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Detection of *Escherichia coli* O157:H7, *Salmonella* spp., *Staphylococcus aureus* and *Listeria monocytogenes* in Kimchi by Multiplex Polymerase Chain Reaction (mPCR)

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We developed an mPCR assay for the simultaneous detection, in one tube, of *Escherichia coli* O157:H7, *Salmonella* spp., *Staphylococcus aureus* and *Listeria monocytogenes* using species-specific primers. The mPCR employed the *E. coli* O157:H7 specific primer Stx2A, *Salmonella* spp. specific primer Its, *S. aureus* specific primer Cap8A-B and *L. monocytogenes* specific primer Hly. Amplification with these primers produced products of 553, 312, 405 and 210 bp, respectively. All PCR products were easily detected by agarose gel electrophoresis, and the sequences of the specific amplicons assessed. Potential pathogenic bacteria, in laboratory-prepared and four commercially available kimchi products, were using this mPCR assay, and the amplicons cloned and sequenced. The results correlated exactly with sequences derived for amplicons obtained during preliminry tests with known organisms. The sensitivity of the assay was determined for the purified pathogen DNAs from four strains. The mPCR detected pathogen DNA at concentrations ranging from approximately 0.45 to 0.05 pM/μl. Thus, this mPCR assay may allow for the rapid, reliable and cost-effective identification of four potentially pathogens present in the mixed bacterial communities of commercially available kimchi.

Keywords: *E. coli* 0157:H7, kimchi, *Listeria monocytogenes*, multiplex PCR, *Salmonella* spp., *Staphylococcus aureus*

Escherichia coli O157:H7, *Salmonella* spp., *Staphylococcus aureus* and *Listeria monocytogenes* are able to survive as potential pathogens in both commercial and laboratory-prepared kimchi (Inatsu *et al.*, 2004). Thus, contamination of kimchi with these organisms at any stage of production or during marketing could pose a potential health risk. For examples, *Salmonella* spp., *S. aureus* and *L. monocytogenes* are among the species currently found in many foods. Furthermore, these bacteria are human pathogens, and have been frequently reported as agents in food poisoning (Gjertsson *et al.*, 2002; Miller *et al.*, 2004; Alarcon *et al.*, 2004; Gasanov *et al.*, 2005; Mora *et al.*, 2005). *E. coli* O157:H7 is an important human pathogen found in the intestines of cattle and shed in their feces (Su and Brandt, 1995; Laegreid *et al.*, 1999). *E. coli* O157 is an important worldwide cause of diarrhea, hemorrhagic colitis and hemolytic-uremic syndrome. In Korea, *E. coli* O157:H7 has been isolated from livestock (Jo *et al.*, 2004). *Salmonella* may cause infections localized to the intestinal epithelium, known as "non- typhoid salmonellosis", as well as the systemic infection "typhoid salmonellosis" (Garcia-Del Portillo *et al.*, 2000). *S. aureus* may cause respiratory disease (Brogden *et al.*, 2005) in susceptible patients, and *L. monocytogenes,* a facultative intracellular pathogen, is able to invade the central nervous system, causing meningoencephalitis and brain abscesses (Join Lambert *et al.*, 2005). Together, these four major bacterial strains have been identified as the most frequent agents in food poisoning. Rapid identification of these pathogenic bacteria can enhance the routine testing of food samples by reducing the time required for testing, as well as the cost of labor and media employed. However, traditional methods of identification requires several days, and are very laborious and time consuming. Alternatively, PCR has been widely accepted as a rapid and sensitive method for the detection and identification of bacteria. The mPCR (multiplex-PCR) method has the additional advantages of a lower economic cost and rapid processing. The detection of three of the bacterial strains, *Salmonella* spp., *S. aureus* and *L. monocytogenes,* has already been described in artificially in-

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oculated raw beef using a multiplex-PCR-CGE-LIF tool (Alarcon *et al.*, 2004). mPCR detection has also been tested on *E. coli* O157:H7 and *Salmonella* spp. (Fratamico and Strobauh, 1998; Jin *et al.*, 2004). For the simultaneous detection of more than one pathogen, the differences in growth requirements and rates must also be considered. Therefore, PCR-based procedures have been favored as rapid and highly specific methods, where the detection and identification can be completed with in 24 h, without the need for isolating pure cultures (Hill, 1996). The mPCR detection also costs less and requires less time for the detection of multiple species than with sequential rounds of uniplex PCR. Moreover, mPCR procedures for the simultaneous detection of two to four bacterial pathogens have been demonstrated (Kong *et al.*, 1995; Brasher *et al.*, 1998; Phuektes *et al.*, 2001; Lim and Lee, 2002; Kong *et al.*, 2002; Gilbert *et al.*, 2003;).

However, the simultaneous detection of *E. coli* O157: H7, *Salmonella* spp., *S. aureus* and *L. monocytogenes* remains to be reported. Moreover, commercially available kimchi has not been investigated with respect to the presence of these potential pathogens, which have the potential to pose substantial health risks.

Here, we report the development of an mPCR technique for the simultaneous detections, in one tube, of four potential pathogens in kimchi using a single step. This mPCR procedure may provide a more cost effective and rapid detection approach than other currently available techniques.

Materials and Methods

Bacterial strains, growth conditions and DNA extraction

The genomic DNA of enterohemorrhagic *E. coli* O157: H7 strain CR-3 was provided by Dr. Lee (College of Veterinary Medicine, Chonbuk National University, Chonju, Korea). The transformants of Stx2A gene of *E. coli* O157:H7 in *E. coli* DH5α. were used as a sample for *E. coli* O157:H7. The *Salmonella* spp. *strain* IFO-3313, *S. aureus* IFO 13276 and *L. monocytogenes* ATCC 43256 were obtained from Dr. Park (College of Veterinary Medicine and School of Agricultural Biotechnology, Seoul National University, Seoul, Korea). The *L. monocytogenes* strain was grown on Brain Heart Infusion (BHI), and the other strains was grown on tryptic soy broth (10 ml) at 37°C, using the method of Inatsu *et. al.* (2004). Organisms were collected by centrifugation (12,000 rpm, 3 min, 4°C). Aliquots of 1- and 10 ml from the cultures and kimchi suspensions, respectively, were used for DNA extraction. DNA was purified using Ultraclean™ Microbial DNA Kits (MoBio Lab, Inc., USA), according to the manufacture's instructions purified, and recovered in 200 μl of elution buffer. The DNA concentration was determined using the Pharmacia GeneQuant RNA/DNA calculator (Pharmacia Biotech, Ltd., USA).

Sample preparation from commercial and inoculated kimchi

Commercially prepared kimchi, produced by different local manufacturers, were purchased randomly from four different stores (a, b, c and d) in the Kwangju area of Korea, and stored at 4°C for a 10 h prior to analysis. Experimental kimchi was inoculated with four bacterial strains, which served as positive controls, as follows: Firstly, 100 μl of each of the cell suspensions and 100 g of each type of laboratory prepared kimchi were added to sterile plastic cups, and the contents thoroughly mixed with a sterile glass rod. Inoculated enumeration plates were incubated at 37°C for 28 h. All samples, including these controls, were subjected to DNA extraction, uniplex PCR, mPCR and sensitivity tests.

mPCR reaction

The primer sequences used for the detection of the four test organisms are shown in Table 1. All primers,

Table 1. Bacterial targets, mPCR primers, PCR product identities, and product sizes

Bacterial target	Primer	PCR Products (size)	Reference
E. coli O157:H7	Stx2Af Stx2Ar	5′-CGAGGGCTTGATGTCTATCAG.-3′ 5′-TCAGTATAACGGCCACAGTCC-3′(553bp)	This work
Salmonella spp.	Itsf Itsr	5′-TATAGCCCCATCGTGTAGTCAGAAC-3′ 5′-TGCGGCTGGATCACCTCCTT-3′(312bp)	Chiu *et al.* (2005)
S. aureus	Cap8A-Bf Cap8A-Br	5′-CCGTTTCATAAGGCGAGTTG-3′ 5′-CTGTTCGGGTATTTGAAGATGG-3′(405bp)	This work
L. monocytogenes	Hlyf Hlyr	5′-CGCAACAAACTGAAGCAAAGG-3′ 5′-TTGGCGGCACATTTGTCAC-3′(210bp)	This work

synthesized by GenoTech Corp (Daejeon, Korea), were designed in our laboratory based on related species-specific genes as follows except Its primer: Stx 2A for the amplification of a 553 bp fragment corresponding to the *stx2A* gene *E. coli* O157:H7 (Kim *et al.*, 2001); Its for the amplification of a 312 bp fragment corresponding to the *Salmonella spp.* (Chiu *et al.*, 2005); Cap8A-B for the amplification of a 405 bp fragment of *cap8* gene of *S. aurus* (Sau *et al.*, 1997); and HlyA for the amplification of a 210 bp corresponding to the *hlyA* gene of *L. monocytogenes* (Join-Lambert *et al.*, 2005).

The mPCR mixture contained 30 μl of 67 mM Tris buffer (pH 8.8), 2.5 mM $MgCl_2$, 0.01% Tween-20, 12% glycerol, 2.5 mM dNTP, 2.5 unit of Taq DNA polymerase (Bioron, Germany), 1 U of uracil-N-glycosylase, to prevent carryover contamination, and 10 pmol of the specific oligonucleotide primer. Amplification was performed in a PCR system (Takara Biomedical Co., Ltd, Japan) on a MicroAmp base, with the following parameters: The initial thermal cycling conditions were initial 94°C for 3 min, followed by 35 cycles of amplification, consisting of denaturation at 94°C for 30 sec, annealing at 61°C for 35 sec and extension (72°C) for 35 sec during each step. Ten samples were run together in each round of amplification. Negative controls, lacking DNA, and genomic DNA were subjected to mPCR. For the PCR with DNA from multiple species, 300 ng of genomic DNA, from each separate species, were analyzed together. For the bacterial DNA from kimchi, 2 μg of DNA was used. The positive control consisted of DNA isolated from each organism grown in a batch culture. The negative controls included PCR mixtures with primers, but with no added DNA. The mPCR was optimized for the simultaneous detections of DNA from all four bacterial species in kimchi, and then compared with individual uniplex PCR assays.

Sensitivity and specificity of specimen-specific mPCR

One picogram of genomic DNAs from the four test organisms were subjected to specimen-specific mPCR. Two and four fold dilutions, from 2.2 pM to 0.05 pM genomic DNA derived from each organism, were then also subjected to species-specific PCR to determine the sensitivity of the test. The specificity of the mPCR was confirmed by sequence analysis of the each primer product from random samples, as previously described (Werle *et al.*, 1994). The sequences were compared with those reported in the NIH-GeneBank database using a BLAST search (http://ncbi.nlm.nih.gov/BLAST/).

Agarose gel electrophoresis

15 μl of the amplified PCR products were subjected to electrophoresis on a 2% agarose gel, containining 0.5 μg of ethidium bromide per ml, and the photographed using an Image master (Pharmacia Biotech. Ltd., USA).

Results

Detection of mPCR products

Each PCR amplification reaction generated a unique DNA fragment of the expected size, without producing PCR products from non-target species, indicating each primer set to be species-specific (Fig. 1). The PCR products were observed by agarose gel electrophoresis. The sequences of the PCR products

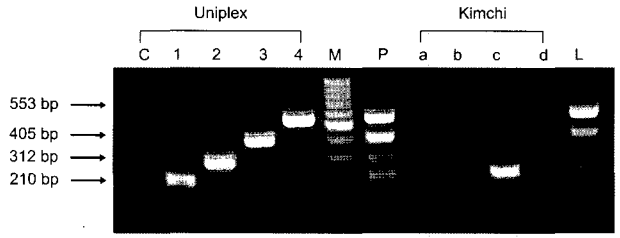

Fig. 1. Uniplex PCR amplification products from species-specific primers for *L. monocytogenes* (lane 1), *Salmonella* spp. (lane 2), *S. aureus* (lane 3), *E. coli* O157:H7 (lane 4), and mPCR based commercial kimchi (Lanes a, b, c and d) and pathogen-inoculated kimchi (lane L). C, negative control; M, 100 bp DNA ladder; P, mixed mPCR products. Numbers on the left indicate the bp of the species-specific amplification products.

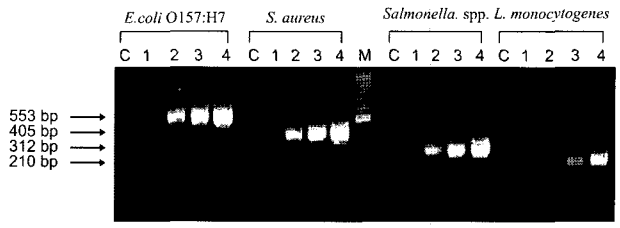

Fig. 2. Sensitivity of mPCR procedures for detecting *E. coli* O157:H7, *Salmonella* spp., *S. aureus*, and *L. monocytogenes* in kimchi. The mPCR reactions contained the following DNA concentrations: *E. coli* O157:H7 (lane 4: mixture of 1.0465 pM/μl, lanes 3, 2 and 1 were diluted 1:2, 1:3 and 1:4, respectively, from the mixture in lane 4). *S. aureus* (lane 4: mixture of 2.2626 pM/μl, lanes 3, 2 and 1 were diluted as with *E. coli*). *Salmonella* spp. (lane 4; mixture of 1.9480 pM/μl, lanes 3, 2, and 1 were diluted as with *E. coli*). *L. monocytogenes* (mixture of 2.2626 pM/μl, lanes 3, 2 and 1 were diluted as with *E. coli*). Each 30 μl mPCR reaction was runs for 35 cycles, withan aliquot of the reaction mixture (10 μl) analyzed by agarose gel electrophoresis andethidium bromide staining. Specific PCR products are indicated by arrows for *E. coli* O157:H7 553 bp, *Salmonella* spp. 312 bp, *S. aureus* 405 bp, and *L. monocytogenes* 210 bp. C, control *E. coli* DNA; M, 100 bp DNA ladder.

were generated by GenoTech Corp., Korea, using individual synthetic primers on a Applied Biosystems 3730xi DNA analyzer (Genotech, Korea); the results were directly comparable to those obtained from the electrophoresis (data not shown).

The primer sets reacted with none of the other five bacteria present as possible pathogens: *E. coli, Bacillus subtilis, Leuconostoc carnosum, Lactobacillus curvatus* and *Lactobacillus kimchi* (data not shown). The different sizes of the amplification products, as indicated in Table 1, were separated using agarose gel electrophoresis. Fig. 1 shows the results of a typical mPCR analysis, along with the four individual PCRs products. The species-specific primers designed in this study successfully differentiated between the four bacterial species in kimchi.

As shown in Fig. 1, while three types of the commercial kimchis (a, b, and d) were not contaminated with a test pathogen, 'c' kimchi was only contaminated with the potential pathogen *L. monocytogenes*. All four pathogens, inoculated into kimchi in the laboratory were detected via our mPCR method.

Sensitivity of species-specific mPCR

Fig. 2 indicates that the minimum concentrations detectable by the species-specific PCR varied in sensitivity according to the species. As determined by the agarose gel electrophoresis, the optimal sensitivity for the detection ranged between 0.057 and 0.452 pg for the bacteria tested. Detection of one of these four species, *L monocytogenes*, was not more sensitive than was detection of the other species.

Discussion

By simultaneously amplifying more than one locus in the same reaction, mPCR has been identified as a rapid and convenient screening assay, with both clinical and research applications. Simultaneous detection of four major potential pathogenic bacteria in kimchi has been demonstrated in the present study by analyzing a single sample using mPCR. The results show that developed mPCR assay was able to to successfully detect *E. coli* O157:H7, *Salmonella* spp., *S. aureus* and *L. monocytogenes* in samples prepared from commercial and inoculated kimchi. Previous work has demonstrated optimal mPCR results using an annealing temperature of 56°C for the detection of *Salmonella* spp., *S. aureus* and *L. monocytogenes* from inoculated meat (Alarcon *et al.*, 2004). However, we obtained an optimum at 61°C for these three strains, as well as for *E. coli* O157:H7. This apparent discrepancy may have been due to the primer composition or concentration, the $MgCl_2$ concentration, or some other PCR variables (Henegariu *et al.*, 1997). The modified primers, shown in Table 1, were somewhat different to those previously published (Su and Brandt., 1995; Kim *et al.*, 2001; Koo and Jaykus, 2003; Chiu *et al.*, 2005). The development of our mPCR assay the species-specific primer construction included additional GC nucleotides at the primer ends. Such issues in the design of a primer should be studied further to ensure consistent results for the efficiency of mPCR between studies. The mPCR reaction mixtures were adjusted according to the method described by Henegariu *et al.* (1997). Three commercially available kimchi, randomly selected from local markets, were not contaminated with three potential tested pathogens, but one sample was infected with *L. monocytogenes*. The sensitivities of detection for the species-specific primers, ranged from 0.05 to 1.0 pM. These differences may have been due to differences in the sequences of the target genes compared with the genomic DNA. These detection sensitivities may agree with those from previously reported uniplex PCR or mPCR, with *Salmonella* spp., *L. monocytogenes, S. aureus* detected at levels above 57, 79, and 260 cfu ml^{-1}, respectively (Alarcon *et al.*, 2004). The sensitivity could be further improved using various approaches, but it is encouraging that the typical amounts of DNA used in any PCR reactions appear to be sufficient for this type of detection. The mPCR products were further analyzed by size comparison with gene standards on agarose gels, and by sequence analysis and comparison with the NIH Genebank database; all of these metthods confirmed the specificity of the mPCR analysis. This mPCR assay should be very useful for the detection of pathogens that may potentially contaminate kimchi and other foods.

With the laboratory inoculated kimchi, all the bacteria studied in this report survived for three weeks after inoculation, but *L. monocytogens* was only detected by mPCR detection in kimchi sample 'c'. This confirms the importance of the routine inspection of commercially produced foods, including kimchi, in order to detect and identify pathogens that potentially pose human health risks.

Finally, we should note that optimal combinations of individual primers are essential for any amplification procedure. In order to optimize an mPCR assay for the detection of many species or strains, critical parameters, as well as the step-by-step protocol (e.g., DNA quality and extraction procedure, adequate primers selection, PCR conditions optimization) suggested by Henegariu *et al.* (1997), should be considered.

In conclusion, this mPCR assay represents a rapid and reliable method for the detection and identification of *E. coli* O157:H7, *Salmonella* spp., *S. aureus* and *L. mococytogenes* in fermented kimchi products.

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