International Journal of Food Microbiology 161 (2013) 134–141

Contents lists available at SciVerse ScienceDirect

International Journal of Food Microbiology

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

Development of a random genomic DNA microarray for the detection and identification of *Listeria monocytogenes* in milk

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ARTICLE INFO

Article history:
Received 4 July 2012
Received in revised form 21 November 2012
Accepted 21 November 2012
Available online 6 December 2012

Keywords:
DNA microarray
Random genomic DNA fragment
Listeria monocytogenes
Milk
Rapid and simultaneous detection

ABSTRACT

We developed a DNA microarray that contains random genomic DNA fragments of *Listeria monocytogenes*, validated its diagnostic abilities using cells grown in laboratory media and milk, and established enrichment conditions for detection of a low population of *L. monocytogenes* in milk. Genomic DNA of *L. monocytogenes* strain ATCC 19111 was fractionated by agarose gel electrophoresis after being cleaved using several different pairs of restriction enzymes. Sixty DNA fragments of different sizes were randomly selected and spotted onto an amine-coated glass slide. To validate diagnostic ability, probes on the DNA microarray were hybridized with genomic DNA extracted from *L. monocytogenes*, other *Listeria* spp., and foodborne pathogenic bacteria belonging to other genera grown in laboratory media. The DNA microarray showed 98–100% positive hybridization signals for the 16 strains of *L. monocytogenes* tested, 7–85% positive signals for 9 strains of other *Listeria* spp., and 0–32% positive signals for 13 strains of other types of foodborne pathogens. In milk, the detection limit of the DNA microarray was approximately 8 log CFU/mL. When milk contained *L. monocytogenes* (3–4 log CFU/mL) with other types of bacteria (*Bacillus* spp., *B. cereus*, *Salmonella* Montevideo, *Peudomonas aeruginosa*, and *Yersinia enterocolitica*; ca. 3 log CFU/mL each), *L. monocytogenes* enriched in UVM modified *Listeria* enrichment broth at 37 °C for 24 h was successfully detected by the DNA microarray. Results indicate that the DNA microarray can detect *L. monocytogenes* and distinguish it from other *Listeria* spp. and other foodborne pathogens in laboratory media and milk. This platform will be useful when developing a DNA microarray to rapidly and simultaneously detect and identify various foodborne pathogens in foods.

1. Introduction

The genus *Listeria* includes eight species: *L. monocytogenes*, *L. ivanovii*, *L. innocua*, *L. welshimeri*, *L. seeligeri*, *L. grayi*, *L. marthii*, and *L. rocourtiae*. *L. marthii* (Graves et al., 2010) and *L. rocourtiae* (Leclercq et al., 2010) were recently described. Among them, *L. monocytogenes* can cause serious diseases (listeriosis) such as meningitis, encephalitis, and sepsis in susceptible individuals, particularly infants, pregnant women, the elderly, and immunocompromised patients (Painter and Slutsker, 2007). Although listeriosis is less common than other foodborne diseases, it remains a public health concern due to its high mortality rate (28%) (Mead et al., 1999).

Various diagnostic techniques such as culture-based and molecular methods have been developed for the detection of *L. monocytogenes* in foods (Jeyaletchumi et al., 2010). Among the culture-based methods, the U.S. Department of Agriculture Food Safety and Inspection Service (USDA-FSIS) and the U.S. Food and Drug Administration (FDA) procedures have been adopted as standard methods in the USA (Donnelly and Nyachuba, 2007). These methods require approximately 7 days to isolate *L. monocytogenes* from foods and should be further improved for more rapid detection (Donnelly and Nyachuba, 2007).

Polymerase chain reaction (PCR) methods are the most widely used molecular methods for rapid detection of foodborne pathogens. However, PCR methods have inherent shortcomings, e.g., they require specific genetic information (Pangallo et al., 2001; Saito et al., 1998) and nonspecific DNA amplification products may be formed (Volokhov et al., 2002). Generation of nonspecific amplification products can cause a significant problem in multiplex PCR assays for the simultaneous detection of bacteria with several genetic markers (Elnifro et al., 2000). Thus, there is a continuing need for improved methods to accurately and simultaneously detect target foodborne pathogens. DNA microarray technology represents an option that may minimize this problem.

A DNA microarray can contain several thousand surface-immobilized DNA probes on a small glass slide. Thus, it can be used as a rapid and parallel high-throughput tool for microbial detection in a single hybridization assay (Fukushima et al., 2003). Functional genes, oligonucleotides, whole genomic DNA, and random genomic DNA fragments have been

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http://dx.doi.org/10.1016/j.ijfoodmicro.2012.11.023

used as probes on DNA microarrays for bacterial identification and microbial community analysis (Cho and Tiedje, 2001; Zhou, 2003). Functional genes and oligonucleotides require sequence information for the specific genes that are used as probes on DNA microarrays. However, whole genomic DNA and random genomic DNA fragments can be used as probes without sequence information, and random genomic DNA fragments are likely to have higher resolution than are whole genomic DNA (Zhou, 2003). We are not aware of reports describing the use of randomly fragmented genomic DNA on microarrays for detecting *L. monocytogenes*.

The ultimate goal of our research is to develop a DNA microarray containing random fragments of genomic DNA of foodborne bacterial pathogens that can be used for rapid and simultaneous detection of these pathogens in a wide variety of foods. Before developing a DNA microarray to detect multiple pathogens in a single assay, it is necessary to fabricate DNA microarray capable of detecting individual foodborne pathogens. In this study, we developed a DNA microarray for the detection of *L. monocytogenes*. The objectives of the study reported here were to fabricate a DNA microarray containing random fragments of genomic DNA originating from *L. monocytogenes*, validate its diagnostic ability in laboratory media and milk, and establish enrichment conditions needed to detect low levels of *L. monocytogenes* in milk.

2. Materials and methods

2.1. Bacterial strains and DNA extraction

The origins and growth conditions of the bacterial strains used in this study are shown in Table 1. The diagnostic ability of the DNA microarray to detect *L. monocytogenes* was determined using genomic DNAs extracted from test strains in laboratory media or milk. Genomic DNAs from cells in laboratory media were extracted using a DNeasy Blood and Tissue Kit (Qiagen, Hombrechtikon, Switzerland; final volume: 30 μL) according to the manufacturer's protocol. Genomic DNAs from cells in milk were extracted using a genomic DNA micro kit (Geneall biotechnology, Seoul, Korea; final volume: 20 μL). Before extracting of genomic DNAs, the bacteria-spiked milk (1.5 mL) was centrifuged (7500 ×*g*, 10 min, 4 °C), and cells were washed with 0.9% NaCl (1 mL). The concentrations of the genomic DNAs were measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

2.2. Fabrication of DNA microarray

Genomic DNA extracted from *L. monocytogenes* strain ATCC 19111 was digested with EcoRI/BamHI, HindIII/XhoI, or HindIII/SacII (New England Biolabs, Beverly, MA, USA). Digested genomic DNA fragments were separated by electrophoresis on a 0.8% agarose gel in Tris-acetate EDTA (TAE) buffer; fragments ranged in size from 100 to 1500 bp. The DNA fragments were eluted from the gels using a QIAquick Gel Extraction Kit (Qiagen) and purified by phenol-chloroform extraction. The purified DNA fragments were ligated into the pPCR-Script Amp vector (Invitrogen, Carlsbad, CA, USA) and transformed into *E. coli* DH5α. The transformed *E. coli* DH5α were spread on Luria–Bertani (LB) agar (BBL, Becton Dickinson) plates supplemented with 100 μg/mL of ampicillin and incubated at 37 °C for 16 h. After incubation, 303 colonies of *E. coli* DH5α were randomly selected from >10,000 colonies on the plates using isopropyl β-D-1-thiogalactopyranoside (IPTG)/X-gal color screening. From those colonies, plasmids were extracted using a QIAprep Spin Miniprep Kit (Qiagen). The plasmids were digested with the same pairs of restriction enzymes (EcoRI/BamHI, HindIII/XhoI, or HindIII/SacII) used for fabricating the plasmids to confirm the sizes of the DNA fragments inserted into the individual plasmids. The DNA fragments properly inserted into the plasmids were amplified using T7 and T3 promoter primer sets and purified using a QIAquick PCR Purification Kit (Qiagen). Finally, 60 fragments of genomic DNA were randomly selected for fabrication of the microarray. The bacteriophage ΦFC1 MJ1 gene was amplified using MJ1 probe F (5′-GGA AGT GGC ATT GTT CCC TT-3′) and MJ1 probe R (5′-TTC AGC TCC GCA TTT ACC AC-3′) and used as an internal standard on the microarray. Sixty fragments of genomic DNA and one amplified MJ1 gene were resuspended (final concentration: 150 ng/μL) in 100% dimethyl sulfoxide (DMSO; Sigma-Aldrich, Milwaukee, WI, USA) and spotted in quadruplicate on an amine-coated glass slide (GAPS™ II coated slides; Corning Inc., Corning, NY, USA) using a Genetix Q-Array Mini (Genetix Inc., Boston, MA, USA). After drying at room temperature, the DNA fragments on the slides were immobilized by UV cross-linking (0.160 J, 254 nm) and stored at room temperature before using the microarray slides in experiments. DNA sequences of the 60 fragments are presented in Supplementary Table S1.

2.3. Labeling of genomic DNA and hybridization

To determine the diagnostic ability of the microarray in laboratory media, 1 μL of genomic DNA (200 ng/μL) of the reference strain (*L. monocytogenes* strain ATCC 19111) and target strains were labeled with Cy5-dCTP and Cy3-dCTP, respectively, with a random priming method using a High Prime DNA Labeling Kit (Roche, Mannheim, Germany). To verify the diagnostic ability of the microarray in milk, 15 μL of genomic DNAs of the target strains was labeled with Cy3-dCTP. The labeling reaction was performed at 37 °C for 2 h. The genomic DNAs labeled with Cy-Dye were purified using a MinElute PCR Purification Kit (Qiagen). DNA mixtures (10 μL) were combined with 2 μL of hybridization buffer (6×saline-sodium citrate [SSC; 1×SSC consists of 0.15 mol/L NaCl plus 0.015 mol/L sodium citrate], 0.2% sodium dodecyl sulfate [SDS], 5×Denhardt's solution, and 0.1 mg/mL of denatured salmon sperm DNA) and denatured at 98 °C for 2 min. Before hybridization, the DNA microarray was placed in 20 mL of prehybridization buffer (1.75×SSC, 0.1% SDS, and 10 mg/mL bovine serum albumin), incubated at 65 °C for 30 min, washed with distilled water for 1 min, treated with 2-propanol (100%) for 30 s, and dried by centrifugation (2231 ×*g*, 5 min, 25 °C). For the hybridization, the denatured DNA mixtures (12 μL) were deposited on the DNA microarray, covered with a cover glass, and incubated at 65 °C for 14–15 h. After the hybridization, the cover glass was removed from buffer 1 (2×SSC), and the DNA microarray containing the hybridized DNA was washed in warm (65 °C) buffer 2 (2×SSC and 0.2% SDS) for 10 min, washed twice in buffer 3 (0.05×SSC) for 5 min, and dried by centrifugation (2231 ×*g*, 5 min, 25 °C). All experiments were performed for two to three times.

2.4. Scanning and data analysis

After hybridization, the DNA microarray was scanned using a GenePix 4000B laser scanner (Axon, Union City, CA, USA) and analyzed using the GenePix Pro 6.0 software (Axon). The intensity value of the local background was subtracted from the median values of each spot. For the intensity values obtained after hybridization of *L. monocytogenes* in laboratory media, the values of fluorescent signals from each spot were normalized by a correction factor calculated from median values (test/reference = 1) of the internal standard. For the *L. monocytogenes* in milk, the values from each spot were normalized by average intensity values of the internal standard. Positive signals were defined as values higher than the average background values plus three times the standard deviation. To show the intensities of hybridization between probes on the DNA microarray and genomic DNA of test strains, the ratios of intensity values (test/reference) were $\log_2$-transformed and visualized on a MultiExperiment Viewer (MeV; http://www.tm4.org). To determine the relatedness between *L. monocytogenes* strains and other bacterial strains, hierarchical cluster analysis (HCA; MeV) and principal components analysis (PCA; R, http://cran.nexr.com/) were performed.