JOURNAL OF
Veterinary
Science

A multiplex real-time PCR for differential detection and quantification of *Salmonella* spp., *Salmonella enterica* serovar Typhimurium and Enteritidis in meats

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Salmonella (*S.*) Typhimurium and *S.* Enteritidis are the major causative agents of food-borne illnesses worldwide. Currently, a rapid detection system using multiplex real-time polymerase chain reaction (PCR) has been applied for other food-borne pathogens such as *Escherichia coli, Staphylococcus aureus* and *Streptococcus* spp. A multiplex real-time PCR was developed for the simultaneous detection of *Salmonella* spp., especially *S.* Typhimurium and *S.* Enteritidis, in beef and pork. For the specific and sensitive multiplex real-time PCR, three representative primers and probes were designed based on sequence data from Genbank. Among the three DNA extraction methods (boiling, alkaline lysis, and QIAamp DNA Mini Kit), the QIAamp DNA Mini Kit was the most sensitive in this study. The optimized multiplex real-time PCR was applied to artificially inoculated beef or pork. The detection sensitivity of the multiplex real-time PCR was increased. The specificity of the multiplex real-time PCR assay, using 128 pure-cultured bacteria including 110 Salmonella isolates and 18 non-Salmonella isolates, was 100%, 100% and 99.1% for *Salmonella* spp., *S.* Typhimurium and *S.* Enteritidis, respectively. The sensitivity was 100%, 100% and 91.7% for *Salmonella* spp., *S.* Typhimurium and *S.* Enteritidis, respectively. The multiplex real-time PCR assay developed in this study could detect up to 0.54 ± 0.09 and $0.65 \pm 0.07 \log_{10}$ CFU/ml for *S.* Typhimurium and *S.* Enteritidis for beef, 1.45 ± 0.21 and $1.65 \pm 0.07 \log_{10}$ CFU/ml for *S.* Typhimurium and *S.* Enteritidis for pork, respectively, with all conditions optimized. Our results indicated that the multiplex real-time PCR assay developed in this study could sensitively detect *Salmonella* spp. and specifically differentiate *S.* Typhimurium from *S.* Enteritidis in meats.

Keywords: multiplex real time-PCR, *Salmonella* Enteritidis, *Salmonella* spp., *Salmonella* Typhimurium

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Introduction

Salmonellosis is one of the major problems causing food-borne bacterial enteritis in many countries. At least 1.3 billion cases of human salmonellosis were reported annually worldwide, and approximately three million patients died from the disease [22]. In the United States of America, an estimated 1.34 million cases of food-borne salmonellosis and 553 deaths are reported annually [20]. In Korea, more than 20 cases were estimated to have occurred annually since 2005 [16].

Salmonella enterica is the representative pathogen causing salmonellosis in humans and animals worldwide and is further classified into more than 2,000 *Salmonella* serotypes. Of the *Salmonella* serotypes, *Salmonella* (*S.*) Typhimurium and *S.* Enteritidis are the most important agents of food-borne Salmonellosis in humans [2,30]. It was estimated that approximately 75% of human salmonellosis cases were due to contaminated food products, such as beef, pork, poultry, and eggs [15].

Salmonella spp. in foods can be detected by various methods such as conventional bacteriological culture [14, 23], serological assays [3], polymerase chain reaction (PCR) [4,21], and more recently, real-time PCR methods [11,29]. Detection of food-borne pathogens using conventional culture techniques takes up to 5 days to get a result. This includes primary and secondary enrichment and serological confirmation of colonies grown on agar plates [14].

To shorten the turnaround time of pathogen detection, PCR has been applied in various stages of the diagnostic procedure, for example, on agar plates having bacterial colonies, in enrichment or selective broths, and in raw materials such as suspect food stuffs. Unlike conventional PCR, real-time PCR assay does not require further analysis by gel electrophoresis to confirm the presence of bacterial pathogens in the sample. More importantly, real-time PCR

assay enables experimenters to obtain both qualitative and quantitative measurement of the targeted pathogen in food samples unlike conventional PCR assay.

In more recent times, real-time PCR assays have been successfully applied in the detection of bacterial pathogens in food products [11,12,24,25]. A single real-time PCR assay was applied for specific detection of major *Salmonella* spp. including *S.* Typhimurium and *S.* Enteritidis [11,27,29]. However, the application of a multiplex real-time PCR assay for the detection of these pathogens is not available.

In the present study, a rapid multiplex real-time PCR assay was developed to identify *Salmonella* spp. and to differentiate *S.* Typhimurim from *S.* Enteritidis in meat samples. For this purpose, various conditions for the assay were optimized, bacterial DNA were amplified using three sets of primer pairs, and the different amplified products were visualized using three unique fluorescent probes.

Materials and Methods

Bacterial strains

A total of 128 bacterial strains (i.e., 110 *Salmonella* spp. and 18 non-Salmonella) were used in this study (Table 1). Salmonella isolates consisted of 13 serotypes and isolated from pig feces by the National Veterinary Research and Quarantine Service, Korea, except serotypes Typhimurium

Table 1. Specificity test of the multiplex real-time PCR detecting *Salmonella* spp., *Salmonella enterica* serovar typhimurium and enteritidis

	Organisms	Source	Number of isolates	Number of detected Sal[‡]	Number of detected ST[§]	Number of detected SE[‖]
Target organisms	Typhimurium	ATCC*14028	1	1	1	0
	Enteritidis	ATCC 13076	1	1	0	1
	Typhimurium	Pig isolate[†]	50	50	50	0
	Enteritidis	Pig isolate	11	11	0	10
	Ardwick	Pig isolate	7	7	0	0
	Bredeney	Pig isolate	7	7	0	0
	Derby	Pig isolate	11	11	0	1
	Illinois	Pig isolate	1	1	0	0
	London	Pig isolate	1	1	0	0
	Montevideo	Pig isolate	5	5	0	0
	Panama	Pig isolate	1	1	0	0
	Ruiru	Pig isolate	5	5	0	0
	Sandiego	Pig isolate	1	1	0	0
	Schwarzengrund	Pig isolate	7	7	0	0
	Senftenberg	Pig isolate	1	1	0	0
Non-target organisms	*Escherichia coli* O157:H7	ATCC 43890	1	0	0	0
	Escherichia coli O26	ATCC 12795	1	0	0	0
	Escherichia coli O111	ATCC 33780	1	0	0	0
	Escherichia coli	NCTC[c] 9001	1	0	0	0
	Yersinia enterocolitica	ATCC 9610	1	0	0	0
	Staphylococcus aureus	ATCC 25923	1	0	0	0
	Staphylococcus aureus	ATCC 29213	1	0	0	0
	Listeria monocytogenes	ATCC 19117	1	0	0	0
	Listeria innocua	ATCC 33090	1	0	0	0
	Listeria ivanovii	ATCC 19119	1	0	0	0
	Clostridium perfringens	ATCC 13124	1	0	0	0
	Rhodococcus equi	ATCC 6939	1	0	0	0
	Campylocbater jejuni	ATCC 33560	1	0	0	0
	Campylobacter coli	ATCC 33559	1	0	0	0
	Campylocbater jejuni	Chicken isolate[†]	2	0	0	0
	Campylobacter coli	Chicken isolate[†]	2	0	0	0

*American Type Culture Collection, [†]National Collection of Type Culture, [‡]Sal: *Salmonella* spp., [§]ST: *Salmonella* Typhimurium, [‖] SE: *Salmonella* Enteritidis.

ATCC 14028 and Enteritidis ATCC 13076. Eighteen non-*Salmonella* spp. also consisted of the various species of 7 genera.

DNA extraction

As a pre-preparation step for the multiplex real-time PCR, DNA extraction was performed using three DNA extraction methods: boiling, alkaline lysis and the QIAamp DNA Mini Kit. One ml of bacterial cells (*S.* Typhimurium ATCC 14028 and *S.* Enteritidis ATCC 13076) was harvested from dilutions of bacterial cultures by centrifugation (14,000 × g, 10 min): Then, the pellets were used for DNA extraction by one of following methods with three replications: i) Boiling method. The pellets were suspended in 300 µl of DNase-RNase-free distilled water (Gibco, USA) by vortexing. The tube was centrifuged at 14,000 × g for 5 min, and the supernatant was discarded carefully. The pellets were re-suspended in 200 µl of DNase-RNase-free distilled water (Gibco, USA) by vortexing. The microcentrifuge tube was incubated for 15 min at 100°C and placed immediately on ice. The tube was centrifuged for 5 min at 14,000 × g at 4°C. The supernatant was carefully transferred to a new microcentrifuge tube and incubated again for 10 min at 100°C and placed immediately on ice. An aliquot of 2 µl of the supernatant was used as the template DNA in the multiplex real-time PCR. ii) Alkaline lysis method: The pellets were suspended in 50 µl of 0.05 N NaOH. The microcentrifuge tube was centrifuged for 5 min at 14,000 × g at 4°C. The supernatant was carefully transferred to a new microcentrifuge tube and supplemented with 8 µl of 1 M Tris-HCl buffer. The microcentrifuge tube was centrifuged for 2 min at 14,000 × g at 4°C. DNase- RNase-free distilled water (Gibco, USA) was then added to adjust to a final volume of 200 µl. An aliquot of 2 µl of the supernatant was used as the template DNA in the multiplex real-time PCR. iii) QIAamp DNA Mini Kit: DNA from bacterial cells were extracted by the QIAamp DNA Mini Kit (Qiagen, Germany) according to the manufacturer's instruction. A volume of 2 µl of aliquot was used as the template DNA in the multiplex real-time PCR.

Primers and dual-labeled probes

Nucleotide sequences for the multiplex real-time PCR primers and dual-labeled probes were designed using Primer 3 version 0.3.0 (Whitehead Institute and Howard Hughes Medical Institute, USA) based on the sequence data available from GenBank [5,6,17]. All primers and probes were synthesized by a commercial company (Operon, Germany). The dual-labeled probes were prepared by labeling reporter dyes to the 5′-terminus and quencher dyes to the 3′-terminus of synthesized oligonucleotides. Three kinds of reporter dyes, i.e., FAM, JOE and Cy5, were used for *Salmonella* spp., *S.* Typhimurium and *S.* Enteritidis, respectively. The quencher dye Black Hole Quencher was used for all probes (Table 2).

Multiplex real-time PCR

Each reaction (20 µl) contained a DNA template (2 µl), 2 × QuantiTect Multiplex PCR NoROX Master Mix (Qiagen, Germany), 0.2 µM of each primer, and 50 nM of dual-labeled probe. The multiplex real-time PCR reactions were performed on a Rotor-Gene 3000 (Corbett Research, Australia). The reaction profile included HotStar*Taq DNA* Polymerase activation (95°C, 10 min), 40 cycles of denaturation (95°C, 10 sec), annealing/extension (64°C, 1 min), followed by an indefinite hold (4°C). Fluorescent data were acquired during the annealing phase. Analysis was performed with Rotor-Gene 3000 Software version 6 with slope correction and reaction efficiency threshold enabled. The negative template control threshold was set to a maximum of 10%.

Table 2. Oligonucleotide sequence of primers and fluorogenic probes for the multiplex real-time PCR

Target species	Target gene	Primer or probe		GenBank Accession number (Nucleotide position)
		Name	Sequence (5′ to 3′)	
Salmonella spp.	16s rRNA	S16R-F	aggccttcgggttgtaaagt	X80676 (415−511)
		S16R -R	gttagccggtgcttcttctg	
		Scom-FAM	FAM-aaccgcagcaattgacgttaccc-BHQ1a	
Salmonella Typhimurium	*fliC*	SfC-F	tgcagaaaattgatgctgct	AY649720 (1226−1325)
		SfC -R	ttgcccaggttggtaatagc	
		ST-JOE	JOE-acctgggtgcggtacagaaccgt-BHQ1a	
Salmonella Enteritidis	*sefA*	SsA-F	ggtaaaggggcttcggtatc	L11008 (244−340)
		SsA-R	tattggctccctgaatacgc	
		SE-Cy5	Cy5-tggtggtgtagccactgtcccgt-BHQ1a	

Detection limit and standard curve of the multiplex real-time PCR

The detection limit and standard curve of the multiplex real-time PCR was determined using *S*. Typhimurium ATCC 14028 and *S*. Enteritidis ATCC 13076. Bacterial count was performed using 10-fold serial dilutions of each bacterial culture (10^{-1} to 10^{-8} dilutions) and standard plate count method with EDDY JET agar (Geneq, Canada). Each DNA extracted by the QIAamp DNA Mini Kit from *S*. Typhimurium ATCC 14028 and *S*. Enteritidis ATCC 13076 (8 $\log_{10}$ CFU/ml) was decimally diluted and subjected to the multiplex real-time PCR.

Preparation of artificially inoculated meat samples

i) Pre-enriched samples: Twenty-five g of meat samples (beef and pork) were homogenized with 225 ml of BPW in a BagMixer (Interscience, France) for 2 min to prepare artificially inoculated samples. Nine ml of the homogenized fluid was then transferred to sterile sample tubes and then 1 ml of each of the bacterial dilutions (*S*. Typhimurium ATCC 14028 and *S*. Enteritidis ATCC 13076, 0 to 8 $\log_{10}$ CFU/ml) was added. Meat samples used in this study were confirmed to be in the absence of *Salmonella* spp. by means of the standard cultural method [1]. The DNA of these samples were extracted by three extraction methods and subjected to the multiplex real-time PCR.
ii) Post-enriched samples: The mixed samples of 9 ml homogenized fluid and 1 ml of the different bacterial dilutions (*S*. Typhimurium ATCC 14028, 0.54 ± 0.09 to 2.61 ± 0.05 $\log_{10}$ CFU/ml and *S*. Enteritidis ATCC 13076, 0.65 ± 0.07 to 2.66 ± 0.05 $\log_{10}$ CFU/ml) were incubated at 37°C for 18 h. The DNA were extracted from the inoculated samples by the QIAamp DNA Mini Kit and subjected to the multiplex real-time PCR. C_T values of the pre-enriched samples and post-enriched samples were compared.

Statistical analysis

Statistical analysis was performed using 2×2 box analysis [18] in order to compare the results of the standard culture method of isolated field and reference strains with those of the multiplex real-time PCR.

Results

Specificity of the multiplex real-time PCR

A total of 128 bacterial strains including 110 *Salmonella* strains (51 *S*. Typhimurium strains, 12 *S*. Enteritidis strains and 47 other *Salmonella* serotype strains) and 18 non-*Salmonella* strains were tested by the multiplex real-time PCR, as shown in Table 1.

S16R and Scom-FAM, primer/probe sets designed for the detection of *Salmonella* spp., were amplified and detected amplicons for all 110 *Salmonella* strains but not from the18 non-*Salmonella* strains. This indicated that S16R and Scom-

FAM could detect all *Salmonella* species, as expected (Table 1).

SfC and ST-JOE, primer/probe sets designed for detection of *S*. Typhimurium strains, were amplified and visualized DNA fragments from all 51 *S*. Typhimurium strains but not from the other 59 Salmonella strains and the 18 non-Salmonella strains, indicating specific detection of *S*. Typhimurium (Table 1).

SsA and SE-Cy5, primer/probe sets used for specific detection of *S*. Enteritidis strains, were amplified and visualized DNA fragments from 11 *S*. Enteritidis and 1 *S*. *derby* strains but not from 1 *S*. Enteritidis, the other 97 other Salmonella strains and the 18 non-Salmonella strains (Table 1).

In the multiplex real-time PCR, *Salmonella* spp., *S*. Typhimurium and *S*. Enteritidis produced mean C_T values of 13.00 ± 0.94, 18.29 ± 2.23 and 16.39 ± 3.38, respectively. For the specificity of the multiplex real-time PCR, the cut-off value was determined at a mean C_T value of 30.

Standard curve using pure cultures

The multiplex real-time PCR assay was performed to determine the level of detectable DNA concentration corresponding to the bacterial concentration. The standard curves were generated using mean C_T values for various concentrations of *S*. Typhimurium ATCC 14028 and *S*. Enteritidis ATCC 13076, ranging from 2 to 7 $\log_{10}$ CFU/ml in the multiplex real-time PCR. The slopes of the standard curves for *S*. Typhimurium on FAM and JOE were -3.37 and -3.37, respectively. The values for *S*. Enteritidis were -3.16 on FAM and -3.27 on Cy5. Therefore, the regression curves for *S*. Typhimurium and *S*. Enteritidis were generated based on the various amounts of bacteria cells, as shown in Figs. 1 and 2, respectively. A good linearity of response ($R^2 = 0.99$) on each respective reaction channel (FAM and JOE for *S*. Typhimurium; FAM and Cy5 for *S*. Enteritidis) was shown between the amount of bacterial DNA and the number of cells.

Our results indicated that the multiplex real-time PCR could successfully detect bacterial DNA corresponding to $\leq 10^2$ CFU/ml of bacteria.

Comparison of sensitivity by DNA extraction methods

For improved performance of the multiplex real-time PCR assay, three DNA extraction methods (boiling, alkaline lysis, QIAamp DNA Mini Kit; Qiagen, Germany) were comparatively tested using *S*. Typhimurium and *S*. Enteritidis from bacterial cultures and artificially inoculated meat samples of beef and pork. The three DNA extraction methods were first optimized using bacterial cultures of *S*. Typhimurium ATCC 14028 and *S*. Enteritidis ATCC 13076. The detection limits of the pure cultures put through boiling, alkaline lysis, and the QIAamp DNA Mini Kit all showed the same results: 0.54 ± 0.09 $\log_{10}$ CFU/ml for *S*. Typhimurim and 0.65 ± 0.07 $\log_{10}$ CFU/ml for *S*. Enteritidis.

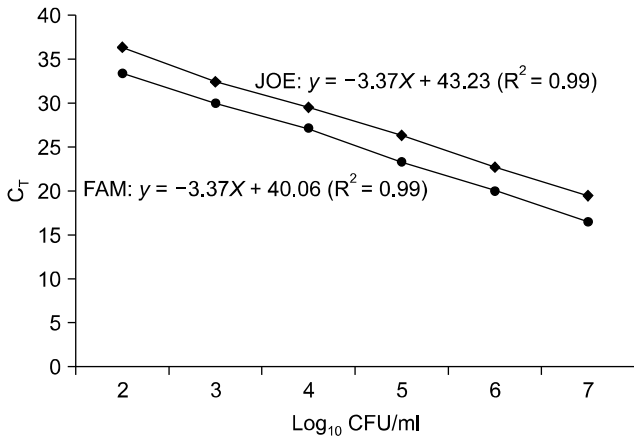When the multiplex real-time PCR, under optimized conditions, was applied to artificially inoculated beef and

Fig. 1. Standard curves for the multiplex real-time PCR for *Salmonella* (S.) Typhimurium. The results of the multiplex real-time PCR were determined using decimal dilution of *S.* Typhimurium ATCC 14028 DNA. The PCR reaction contained primers and probes for all *Salmonella* spp., *S.* Typhimurium and *S.* Enteritidis. Vertical (y) axis, fluorescence intensity; horizontal (χ) axis, PCR cycle numbers. Standard curves for the multiplex real-time PCR of *S.* Typhimurium. The reactions of *S.* Typhimurium were always positive at 555 nm (JOE) and 510 nm (FAM). The threshold values (C_T) were plotted against the corresponding bacterial cell number ($\log_{10}$ CFU/ml).
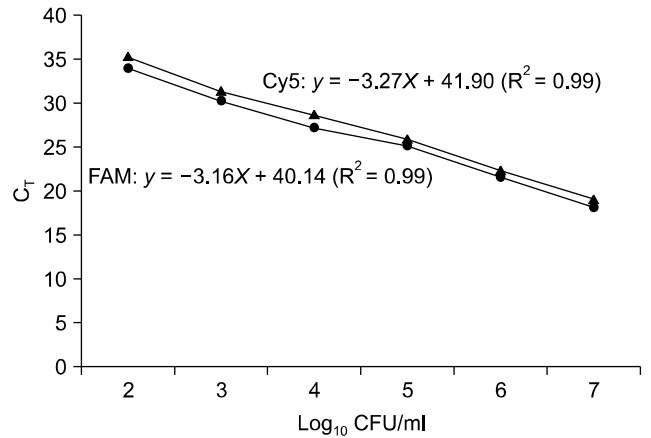
Fig. 2. Standard curves of the multiplex real-time PCR for *Salmonella* (*S.*) Enteritidis. The results of the multiplex real-time PCR were determined using decimal dilution of *S.* Enteritidis ATCC 13076 DNA. The PCR reaction contained primers and probes for all *Salmonella* spp., *S.* Typhimurium and *S.* Enteritidis. Vertical (y) axis, fluorescence intensity; horizontal (χ) axis, PCR cycle numbers. Standard curves for the multiplex real-time PCR of *S.* Enteritidis. The reactions of *S.* Enteritidis were always positive at 665 nm (Cy5) and 510 nm (FAM). The threshold values (C_T) were plotted against the corresponding bacterial cell number ($\log_{10}$ CFU/ml).
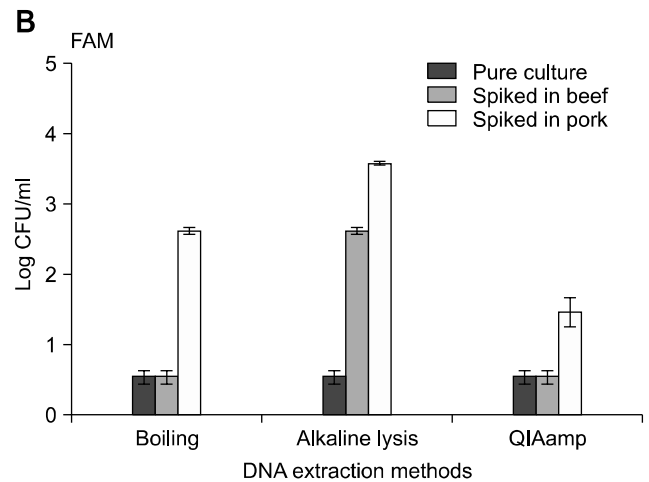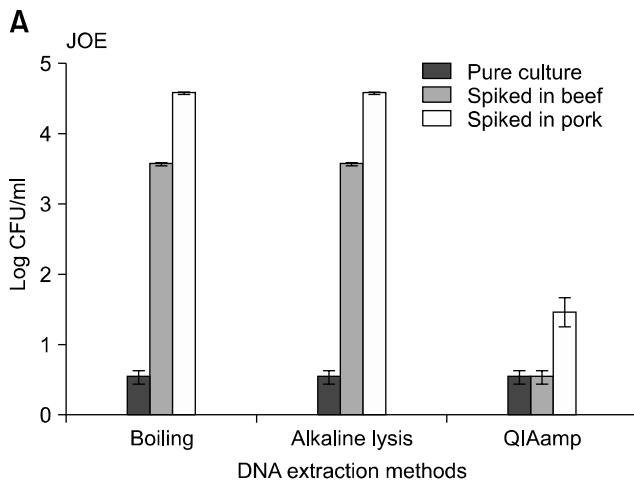

Fig. 3. Comparison of sensitivity of the multiplex real-time PCR on *Salmonella* Typhimurium ATCC 14028 using the three DNA extraction methods. (A) The results at 555 nm (JOE). (B) The results at 510 nm (FAM).

pork, the DNA extraction method using the QIAamp DNA Mini Kit was the most effective (0.54 ± 0.09 and 0.65 ± 0.07 $\log_{10}$ CFU/ml for *S.* Typhimurium and *S.* Enteritidis in beef, 1.45 ± 0.21 and 1.65 ± 0.07 $\log_{10}$ CFU/ml for *S.* Typhimurium and *S.* Enteritidis in pork, respectively).

The detection limits for the alkaline lysis method were 3.57 ± 0.03 and 4.56 ± 0.03 $\log_{10}$ CFU/ml for *S.* Typhimurium and *S.* Enteritidis in beef, and 4.57 ± 0.02 and 2.26 ± 0.05 $\log_{10}$ CFU/ml for *S.* Typhimurium and *S.* Enteritidis in pork.

The detection limits for boiling method were 3.57 ± 0.02

and 3.57 ± 0.03 $\log_{10}$ CFU/ml for *S.* Typhimurium and *S.* Enteritidis in beef, and 4.57 ± 0.02 and 2.26 ± 0.05 $\log_{10}$ CFU/ml for *S.* Typhimurium and *S.* Enteritidis in pork (Figs. 3 and 4).

Our results indicated that the QIAamp DNA Mini Kit was the most effective in extraction and amplification of bacterial DNA from artificially inoculated meats for the multiplex real-time PCR.
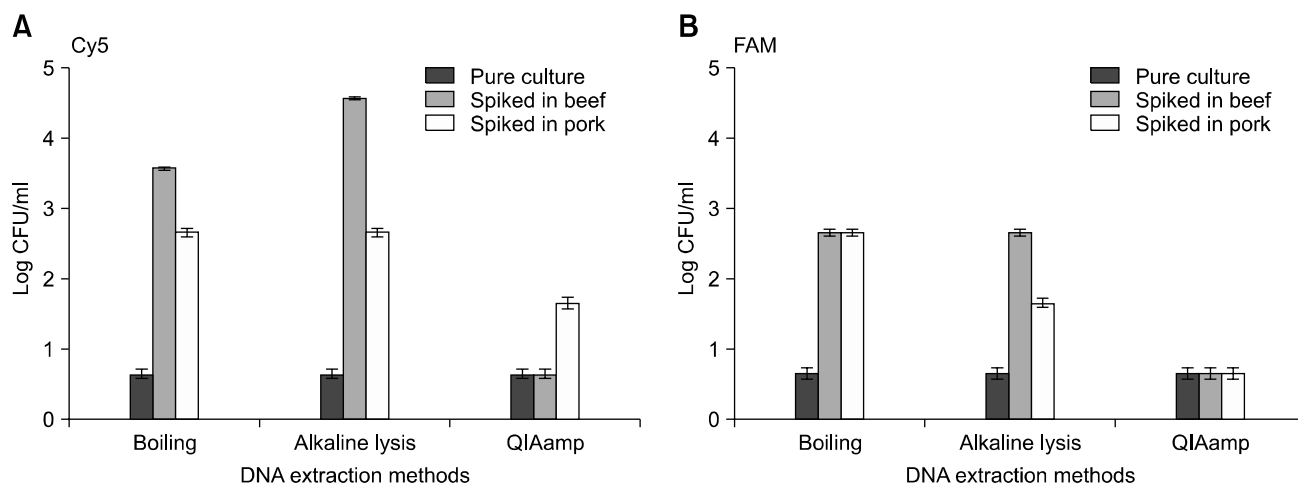
Fig. 4. Comparison of sensitivity of the multiplex real-time PCR on *Salmonella* Enteritidis ATCC 13076 using the three DNA extraction methods. (A) The results at 555 nm (JOE). (B) The results at 510 nm (FAM).

Comparison of C_T value between pre-enriched and post-enriched meat samples

The multiplex real-time PCR assay was applied to determine whether bacterial enrichment conditions affect sensitivity of the assay. For this purpose, *S.* Typhimurium ATCC 14028 or *S.* Enteritidis ATCC 13076 at low initial cell concentrations (0.54 ± 0.09 to 2.61 ± 0.05 log$_{10}$ CFU/ml for *S.* Typhimurium and 0.65 ± 0.07 to 2.66 ± 0.05 log$_{10}$ CFU/ml for *S.* Enteritidis) were spiked into beef and pork. The meat samples were taken for the multiplex real-time PCR either immediately after spiking (pre-enrichment condition) or put in incubation at 37°C for 18 h after spiking (post-enrichment condition).

When 0.54 ± 0.09 log$_{10}$ CFU/ml of *S.* Typhimurium and 0.65 ± 0.07 log$_{10}$ CFU/ml of *S.* Enteritidis were spiked into meats, the multiplex real-time PCR assay could detect bacteria in the spiked beef but not in the spiked pork, both from a pre-enrichment condition. The multiplex real-time PCR assay detected bacteria that underwent a post-enrichment condition. Pre-enriched meats had C_T values of 35.32 or more, while post-enriched meats had C_T values of 14.41 to 22.23.

When 1.45 ± 0.21 log$_{10}$ CFU/ml of *S.* Typhimurium and 1.65 ± 0.07 log$_{10}$ CFU/ml of *S.* Enteritidis were spiked into meats, the multiplex real-time PCR assay detected bacteria in all conditions (beef, pork, pre-enriched, and post-enriched). However, pre-enriched meats had C_T values from 30.94 to 36.94, while post-enriched meats had C_T values ranging from 14.52 to 22.06.

When 2.61 ± 0.05 log$_{10}$ CFU/ml of *S.* Typhimurium and 2.66 ± 0.05 log$_{10}$ CFU/ml of *S.* Enteritidis were spiked into meats, the multiplex real-time PCR assay detected bacteria in all conditions (beef, pork, pre-enriched, and post-enriched). However, pre-enriched meats had C_T values from 26.65 to 32.06, while post-enriched meats had C_T values from 14.47 to 20.75 (Table 3).

Table 3. Comparison of mean C_T values between pre-enrichment and post-enrichment

(A) Beef

| log$_{10}$ CFU/ml | Beef | | | | | |
| | Pre-enrichment | | | Post-enrichment | | |
	FAM	JOE	Cy5	FAM	JOE	Cy5
ST* 2.61 ± 0.05	26.99	31.91	−‡	14.47	20.23	−
1.45 ± 0.21	32.23	36.94	−	14.52	20.31	−
0.54 ± 0.09	35.32	37.83	−	16.00	21.40	−
SE† 2.66 ± 0.05	32.06	−	29.95	17.01	−	19.83
1.65 ± 0.07	34.70	−	32.35	17.85	−	21.27
0.65 ± 0.07	38.80	−	38.97	18.20	−	22.23

(B) Pork

| log$_{10}$ CFU/ml | Pork | | | | | |
| | Pre-enrichment | | | Post-enrichment | | |
	FAM	JOE	Cy5	FAM	JOE	Cy5
ST* 2.61 ± 0.05	26.65	31.67	−	15.54	20.75	−
1.45 ± 0.21	30.94	35.25	−	16.40	22.06	−
0.54 ± 0.09	−	−	−	14.41	20.42	−
SE† 2.66 ± 0.05	32.03	−	28.65	18.76	−	20.50
1.65 ± 0.07	34.41	−	32.62	18.63	−	20.92
0.65 ± 0.07	−	−	37.60	21.06	−	21.55

Salmonella Typhimurium, †*Salmonella* Enteritidis, ‡not tested.

Table 4. The evaluation of the multiplex real time PCR developed in this study

Analysis item	Values for each serotype		
	Salmonella spp.	*Salmonella* Typhimurium	*Salmonella* Enteritidis
Specificity (%)	100	100	99.1
Sensitivity (%)	100	100	91.7
Efficiency (%)	100	100	98.4
Positive predictive value (%)	100	100	91.7
Negative predictive value (%)	100	100	99.1
Probability of false positive result (%)	0	0	0.9
Probability of false negative result (%)	0	0	8.3

Our results indicated that the multiplex real-time PCR under a post-enriched condition is more available and more sensitive than under a pre-enriched condition to detect small amounts of bacteria in meat.

The evaluation of the multiplex real-time PCR

For evaluation, the multiplex real-time PCR assay developed herein was tested using 128 field and reference bacterial isolates prepared by conventional bacteriological tests. Results obtained from both tests were analyzed by statistical analysis using 2 × 2 box analysis [18], as shown in Table 4.

The multiplex real-time PCR assay showed 100% specificity for *Salmonella* spp. and *S.* Typhimurium and 99.1% for *S.* Enteritidis. The multiplex real-time PCR assay showed 100% sensitivity for *Salmonella* spp. and *S.* Typhimurium and 91.7% for *S.* Enteritidis. The probability of false positive results was 0, 0, and 0.9% for *Salmonella* spp., *S.* Typhimurium and *S.* Eneritidis, respectively. The probability of false negative results was 0, 0, and 8.3% for *Salmonella* spp., *S.* Typhimurium and *S.* Eneritidis, respectively.

Discussion

The multiplex real-time PCR developed in this study was the first to detect all *Salmonella* spp. possibly related with meats and to differentiate simultaneously *S.* Typhimurium from *S.* Enteritidis in meats. Previously, real-time PCR assays had been applied for *Salmonella* spp. and other food-borne pathogens [9,10,19,24,25]. Furthermore, real-time PCR assays for *Salmonella* spp. were limited to detect a specific single strain of *Salmonella* spp., for example, *S.* Typhimuirum [8] or *S.* Enteritidis [26]. The detection limits of the multiplex real-time PCR assay were $0.54 \pm 0.09 \log_{10}$ CFU/ml for *S.* Typhimurim and $0.65 \pm 0.07 \log_{10}$ CFU/ml for *S.* Enteritidis in bacterial cultures, indicating that the multiplex real-time PCR assay developed in this study had enough sensitivity to apply the assay to a diagnostic purpose compared to previous real-time PCR assays [8,27]. These advantages improved the multiplex real-time PCR assay in terms of shortening turnaround time for bacterial detection and reducing the risk of cross-contamination during the experiment. If so, the multiplex real-time PCR assay can rapidly detect and identify *Salmonella* spp., *S.* Typhimurium and *S.* Enteritidis, within a turnaround time of $\leq$ 5 h; the conventional culture method requires 4 to 5 days for identification of bacteria present in meat [14].

A number of methods for extracting bacterial DNA directly from meat have been reported and utilized substances such as Chelex-100 [19], phenol-chloroform [32], boiling, and alkaline lysis [7]. In this study, three methods were compared for the multiplex real-time PCR. Although boiling and alkaline methods were faster and more convenient than the QIAamp DNA Mini Kit, the results of the QIAamp DNA Mini Kit showed the best efficacy for bacterial DNA extraction from spiked meats compared to the other two DNA extraction methods. It was indicated that the QIAamp DNA Mini Kit may be the most efficient in harvesting bacterial DNA and reducing the remaining PCR inhibitors. Therefore, the QIAamp DNA Mini Kit was utilized for improved efficacy of the multiplex real-time PCR assay in this study.

Next, an additional enrichment step was applied to increase the sensitivity of the multiplex real-time PCR on artificially inoculated meat samples. With the enrichment step, the multiplex real-time PCR was able to detect up to $0.54 \pm 0.09 \log_{10}$ CFU/ml of *S.* Typhimurium and $0.65 \pm 0.09 \log_{10}$ CFU/ml of *S.* Enteritidis. The detection limits of the multiplex real-time PCR reported herein were more sensitive than previous real-time PCR assays, which reported detection limits of 10^3 CFU/ml after a 10 h enrichment step [26].

Three genomic sites, 16s rRNA, fliC gene and *sefA* gene, used in this study have reported as candidates suitable for common or specific detection of *Salmonella* spp. in real-time PCR [13,17,28,31]. As expected, the multiplex real-time PCR showed high sensitivity (91.7% to 100%) and specificity (99.1% to 100%).

In conclusion, the multiplex real-time PCR assay would be useful for the simultaneous detection of *Salmonella* spp., *S.* Typhimurium and *S.* Enteritidis in meats, also taking into consideration its high sensitivity and specificity. If utilizing the additional enrichment step, the multiplex real-time PCR would have more improved detection limits ($0.54 \pm 0.09 \log_{10}$ CFU/ml for *S.* Typhimurium and $0.65 \pm$

0.09 $\log_{10}$ CFU/ml for *S.* Enteritidis).

Although the multiplex real-time PCR assay was demonstrated as an applicable assay in artificially inoculated meats, it needs further research for natural meat cases and other types of food and environmental samples such as litter, feces or feed.

Acknowledgments

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