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Simultaneous Detection of *Yersinia enterocolitica*, *Staphylococcus aureus*, and *Shigella* spp. in Lettuce Using Multiplex PCR Method

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Abstract The development of rapid, infallible, and sensitive methods of detecting foodborne pathogens has received much impetus in recent years owing to an increased public awareness of the health hazards. For the rapid and simultaneous detection of these foodborne pathogens, a multiplex PCR method was developed. *Yersinia enterocolitica*, *Staphylococcus aureus*, and *Shigella* spp. are bacteria of concern because of their specific growing condition that enables them to live at low temperatures. In order to detect each pathogenic bacterium, specific primers from *Y. enterocolitica*, *St. aureus*, and *Sh. flexneri* were selected and validated successfully. To apply this method to food stored at low temperature, *Y. enterocolitica*, *St. aureus*, and *Sh. flexneri* were artificially inoculated in lettuce and incubated for enrichment. The multiplex PCR assays were able to simultaneously detect three pathogens, and the presence of three bands was observed at initial inoculation levels of approximately 1×10^1 CFU/g in lettuce. Therefore, this method could be used for simultaneous detection of *Y. enterocolitica*, *St. aureus*, and *Shigella* spp. contaminated in lettuce during cultivation, transportation, preservation, and storage.

Key words: Multiplex PCR, *Shigella* spp., *Staphylococcus aureus*, *Yersinia enterocolitica*

Yersinia enterocolitica, *Staphylococcus aureus*, and *Shigella* spp. are foodborne pathogens and are able to live at low (4–8°C) temperature. Recently, social well-being trends are promoting consumption of raw vegetables such as salads. Consequently, those pathogens are of much concern, because of their specific growing condition to be able to live at a low temperature. To the best of our knowledge, there has been no study done so far to detect these pathogens simultaneously from raw vegetables. The number of outbreaks of human infections associated with consumption of raw fruits and vegetables has increased in recent years. Advances in processing, preservation, and distribution technologies have enabled the supply of nearly all types of fresh fruits and vegetables in season and out of season. However, some aspects of these technologies also cause risk of human illness associated with a wide range of pathogenic microorganisms [2, 3].

Y. enterocolitica occurs in a wide range of ecosystems and is found in the intestinal tracts of birds, fishes, and various types of foods from several countries [4, 7]. Recently, the presence of pathogenic strains of *Y. enterocolitica* on raw produce has increased concern about the potential of salad as cause vehicles of yersiniosis in humans. *Y. enterocolitica* can grow at refrigeration temperature up to 5 days, which is commonly used during transport and storage of fresh produce.

St. aureus has been detected in both fresh foods and ready-to-eat vegetable salads [1]. Previous studies have approached the detection of *St. aureus* strains on the basis of *femA* and *mecA* gene amplification [6, 8, 12]. The genus *Shigella* is composed of four species, *Shigella dysenteriae*, *Shigella flexneri*, *Shigella sonnei*, and *Shigella boydii*, which are all pathogenic to humans [16]. Many foodborne outbreaks of shigellosis are reported each year. In the early 20th century, *Sh. dysenteriae* strains were the primary cause of shigellosis. However, in recent years, *Sh. dysenteriae* has almost disappeared in developed countries, but is replaced by *Sh. flexneri* and *Sh. sonnei* [14]. Symptoms caused by *Shigella* species continue to be an important cause of diarrheal disease. *Shigella* strains are well known to cause shigellosis in humans who take low dose of 10^1 to 10^2 cells [14]. Specifically, *Sh. sonnei* can survive on lettuce at 5°C for 3 days without reduction in number, and also increase by more than 1,000-fold at 22°C [21].

The detection methods of each *Y. enterocolitica*, *St. aureus*, and *Shigella* spp. strain include restriction fragment length polymorphism (RFLP) analysis, field-inversion gel electrophoresis (FIGE), pulsed-field gel electrophoresis

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(PFGE) [22], random amplification of polymorphic DNA (RAPD) [5], and ribotyping [9]. However, there is no method available to detect the above three bacteria simultaneously. Each method demonstrates certain disadvantages with limited reproducibility, and the need for highly developed techniques or high cost. However, the multiplex PCR method could overcome these disadvantages and has the potential for rapid and sensitive detection of foodborne pathogens.

The purpose of this study was to develop a multiplex PCR assay that is specific, sensitive, simple, and highly reproducible for simultaneous detection of *Y. enterocolitica*, *St. aureus*, and *Shigella* spp.

MATERIALS AND METHODS

Bacterial Strains

The 22 bacterial strains tested in this study are shown in Table 1. Reference strains were collected from the American Type Culture Collection (ATCC). All strains were cultivated overnight with shaking. *Y. enterocolitica* and *St. aureus* were grown in tryptic soy broth (Difco Laboratories, Detroit, MI, U.S.A.), and *Sh. flexneri* was grown in nutrient broth (Difco Laboratories, Detroit, MI, U.S.A.) at 37°C.

Table 1. Bacterial strains used for the evaluation of specificity of PCR primers in this study.

Species	Strain	Amplicon size (bp)		
		359	482	600
Staphylococcus aureus	ATCC 6538	-	+	-
Staphylococcus aureus	ATCC 29737	-	+	-
Staphylococcus aureus	ATCC 6538P	-	+	-
Staphylococcus aureus	ATCC 25923	-	+	-
Staphylococcus parahaemolyticus	ATCC 29970	-	-	-
Staphylococcus epidermidis	ATCC 14990	-	-	-
Shigella flexneri	ATCC 12022	-	-	+
Shigella sonnei	ATCC 25931	-	-	+
Shigella boydii	ATCC 8700	-	-	+
Yersinia enterocolitica	ATCC 23715	+	-	-
Salmonella typhimurium	ATCC 14028	-	-	-
Escherichia coli O157 : H7	ATCC 43894	-	-	-
Escherichia coli	ATCC 27325	-	-	-
Escherichia coli	ATCC 23736	-	-	-
Escherichia coli	ATCC 25922	-	-	-
Escherichia coli	ATCC 11775	-	-	-
Vibrio parahaemolyticus	ATCC 17802	-	-	-
Campylobacter jejuni	ATCC 33560	-	-	-
Bacillus cereus	ATCC 14579	-	-	-
Bacillus cereus	ATCC 10876	-	-	-
Listeria monocytogenes	ATCC 19113	-	-	-
Listeria monocytogenes	ATCC 19114	-	-	-

+, Positive; -, Negative.

Isolation of Genomic DNA from Bacterial Culture

Genomic DNA was isolated from *Y. enterocolitica*, *St. aureus*, and *Sh. flexneri* using the DNeasy Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction. The concentration of extracted DNA was measured using a UV-spectrophotometer (Model UV-1700, Shimadzu, Tokyo, Japan). The purity of genomic DNA used in this study was within the 1.8 to 2 ratio (A_{260}/A_{280}).

Oligonucleotide Primers

The primers ail-F and -R produced a 359-bp amplicon from the attachment invasion locus gene of *Y. enterocolitica* [17]. The primers nuc-F and -R yielded a 482-bp amplicon from the nuclease gene of *St. aureus* [20]. The primers ipaH-F and -R yielded a 600-bp amplicon from the invasion plasmid antigen H gene of *Shigella* spp. [10]. The sequences of these primers are shown in Table 2.

Multiplex PCR Condition

Multiplex PCR was performed with a PCR PC 808 (ASTEC, Tokyo, Japan) [11]. A total 25-μl reaction volume of PCR mixture contained 1×PCR buffer, 0.2 mM each of dNTP, 1 mM $MgCl_2$, primers (400 nM ail-F and -R; 2 μM nuc-F and -R; 8 nM ipaH-F and -R), 25 ng of template DNA, and 1 U of TaKaRa Ex *Taq* polymerase (TaKaRa, Tokyo, Japan). Reaction parameters included initial denaturation at 94°C for 5 min, 35 cycles of amplification with denaturation at 94°C for 1 min, annealing at 65°C for 1 min, extension at 72°C for 1 min, and final extension of the incompletely synthesized DNA at 72°C for 5 min.

Agarose Gel Electrophoresis

The PCR amplicons were analyzed by agarose gel electrophoresis. Ten μl of PCR products was loaded onto a 1.5% agarose gel and subjected to electrophoresis for 20 min at 100 V in 0.5×TAE buffer (Bionics Corp, Seoul, Korea). The PCR products were visualized on a transilluminator and photographed by a digital camera (Nikon Coolpix 4300, Japan).

Sample Preparation from Lettuce

Air-packaged lettuce samples were purchased from a local retail market and transferred to a sterile bag for analysis on the same day. Unwashed and cut lettuce (25 g) in 225 ml of buffered peptone water (BPW) was inoculated with *Y. enterocolitica*, *St. aureus*, and *Sh. flexneri*. For enumeration of the above bacteria, 1 ml aliquots of grown bacteria broth were serially diluted in BPW and inoculated, at 2×10^0–10^5 CFU/g for *Y. enterocolitica*, 4×10^0–10^5 CFU/g for *St. aureus*, and 1×10^0–10^5 CFU/g for *Sh. flexneri*, into the sample. A negative control was prepared by seeding 1 ml of buffered peptone water into the corresponding sample. The samples in a sterile filter bag were homogenized in a stomacher (Stomacher Lab Blender 400, Seward

Table 2. Sequences of oligonucleotide primers used in this study.

Species	Primer	Sequence (5'-3')	Target gene	Amplicon size
Yersinia enterocolitica	ail-F	CTA TTG GTT ATG CGC AAA GC	*ail*	359 bp
	ail-R	TGC AAG TGG GTT GAA TTG CA		
Staphylococcus aureus	nuc-F	GAA AGG GCA ATA CGC AAA GA	*nuc*	482 bp
	nuc-R	TAG CCA AGC CTT GAC GAA CT		
Shigella spp.	ipaH-F	GTT CCT TGA CCG CCT TTC CGA TAC CGT C	*ipaH*	600 bp
	ipaH-R	GCC GGT CAG CCA CCC TCT GAG AGT AC		

Laboratories, London, U.K.) for 30 s. The resulting mixture was incubated at 37°C for 24 h until stationary enrichment. One ml aliquots of enriched samples were stored at −20°C until total DNA was extracted.

DNA Extraction from Lettuce Sample

The four kinds of DNA extraction were performed to compare the sensitivity and reproducibility of PCR detection. Aliquots of samples were harvested by centrifugation at 16,000 ×*g* for 3 min.

Boiling Method. The harvested cells were resuspended with 200 µl of TE (Tris-EDTA, pH 8.0) buffer and sustained in a boiling water bath for 20 min. After cooling at room temperature, the samples were centrifuged at 16,500 ×*g* for 10 min and 5 µl of the supernatant was used as template DNA [13].

Guanidine Thiocynate EDTA Sacosyl Method. The harvested cells were resuspended in 100 µl of TE (Tris-EDTA, pH 8.0) buffer and 500 µl of guanidine thiocynate EDTA sacosyl solution, and then incubated for 30 min at 65°C. After cooling on ice for 5 min, 250 µl of 7.5 M ammonium acetate was added and mixed in carefully. After cooling on ice for 10 min, the suspension was vortexed vigorously with 500 µl of phenol:chloroform:isoamyl alcohol (25:24:1) and then centrifuged at 16,000 ×*g* for 10 min. A portion (500 µl) of the supernatant was transferred to a new 1.5 ml tube containing 250 µl of 100% isopropanol. After mixing well, the mixture was centrifuged at 16,000 ×*g* for 2 min, the supernatant was discarded, and then the DNA pellet was rinsed with 70% ethanol. The pellet was eluted in 100 µl of distilled water and 5 µl of the solution was used as template DNA.

PrepMan Ultra Sample Preparation Reagent. The harvested cells were resuspended with 200 µl of PrepMan Ultra Sample Preparation Reagent (Applied Biosystems, U.S.A.) and kept in a boiling water bath for 15 min. After cooling at room temperature for 10 min, the samples were centrifuged at 16,500 ×*g* for 10 min. Fifty µl of supernatant was transferred to a clean 1.5 ml tube containing 400 µl of TE buffer (10 mM Tris, 1 mM EDTA). After mixing, 50 µl of 3 M sodium acetate and 500 µl of isopropanol were added. After vortexing vigorously, the samples were left at room temperature for 30 min and then centrifuged at 16,500 ×*g* for 10 min. The supernatant was discarded, and DNA pellet was dissolved in 100 µl of distilled water by heating for 10 min at 37°C. Five µl of the solution was used as DNA template for PCR.

DNeasy Tissue Kit. Genomic DNA was isolated from samples using the DNeasy Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction.

RESULTS AND DISCUSSION

Primer Selection and Specificity

In the present study, three pairs of primers were selected to simultaneously detect different types of food pathogens using the multiplex PCR method. Each primer was synthesized at a species-specific region of the attachment invasion locus (*ail*) gene in *Y. enterocolitica*, the nuclease (*nuc*) gene in *St. aureus*, and the invasion plasmid antigen H (*ipaH*) gene in *Sh. flexneri*. In order to verify and evaluate the specificity of the primers used in this study, PCR was performed with DNA templates prepared from 22 different bacterial type strains, including positive control strains, as shown in Table 1. The primers ail-F and -R produced a 359-bp amplicon from the attachment invasion locus gene of *Y. enterocolitica* [17], the Primers nuc-F and -R yielded a 482-bp amplicon from the nuclease gene of *St. aureus* [20], and the primers ipaH-F and -R produced a 600-bp amplicon from the invasion plasmid antigen H gene of *Shigella* spp. [10]. The selected primers were tested to examine the possible cross-reactions of primers by homology searches using the basic local alignment searching tool (BLAST) program and reactivity of 22 other pathogenic bacteria. No false positives and negatives were recorded. PCR amplicons of different genes also yielded the products of expected sizes.

Comparison and Sensitivity of DNA Extraction Methods

To simultaneously detect three kinds of bacterial strains using the multiplex PCR method, the DNA extraction procedure is crucial to increase the sensitivity and capability to detect a few numbers of bacteria cells in foods. Establishment of a proper DNA extraction method is important to confirm high sensitivity of the multiplex PCR assay [15]. In this study, the four different DNA extraction methods, boiling method [21], guanidine thiocynate EDTA sacosyl method

[18], PrepMan Ultra Sample Preparation Reagent (Applied Biosystems, U.S.A.), and DNeasy Tissue Kit (Qiagen, Hilden, Germany), were used to compare the efficacy of PCR amplification. The boiling method has been reported to be an effective way to obtain a pathogenic bacterial genomic DNA [18]. The guanidine thiocynate EDTA sacosyl method is applicable to both Gram-positive and Gram-negative bacteria and eliminates endogenous nuclease activity without phenol, RNase, and protease treatments [19]. The PrepMan Ultra Sample Preparation Reagent (Applied Biosystems, U.S.A.) as a commercial product has an advantage of saving time. Only DNA extraction with the DNeasy Tissue Kit showed clear PCR products from food pathogens in lettuce. Furthermore, the DNeasy Tissue Kit contains a filter to eliminate PCR inhibitors, since the constituents of lettuce, such as phenolic and organic compounds, might interfere with DNA amplification [23]. Thus, we decided to use the DNeasy Tissue Kit for the DNA extraction in this multiplex PCR assay.

Optimization of the Multiplex PCR Assay

Following careful comparison of various reactions, the concentrations of the *ail*, *nuc*, and *ipaH* primers were set up as 400 nM, 2 μM, and 8 nM, respectively. Under the optimized multiplex PCR condition, the PCR assay yielded a 359-bp product in *Y. enterocolitica*, a 482-bp product in *St. aureus*, and a 600-bp product in *Shigella* spp. (lanes 2 to 4 in Fig. 1). Two or three corresponding amplicons of 2 or 3 different pathogens were detected (lanes 5 to 8 in Fig. 1). The size of each amplicon corresponded to the expected size and no additional or nonspecific bands were observed. This multiplex PCR method showed high specificity of the assay.

Optimization of the Multiplex PCR Assay on Artificially Contaminated Lettuce

The multiplex PCR assay was evaluated using lettuce artificially contaminated with *Y. enterocolitica*, *St. aureus*, and *Sh. flexneri*. After 24 h of enrichment, all three kinds

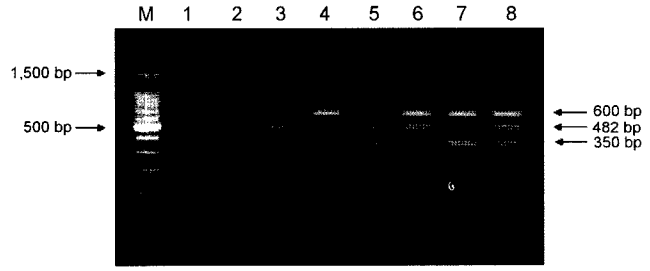

Fig. 1. Multiplex PCR products from pure DNA of *Yersinia enterocolitica*, *Staphylococcus aureus*, and *Shigella flexneri*.
M, molecular weight marker (100-bp ladder, TaKaRa); lane 1, no template; lane 2, *Y. enterocolitica*; lane 3, *St. aureus*; lane 4, *Sh. flexneri*; lane 5, *Y. enterocolitica*, *St. aureus*; lane 6, *St. aureus*, *Sh. flexneri*; lane 7, *Y. enterocolitica*, *Sh. flexneri*; lane 8, *Y. enterocolitica*, *St. aureus*, and *Sh. flexneri*.

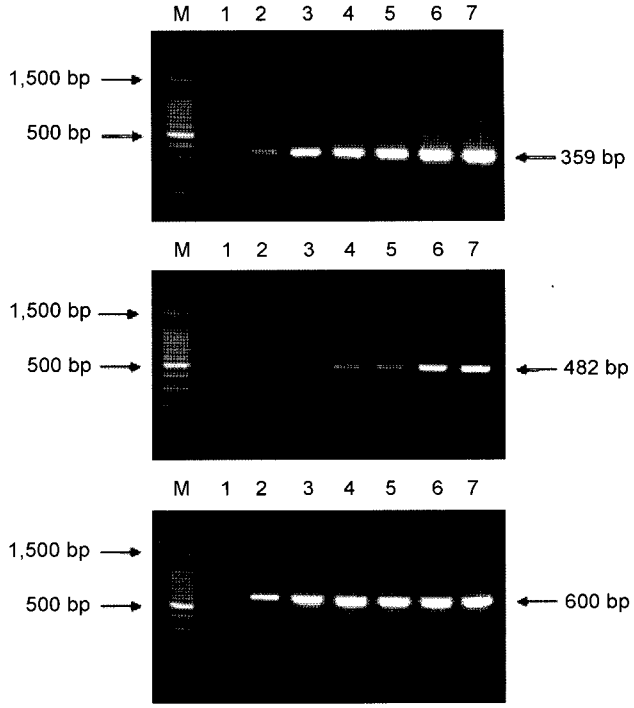

Fig. 2. Sensitivity of the single PCR reaction tested in artificially inoculated lettuce after enrichment.
M, molecular weight marker (100-bp ladder, TaKaRa); lane 1, no template; lanes 2–7, lettuce inoculated with 2×10^{0}–10^{5} CFU/g of *Y. enterocolitica*, 4×10^{0}–10^{5} CFU/g of *St. aureus*, 1×10^{0}–10^{5} CFU/g of *Sh. flexneri*.

of bacteria were detected at initial inoculation level of 2×10^{1}, 4×10^{1}, and 1×10^{1} CFU/g, respectively (lane 3 in Fig. 3). The bands corresponding to the expected amplicons were clearly visible, and intensities were comparable with

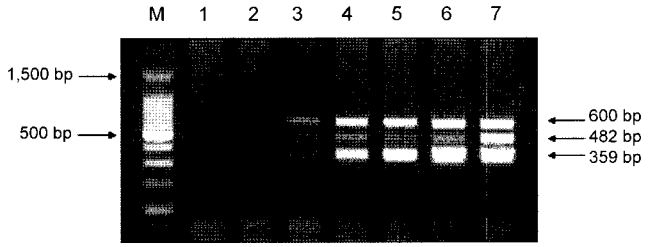

Fig. 3. Multiplex PCR products from enrichment cultures of lettuce artificially inoculated with *Yersinia enterocolitica*, *Staphylococcus aureus*, and *Shigella flexneri*.
M, molecular weight marker (100-bp ladder, TaKaRa); lane 1, no template; lane 2, lettuce inoculated with 2×10^{0} CFU/g of *Y. enterocolitica*, 4×10^{0} CFU/g of *St. aureus*, and 1×10^{0} CFU/g of *Sh. flexneri*; lane 3, lettuce inoculated with 2×10^{1} CFU/g of *Y. enterocolitica*, 4×10^{1} CFU/g of *St. aureus*, and 1×10^{1} CFU/g of *Sh. flexneri*; lane 4, lettuce inoculated with 2×10^{2} CFU/g of *Y. enterocolitica*, 4×10^{2} CFU/g of *St. aureus*, and 1×10^{2} CFU/g of *Sh. flexneri*; lane 5, lettuce inoculated with 2×10^{3} CFU/g of *Y. enterocolitica*, 4×10^{3} CFU/g of *St. aureus*, and 1×10^{3} CFU/g of *Sh. flexneri*; lane 6, lettuce inoculated with 2×10^{4} CFU/g of *Y. enterocolitica*, 4×10^{4} CFU/g of *St. aureus*, and 1×10^{4} CFU/g of *Sh. flexneri*; lane 7, lettuce inoculated with 2×10^{5} CFU/g of *Y. enterocolitica*, 4×10^{5} CFU/g of *St. aureus*, and 1×10^{5} CFU/g of *Sh. flexneri*.

other bands. To evaluate the sensitivity of the multiplex PCR assay, single PCR was also performed with each primer. After 24 h of enrichment, *Y. enterocolitica*, *Sh. flexneri*, and *St. aureus* were detected up to the sample initial inoculation level of 2×10^0, 1×10^0, and 4×10^1 CFU/g, respectively (Fig. 2). This might have been due to the difference of each bacterial growth rate and the presence of competing bacteria that might restrict the growth of the other bacterium.

In conclusion, multiplex PCR was evaluated for rapid and simultaneous detection of *Y. enterocolitica*, *St. aureus*, and *Shigella* spp. in lettuce. This approach was able to simultaneously detect these food pathogens that grow at a low temperature. This simple procedure can be a valuable tool to detect various food pathogens in one tube.

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