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Short Communication

Simple and rapid detection of *Listeria monocytogenes* in fruit juice by real-time PCR without enrichment culture

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ABSTRACT

We developed a simple, rapid procedure for the detection of *Listeria monocytogenes* in unpasteurized fruit juice using real-time PCR without enrichment culture. PCR inhibitors were removed from fruit juices using Chelex resin followed by gel filtration with a Sephadex column. The purification step can be completed in 20 min, and purified juice samples can be used directly as PCR templates without further dilution. PCR conditions were optimized and maximum sensitivity (ca. 1 cell/reaction) was achieved. This convenient method should prove useful for high-throughput surveillance of *L. monocytogenes* as well as other food-borne pathogens that may contaminate fruit juices.

1. Introduction

Despite the seemingly minimal bacterial contamination risks of fruit juice, due to its high concentration of organic acids (Fisher & Golden, 1998; US Department of Health & Human Service, 1995), contaminated fruit juices have caused numerous outbreaks of food-borne disease (Vojdani, Beuchat, & Tauxe, 2008). Indeed, some juice manufacturers produce unpasteurized fruit juices, because thermal treatment can degrade the sensory and nutritional qualities of juice. *Escherichia coli* O157:H7, *Salmonella* spp., and *Listeria monocytogenes* can develop adaptations enabling them to survive in acidic environments (Gahan, O'Driscoll, & Hill, 1996; Hill, O'Driscoll, & Booth, 1995; Leyer, Wang, & Johnson, 1995; O'Driscoll, Gahan, & Hill, 1996; Yuk & Schneider, 2006) such as apple, orange, and grape juices (pH 3.6–3.7) for as long as 12 weeks (Oyarzabal, Nogueira, & Gombas, 2003).

L. monocytogenes poses especial health risks associated with fruit juices because of its ability to grow at conventional refrigeration temperatures (4 °C) under acidic conditions (Dykes & Dworaczek, 2002; George, Lund, & Brocklehurst, 1988). *L. monocytogenes* causes listeriosis, a serious disease with complications including meningitis, septicemia, and spontaneous abortion in immunocompromised individuals and pregnant women (Kunh & Goebel, 1999). Compared to other common food-borne infections, listeriosis has a relatively high mortality rate (20–30%) (Bula, Bille, & Glauser, 1995; Goulet et al., 1998; Lyytikainen et al., 2000). Most cases of listeriosis are caused by the ingestion of *L. monocytogenes*-contaminated ready-to-eat foods that do not require heating prior to consumption (Farber & Peterkin, 1991; Gasanov, Hughes, & Hansbro, 2005). For the safety of consumers, juice manufacturers as well as food safety supervising agents need a fast and simple surveillance method to test the sterility of their products and the microbiological quality of juices in the market.

We developed a real-time PCR-based method for detecting *L. monocytogenes* in unpasteurized fruit juice. Our convenient method allows for the pre-PCR treatment of fruit juice without enrichment culture (Fig. 1), and utilizes optimized real-time PCR (RTi-PCR) conditions to achieve maximum sensitivity (approximately one CFU per reaction). This paper describes the design, sensitivity, and quantitative properties of our method.

2. Materials and methods

2.1. Bacterial strains and DNA extraction

Eleven strains of *Listeria* (nine strains of *L. monocytogenes* [ATCC 19111–19118 and ATCC 15313], *L. ivanovii* ATCC 19119, and *L. innocua* ATCC 33090) were obtained from the American Type Culture Collection (ATCC) and used to evaluate primer specificity in our initial experiments. All *Listeria* strains were cultured in Brain Heart Infusion (BHI) broth (Difco, Detroit, USA) at 37 °C. Non-*Listeria* strains (*Pseudomonas*, *Escherichia*, *Vibrio*, *Alcaligenes* and *Bacillus*) were obtained from laboratory stocks and were cultured in nutrient broth (Difco) at 37 °C. Genomic DNA was extracted from the bacterial strains according to standard procedures (Sambrook

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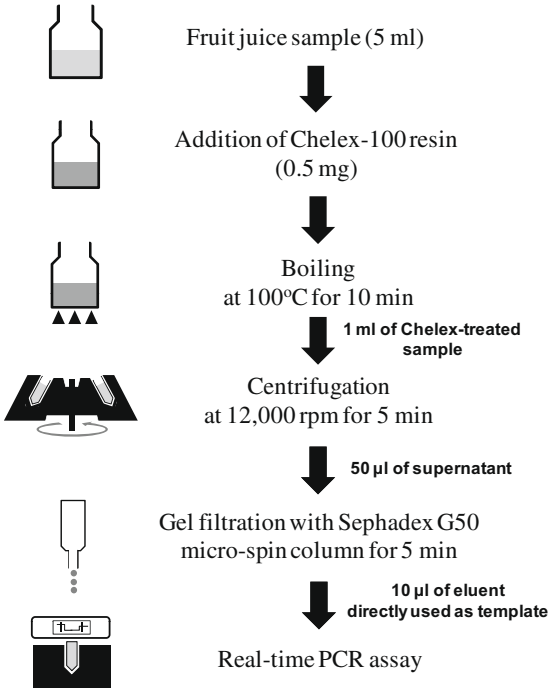

Fig. 1. Schematic diagram showing the suggested procedure for the purification of bacterial genomic DNA from fruit juice without enrichment culture.

& Russell, 2001). DNA concentrations were determined using a DNA quantification kit (Sigma, St. Louis, USA) and a fluorometer according to the manufacturers' protocols.

2.2. Oligonucleotide primers

PCR primers were designed based on the *L. monocytogenes*-specific *hly* gene encoding listeriolysin (Mengaud et al., 1988). The *hly* sequences (accession numbers AF253320, M24199, U25446, U25452, and U25449) were obtained from the GenBank database (http://www.ncbi.nlm.nih.gov) and were aligned using Clustal W (Thompson, Higgins, & Gibson, 1994). A consensus sequence was subjected to primer searches with ArrayDesigner software (Premier Biosoft, Palo Alto, USA). After assessing the specificity of primer sequences *in silico* via NCBI-BLAST (http://blast.ncbi.nlm.nih.gov) searches against available GenBank sequences, a *L. monocytogenes*-specific PCR primer pair was designed.

The specificity of the primers was tested using nine strains of *L. monocytogenes* (listed above) as positive controls and *L. ivanovii*, *L innocua*, and five non-*Listeria* strains from laboratory stocks as negative controls. The PCR reaction mixture included 25 µl of *Taq* PreMix with $MgCl_2$ (Takara, Shiga, Japan), 1 µl each of the forward and reverse primers (stock concentration, 20 µM), 10 ng of template DNA, and sterile distilled water to a 50-µl total final volume. The PCR thermal profile was as follows: initial denaturation at 95 °C for 5 min, 30 cycles consisting of denaturation at 95 °C for 1 min, primer annealing at 53 °C for 1 min, extension at 72 °C for 2 min, and a final elongation step at 72 °C for 20 min. Thermocycling was performed on a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, USA).

2.3. Real-time PCR assay

Real-time PCR (RTi-PCR) was optimized by hot-start condition using SYBR Premix Ex *Taq* Kit (Takara, Shiga, Japan). SYBR Green I and ROX were used as reporter and passive reference dyes, respectively. Reactions were carried out in MicroAmp optical eight-tube strips (Applied Biosystems) using a ABI Prism 7300 sequence detection system (Applied Biosystems). Each reaction contained 10 µl of template DNA, 25 µl of SYBR Premix Ex *Taq*, 1 µl of ROX dye, 1 µl (20 pmol) of each primer, 1 µl of BSA (20 mg/ml, Bovine Serum Albumin, Roche, Mannheim, Germany), and sterile water to bring the total reaction volume to 50 µl. Thermal-cycling parameters were as follows: 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s, 53 °C for 15 s, and 72 °C for 1 min. Fluorescent signals were collected at the extension step. RTi-PCR-amplifications were performed in quadruplicate with a range of concentrations from 2.5 to 2.5×10^6 GE (see below) of the *L. monocytogenes* genomic DNA.

The specificity of RTi-PCR was evaluated using the melting temperature (T_m) calculated from the melting curve of the PCR product, which was obtained after completion of the PCR cycles using an additional thermal step (95 °C for 15 s, 60 °C for 1 min, 95 °C for 15 s, and 60 °C for 15 s). The T_m peak of the PCR product was calculated based on the initial fluorescence curve (F/T) by plotting the negative derivative of the fluorescent signal over temperature versus temperature ($-dF/dT$ versus T).

2.4. Assignment of ΔR_n and C_T

The collected fluorescent signals were analyzed with ABI Sequence Detection Software ver1.4 (Applied Biosystems). The fluorescent signal intensity of the reporter dye was normalized using the passive reference dye to correct for fluctuations in the fluorescent signal due to the changes in concentration and volume of the reaction mixture. Normalization of the reporter dye signal was achieved by dividing its signal intensity by the signal intensity of the passive reference dye, resulting in a ratio defined as the R_n (normalized reporter) for a given reaction. The ΔR_n is the difference between R_n^+ and R_n^-, where R_n^+ is the R_n of a reacted sample and R_n^- is the R_n of an un-reacted sample obtained during early PCR cycles prior to a detectable increase in fluorescent signal. The C_T (threshold cycle) indicates the PCR cycle at which a significant increase in ΔR_n was first detected. The threshold level was defined as the mean baseline signal calculated for PCR cycles 3–15 plus $10 \times$ SD (standard deviation) and was determined automatically by the detection system with default settings.

2.5. Detection of L. monocytogenes in artificially contaminated fruit juices

We collected samples of apple, grape, watermelon, and orange juices that were unpasteurized and contained no additives (e.g., sugar, colorant, or preservatives) from local manufacturers and checked for prior contamination with *L. monocytogenes* by conventional culture methods. The juice samples were then spiked with decreasing amounts of *L. monocytogenes* cells and immediately subjected to the Chelex-based treatment described above. Chelex-100 resin (0.5 mg/5 ml, BioRad) was added to the spiked juice samples, and the sample was then boiled at 100 °C for 10 min. An aliquot (1 ml) of each sample was centrifuged at 12,000 rpm for 5 min (Eppendorf AG 22331). After centrifugation, 50 µl of the supernatant was subjected to further purification with a Sephadex column (Microspin G-50, GE Healthcare, Buckinghamshire, UK) and 10 µl ($3.2–3.2 \times 10^3$ cells per reaction) of the eluent was directly used as template for RTi-PCR to obtain C_T values. Distilled water samples were subjected to the same conditions and evaluated as a control.