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Rapid and Sensitive Detection of *Listeria monocytogenes* Using a PCR-Enzyme-Linked Immunosorbent Assay

Kim, Hye-Jin and Jae-Chang Cho*

Institute of Environmental Science and Engineering, Department of Environmental Sciences, Hankuk University of Foreign Studies, Yongin 449-791, Korea

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A PCR-enzyme-linked immunosorbent assay (PCR-ELISA) was developed for the rapid and sensitive detection of *L. monocytogenes*. PCR primers generating a 132-bp amplicon and a capture probe able to hybridize to the PCR amplicon were designed based on the *L. monocytogenes*-specific *hly* gene encoding listeriolysin. The detection limit of PCR-ELISA for *L. monocytogenes* was determined to be as low as 10 cells per PCR reaction, and this level of detection was achieved within 5 h. These results indicate that the PCR-ELISA provides a valuable tool for the rapid and sensitive detection of *L. monocytogenes* for the ready-to-eat food industry.

Keywords: PCR-ELISA, *Listeria monocytogenes*

Listeria monocytogenes is a significant foodborne pathogen that is widely distributed in both natural and human-made environments [9, 11]. In immunocompromised individuals and pregnant women, it is capable of causing listeriosis, a serious disease that may result in severe complications such as meningitis, septicemia, and spontaneous abortion [20]. Compared with other common foodborne infections, listeriosis has a relatively high mortality rate (20–30%) [5, 13, 24]. Most cases of listeriosis are caused by the ingestion of *L. monocytogenes*-contaminated ready-to-eat foods that do not require heating before consumption [9, 11], such as raw and smoked fish, bean curd (*tofu*), raw and fermented vegetables (*e.g.*, salads and *kimchi*), cheese, milk, raw-meat sausage, and ice cream. Because *L. monocytogenes* is ubiquitous and capable of growth at refrigeration temperature (4°C) [8, 9, 11], it may contaminate foods at any point of production, distribution, or storage. The prevalence of *L. monocytogenes* contamination in a variety of ready-to-eat foods and food processing environments has been reported from 6–36% up to 78% of tested samples [1–3, 16, 21, 28, 30, 32]. Since food safety regulations have tended to adopt a zero tolerance attitude for the presence of *L. monocytogenes* in ready-to-eat foods [10], it is important to have a rapid test with high sensitivity for detecting *L. monocytogenes*.

In this study, a PCR-enzyme-linked immunosorbent assay (PCR-ELISA) for the rapid and sensitive detection of *L. monocytogenes* was developed. PCR–ELISA involves incorporation of chemically tagged nucleotides into the PCR amplicon that may be detected later with an enzyme-conjugated antibody [23]. PCR-ELISA has been used for the detection of pathogenic bacteria from food, clinical, and environmental samples [4, 6, 7, 12, 14, 15, 26, 27, 31, 35], but few studies evaluated the utility of PCR-ELISA for detecting *L. monocytogenes*. For PCR-ELISA detection of *L. monocytogenes*, *L. monocytogenes*-specific PCR primers and a capture probe were designed, validated, and implemented based on the *hly* virulence gene encoding listeriolysin [25]. Here, the specificity, sensitivity, and convenience of the developed method are described.

Bacterial Strains and DNA Extraction

Eleven strains of *Listeria* (9 strains of *L. monocytogenes* [ATCC 19111–19118 and ATCC 15313], *L. ivanovii* ATCC 19119, and *L. innocua* ATCC 33090) from the American Type Culture Collection (ATCC) were used to evaluate primer specificity in initial PCR optimization experiments. All *Listeria* strains were cultured in brain heart infusion (BHI) broth (Difco, Detroit, MI, U.S.A.) at 37°C. Non-*Listeria* strains (*Pseudomonas, Escherichia, Vibrio, Acidobacteria,* and *Bacillus*) were obtained from laboratory stocks and cultured in nutrient broth (Difco). Genomic DNAs from the bacterial strains were extracted according to the standard procedures described by Sambrook and Russell [33]. DNA concentrations were determined by using a DNA Quantification kit (Sigma, St. Louis, MO, U.S.A.) and fluorometer according to the manufacturer's protocols.

*Corresponding author
Phone: +82-31-330-4350; Fax: +82-31-330-4529;
E-mail: chojc@hufs.ac.kr

Oligonucleotide Primers and Capture Probe

PCR primers and a capture probe were designed based on the *L. monocytogenes*-specific *hly* gene encoding listeriolysin [25]. The *hly* sequences (Accession Nos. AF253320, M24199, U25446, U25452, and U25449) were obtained from the NCBI (National Center for Biotechnology Information) GenBank database and aligned with the CLUSTAL W software [34]. A consensus sequence from the aligned *hly* sequences was subjected to primer and probe searches with the ArrayDesigner software (PREMIER Biosoft, Palo Alto, CA, U.S.A.). After checking the specificity of primer and probe sequences *in silico* using the NCBI BLAST (Basic Local Alignment Search Tool), searches against sequences available in the GenBank database, PCR primers [L-JH-f (5'-TCCGCCTGCAAGTCC-TAAG-3') and L-JH-r (5'-GGCGGCACATTTGTCACTG-3')] generating a 132-bp amplicon and a capture probe [L-JH-c (5'-CAAGTCCTAAGACGCCAATCG-3')] able to hybridize to the PCR amplicon were designed. The capture probe was synthesized with an incorporated 5' biotin.

PCR–ELISA Detection of *L. monocytogenes*

The *L. monocytogenes*-specific *hly* gene was amplified using primers L-JH-f and L-JH-r. The reaction mixture included 25 μl of *Taq* PreMix with $MgCl_2$ (TaKaRa, Shiga, Japan), 1 μl of each of the forward and reverse primers (stock concentration, 20 μM), 10 ng of template DNA, and sterilized 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, and 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 Biosystem, Foster City, CA, U.S.A.).

The specificity of the primers was tested with 9 strains of *L. monocytogenes* as positive controls. *L. ivanovii*, *L. innocua*, and 5 non-*Listeria* strains from laboratory stocks were used as negative controls. PCRs were applied to undiluted

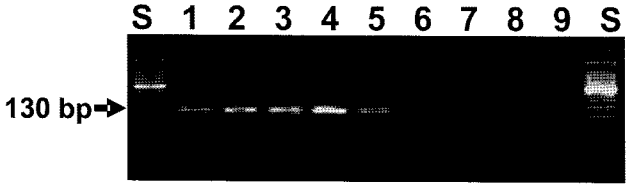

Fig. 1. Agarose gel electrophoretogram of PCR-amplified *hly* sequences.

PCR products were characterized by comparison with a standard molecular-size marker (100-bp ladder). Five of 9 positive control strains and 4 of 9 negative control strains are included in the electrophoretogram. Lane S, standard size marker; Lane 1, *Listeria monocytogenes* ATCC 19111; lane 2, *L. monocytogenes* ATCC 19113; lane 3, *L. monocytogenes* ATCC 19115; lane 4, *L. monocytogenes* ATCC 19117; lane 5, *L. monocytogenes* ATCC 15313; lane 6, *L. ivanovii* ATCC 19119; lane 7, *L. innocua* ATCC 33090; lane 8, *Escherichia coli* strain B; lane 9, *Pseudomonas aeruginosa* PAO1.

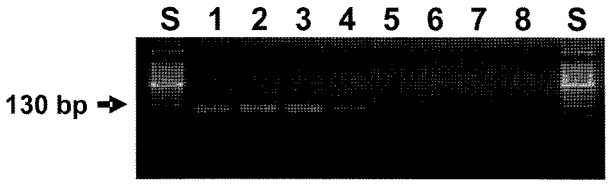

Fig. 2. Agarose gel electrophoretogram of PCR products from serial dilutions of genomic DNA extracted from *Listeria monocytogenes* ATCC 19117.

Lane S, standard molecular size marker (100-bp ladder); Lanes 1 to 7, 10-fold serial dilutions (10^6–10^0 genome equivalents per reaction); Lane 8, undiluted *E. coli* genomic DNA as a negative control.

genomic DNAs from positive and negative control strains. PCR products of the expected size of 132 bp were amplified for all *L. monocytogenes* strains, and no products were amplified for any of the negative control strains (Fig. 1). To evaluate the sensitivity of PCR, serial dilutions of the genomic DNA from *L. monocytogenes* were prepared. The molecular weight of the *L. monocytogenes* genome was calculated based on the average size of the genome (2.96 Mb) (http://cmr.tigr.org). The amount of genomic DNA in each of the serial dilutions was converted to the number of genomes equivalent to the number of *L. monocytogenes* cells. After the 10-fold serial dilutions of the genomic DNA were made, PCR was performed on each diluted DNA sample (10^6–10^0 genome equivalents [GE] per reaction) using the L-JH-f and L-JH-r primers (Fig. 2). The assay detected up to 10^3 *L. monocytogenes* GE (visible band in ethidium bromide-stained agarose gel). However, in the PCR assays, the amplification products are examined by gel electrophoresis, which may lack sensitivity and specificity, or by Southern hybridization, which is not convenient for processing large numbers of samples. Alternatively, an immunological method (ELISA) was applied for examining the PCR-amplification products with the newly designed capture probe in this study, thus facilitating specific and sensitive detection of the PCR products.

PCR-ELISA was conducted on 10-fold serial dilutions of *L. monocytogenes* genome in triplicate. PCR products were directly labeled with digoxigenin (DIG) during the PCR-amplification step described above. DIG-11-dUTP was incorporated into the PCR products by using a PCR-DIG Labeling kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol. After PCR-amplification, the DIG-labeled PCR products (10 μl) were denatured with 20 μl of denaturation solution (Roche Diagnostics) at room temperature (25°C) for 10 min. Denatured PCR products were detected in a sandwich hybridization assay using streptavidin-coated microtiter plates (Roche Diagnostics). For each hybridization, a 30-μl aliquot of denatured PCR product was transferred to each well of a microtiter plate and incubated at 37°C for 1 h with 220 μl of hybridization solution (Roche Diagnostics) containing 22 pmole of biotinylated capture probe per mL. The wells were washed three times at room temperature with 250 μl

of washing solution (Roche Diagnostics). Then, 200 μl of anti-DIG-POD conjugate solution [10 mU/ml peroxidase-conjugated anti-digoxigenin antibody (Roche Diagnostics)] was added per well and incubated at 37°C for 30 min. The wells were washed five times with 250 μl of washing solution at room temperature, and 200 μl of ABTS (2,2'azinobis[3-ethylbenzthiazolinesulfonic acid], 1 mg/ml) solution (Roche Diagnostics) was added to each well to detect bound hybrid. Spectrophotometric analysis (OD_{405}) was performed using a microtiter plate reader (BioTek, Winooski, VT, U.S.A.) and measured against the reference wavelength at 492 nm during color development. Negative controls (sterilized distilled water and PCR reaction mix with no primers) were included with each set of tests, and all tests were performed in triplicate. Positive reactions were determined by calculating the cutoff values (the mean of 24 replicates of the negative controls plus 2× standard deviation) for each test.

For colorimetric analysis, OD_{405} readings were recorded, averaged, and plotted against color development time for each dilution series (Fig. 3). Negative controls showed absorbances of 0.069±0.008, and thus a cutoff level of OD_{405}>0.085 was applied to determine a positive result. The detection limit of PCR–ELISA on *L. monocytogenes* was determined to be as low as 32.0 fg, the equivalent of 10 CFUs per PCR reaction. The intensity of the color in positive samples could be observed with the naked eye (OD_{405}>0.1) after 10–15 min of the color development. Additionally, a linear correlation (r^2=0.65, P<0.1) between OD_{405} and the cell density of *L. monocytogenes* was observed. PCR-ELISA has been reported to increase the sensitivity of PCR product detection more than electrophoresis-based methods [15, 22, 29]. Additionally, PCR-ELISA increases the specificity the of PCR by avoiding false positives due to nonspecific PCR products [15, 17–19, 36]. Increased specificity of PCR-ELISA is achieved through the use of a capture probe, which is equivalent to a *Taq*Man probe in real-time PCR.

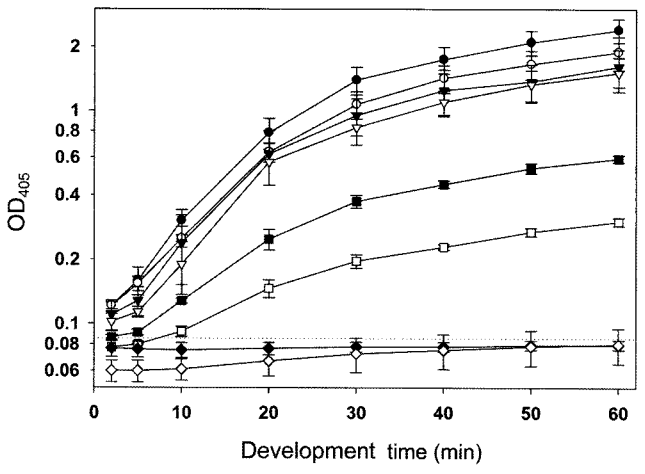

Fig. 3. Spectrophotometric analysis (OD_{405}) of PCR–ELISA for detecting *Listeria monocytogenes*.
OD_{405} values were plotted against ELISA color development time for each dilution series of *L. monocytogenes*. The dotted line indicates the average absorbance +(2× standard deviation) of negative controls.

Concluding Remarks

Using specifically designed primers and a capture probe, the PCR-ELISA method described was sensitive enough to detect *L. monocytogenes* at levels as low as 10 CFUs (genome equivalents) per reaction, and this level of detection was achieved within 5 h. PCR-ELISA has the time advantages of the DNA-based techniques, the specificity and sensitivity of Southern hybridization, and the convenience of being able to accommodate the 96-well microtiter plate format allowing a larger sample size to be analyzed simultaneously. Another advantage of this method is that PCR-ELISA can be easily implemented in portable detection systems without special equipment such as spectrophotometers and real-time thermocyclers. For the rapid and sensitive detection of *L. monocytogenes*, PCR-ELISA will be a valuable tool in the ready-to-eat food industry.

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