due to the presence of multiple copies of the genes in the cell, to maintain adequate concentration of ribosomes (Alberts et al., 2002).

The aim of the present study was to evaluate the MPN method combined with RTi-PCR based on 16S rRNA genes for enumeration of *L. monocytogenes* in naturally and artificially contaminated minimally processed leafy vegetables.

2. Materials and methods

2.1. Food samples

From September 2007 to August 2008, a total of 162 samples of minimally processed leafy vegetables were randomly acquired from six grocery stores in the city of Ribeirão Preto, São Paulo, Brazil. Ten different types of samples were examined: collard green ($n = 30$), cabbage ($n = 28$), lettuce ($n = 26$), mixed parsley and spring onion bunches ($n = 22$), chinese cabbage ($n = 13$), arugula ($n = 6$), chicory ($n = 11$), wild chicory ($n = 13$), spinach ($n = 9$) and watercress ($n = 4$).

2.2. Detection of Listeria spp

Detection of *Listeria* spp. was accomplished with the immunoassay *Listeria* Rapid Test (Oxoid Ltd, Basingstoke, Hampshire, England) according to manufacturer's instructions (Oxoid, 2007). Aliquots of all samples were kept frozen and positive samples by the immunoassay were further tested for *L monocytogenes* by conventional identification tests.

2.3. Enumeration of L. monocytogenes

L monocytogenes was enumerated in two naturally and six artificially contaminated samples of leafy minimally processed vegetables, using the Most Probable Number (MPN) technique with classical and real-time PCR detection (De Martinis et al., 2007; Hitchins, 2007).

For non-inoculated samples, each vegetable (50 g-portion) was mixed in a sterile plastic bag for 2 min with 450 ml of BLEB in a piston homogenizer (MA 440, Marconi, Brazil). For experimental inoculation studies, 50 g-portions of each vegetable were sampled in sterile plastic bags and added of 450 ml of Buffered *Listeria* Enrichment Broth (BLEB, Oxoid Ltd, Basingstoke, Hampshire, England) and added of 1-ml inoculum prepared in phosphate buffered saline (PBS) to yield ca. 1, 2 or 3 $\log_{10}$ CFU of *L. monocytogenes* ATCC 19114 per gram of food.

Triplicate series of tubes for MPN enumeration were prepared to contain 10, 1, 0.1, 0.01, 0.001, 0.0001, 0.00001 and 0.000001 g of food. After incubation at 30 °C for 4 h, cultures were added of BLEB selective supplement (Oxoid, SR 0141E) and further incubated for 44 h at 30 °C. Aliquots of enrichment broths were streaked on plates containing Oxford (OX) and PALCAM (PAL) agars (Oxoid Ltd) and incubated for two days at 35 °C, for isolation of presumptive *Listeria* spp. colonies. Three to five typical black colonies from each agar were picked for purification on TSA-YE plates and colonies with blue appearance under transilumination were selected for *L. monocytogenes* identification by biochemical tests (Hitchins, 2007).

2.4. Extraction of L. monocytogenes DNA

DNA from cultures of each MPN tube was extracted by the boiling method without Triton X-100 (Hitchins et al., 2004). For that, samples were centrifuged for 15 min at 10 °C and 8000 g (Sorvall, Legend™ MACH 1.6/R, Germany) and the supernatants were discarded. The pellets were res-suspended in 5 ml PBS and re-centrifuged for 10 min. The supernatants were discarded again and the pellets were res-suspended in 2 ml of purified water (Milli-Q, Millipore, USA). The suspensions were heated for 10 min in a boiling water bath and immediately cooled on ice. After centrifugation for 10 min, the supernatants were retained and the sediments were discarded. The DNA extracted was stored at −20 °C until further analysis.

2.5. Real-time PCR assay

PCR amplification of DNA from extracts of each MPN enrichment culture was done according to Wang et al. (2007) with primers for 16S rRNA purchased from Invitrogen, Carlsbad, USA (L-1: 5′-CACGTGCTACAATGGATAG-3′ and L-2: 3′-GATTAGGGTATTTTGA-TAAGA-5′). The final reaction mixture of 25 μl was composed by 12.5 μl of Absolute^{M} QPCR SYBR® Green Mix (ABgene, Blenheim Road, Epson, Surrey, UK), 4 μl of extract of DNA (or appropriate dilution, corresponding to ca. 39 ng of DNA) and 1.25 μl of primer solution (0.25 mM of each primer). The amplification of target DNA and detection of PCR products were performed with the real-time PCR system MiniOpticon™ (Bio-Rad Laboratories, Hercules, CA, USA) equipped with the Opticon Monitor Analysis Software - version 3.1 (Bio-Rad Laboratories) for data acquisition and analysis of results. The amplification of the 16S rRNA genes was performed at 95 °C for 15 min followed by 45 cycles of 2 s at 95 °C, 10 s at 55 °C and 10 s at 72 °C. After the final amplification, the specificity of PCR was checked by the melting temperature of amplicon (Tm), calculated from a dissociation curve obtained by increasing the temperature from 60 to 95 °C at a rate of 0.3 °C/s (De Martinis et al., 2007). SYBR® Green allowed the continuous monitoring of amplicon concentration by the fluorescence characteristic of the SYBR® Green: DNA complex.

Real-time PCR amplification products were also analyzed by electrophoresis in agarose gel at 3% in TAE 1X (Tris base 0.04M, 0001M EDTA, 0.04M sodium acetate, pH 8.5) and 0.5 μg/ml of ethidium bromide (Vetec, Brazil). Each reaction mixture (5 μl) plus 3 μl of the loading buffer Bromophenol blue 0.25% (Vetec, Brazil) and 30% glycerol (Synth, Brazil) were applied in the gel. Electrophoretic separation was performed at 110 V, 1.81 A (FB 200, Fisher Scientific, USA) for 1 h and 15 min, the gel was observed under ultraviolet light and photos were taken for documentation of results (MiniBio UV, DNR Bio-Imaging Systems, Israel). The molecular weight of the PCR product (70pb) was determined by comparison with a standard of 50 bp molecular weight marker (Invitrogen, Carlsbad, USA).

2.5.1. Inclusivity and exclusivity tests for real-time PCR assay

DNA of pure cultures were extracted as previously described (Hitchins et al., 2004) and the bacterial strains used for inclusivity