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Direct and Quantitative Analysis of *Salmonella enterica* Serovar Typhimurium Using Real-Time PCR from Artificially Contaminated Chicken Meat

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For quantitative PCR assay of *Salmonella enterica* serovar Typhimurium in food samples, a real-time PCR method was developed, based on DNA genome equivalent. Specific primers and probe designed based on the STM4497 gene of *S.* Typhimurium LT2 showed the specificity to *S.* Typhimurium. Threshold cycle (Ct) values of real-time PCR were obtained from a quantitative standard curve with genomic DNA of *Salmonella* Typhimurium. In addition, the recovery of *S.* Typhimurium inoculated artificially to chicken samples with 4.5×10^5 to 4.5 CFU/ml was evaluated by using real-time PCR and plate-count methods. Result showed that the number of cells calculated from the real-time PCR method had good correlation with that of the plate-count method. This real-time PCR method could be applicable to the detection and quantification of *S.* Typhimurium in food samples.

Keywords: Real-time PCR, *Salmonella* Typhimurium, quantitative analysis

The *Salmonella* genus is a Gram-negative bacterium and consists of 2 species, *Salmonella enterica* and *Salmonella bongori* (subspecies V). *Salmonella enterica* is divided into 6 subspecies including I, II, IIIa, IIIb, IV, and VI. *Salmonella* is classified into over 2,500 serovars based on the Kauffmann-White scheme [22, 23]. Among *Salmonella* subspecies I, *Salmonella enterica* serovar Typhimurium is the most frequently isolated serovar from global foodborne outbreaks of salmonellosis [14, 17], and therefore, rapid detection and identification for this serovar are necessary in the food industry.

Detection of *Salmonella* by bacteriological methods generally takes 3 to 4 days, and these methods are labor-intensive, expensive, complicated, and time-consuming. The rapid and accurate identification of bacterial pathogens from food is important in the food industry and for public health. PCR has the potential of being a powerful method in microbiological diagnostics because of its simplicity, rapidity, and accuracy [10, 20, 21, 24, 25, 27, 29]. Recently, a real-time PCR method has been reported to detect foodborne pathogens, including *Salmonella*, with advantages of quantification, sensitivity, and rapidity. Most primer sets and probes for real-time PCR were based on various target genes, and reacted for *Salmonella* spp. and *Salmonella enterica* serovars [3, 5, 9, 18]. Furthermore, the real-time PCR method was used along with an enrichment step before bacterial genomic DNA extraction for detection of *Salmonella* in food [2, 5, 9, 15].

We recently designed a specific primer based on the STM4497 gene of *S.* Typhimurium LT2 using comparative genomics, and suggested for specific detection of *S.* Typhimurium among various *Salmonella* serovars [12]. In addition, *Salmonella* serovars were genotyped using comparative genomics of various genomic sequences of *Salmonella* serovars, and a new identification scheme of major pathogenic *Salmonella* serovars has been suggested, demonstrating the potential application of genomics and bioinformatics technologies to the detection and identification of foodborne pathogen [11].

In this study, a quantitative detection method of *S.* Typhimurium was developed using a real-time PCR method. In addition, the recovery efficiency of *S.* Typhimurium, which was artificially inoculated to chicken samples, was evaluated by using the real-time PCR method and plate-count method.

Materials and Methods

Bacterial Strains and Growth Condition

Forty-eight bacterial strains, from the American Type Culture Collection (ATCC) including major foodborne pathogens and other related bacteria, were used in this study, and the strains are listed in Table 1. Standard culture conditions were used. *Salmonella* serovar Typhimurium ATCC 19585 was grown as a reference strain in

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Table 1. Strains used for selectivity real-time PCR tests and results.

Species	Strain	Result
Salmonella serovar Typhimurium	ATCC 19585	+
Salmonella serovar Typhimurium	ATCC 14028	+
Salmonella serovar Typhimurium	ATCC 13311	+
Salmonella serovar Typhi	ATCC 6539	-
Salmonella serovar Typhi	ATCC 33459	-
Salmonella serovar Paratyphi C	ATCC 13428	-
Salmonella serovar Paratyphi B	ATCC 10719	-
Salmonella serovar Enteritidis	ATCC 4931	-
Salmonella bongori	ATCC 43975	-
Salmonella enterica salamae	ATCC 15793	-
Salmonella enterica arizonae	ATCC 13314	-
Salmonella enterica diarizonae	ATCC 43973	-
Salmonella enterica houtenae	ATCC 43974	-
Salmonella enterica indica	ATCC 43976	-
Salmonella serovar Choleraesuis	ATCC 13312	-
Salmonella serovar Gallinarum	ATCC 9184	-
Salmonella serovar Pullorum	ATCC 9120	-
Escherichia coli O157:H7	ATCC 43894	-
Escherichia coli	ATCC 27325	-
Yersinia enterocolitica	ATCC 23715	-
Bacillus cereus	ATCC11778	-
Bacillus subtilis	ATCC 6633	-
Enterococcus faecalis	ATCC 19433	-
Klebsiella pneumoniae subsp. *Pneumoniae*	ATCC 8724	-
Bacillus thuringiensis	ATCC 10792	-
Bacillus anthracis	ATCC 14578	-
Listeria monocytogenes	ATCC 19118	-
Listeria ivanovii	ATCC 19119	-
Listeria welshimeri	ATCC 35897	-
Listeria inocua	ATCC 33090	-
Listeria grayi	ATCC 25401	-
Listeria seeligeri	ATCC 35967	-
Staphylococcus aureus	ATCC 6538	-
Staphylococcus haemolyticus	ATCC 29970	-
Staphylococcus epidermidis	ATCC 14990	-
Vibrio parahaemolyticus	ATCC 17802	-
Vibrio cholerae	ATCC 14547	-
Vibrio vulnificus	ATCC 33815	-
Campylobacter jejuni	ATCC 43429	-
Clostridium botulinum	ATCC 3502	-
Clostridium perfringens	ATCC 3624	-
Citrobacter freundii	ATCC 8090	-
Enterobacter aerogenes	ATCC 13048	-
Enterobacter cloacae	ATCC 13047	-
Enterobacter sakazakii	ATCC 29544	-
Proteus vulgaris	ATCC 29905	-
Shigella flexneri	ATCC 12022	-
Shigella sonnei	ATCC 25931	-

Luria-Bertani broth medium (Difco Laboratories, Detroit, MI, U.S.A.) overnight at 37°C.

Sample Preparation from Chicken and Genomic DNA Extraction

Air-packaged breast meat of chicken was purchased from a local retail market and transferred in a sterile bag for analysis on the same day. The chicken meat was washed under running water and chopped into pieces. Then, 25 g of meat was mixed with 225 ml of phosphate buffered saline (PBS) in a sterile filter bag (BA6141/STR filter bag; Seward Laboratories, West Sussex, U.K.). One ml of bacterial culture, having an optical density of 0.66 (at 600 nm) and corresponding to 10^8 CFU/ml, was serially 10-fold diluted into 9 ml of PBS solution. The chicken sample and PBS solution were artificially inoculated with 1 ml of the 10-fold diluted bacteria-PBS solution. Six samples ranging from 4.5×10^5 CFU/ml to 4.5 CFU/ml of chicken-PBS solution were prepared. A negative control (not inoculated) sample was prepared by seeding 1 ml of PBS buffer into corresponding chicken sample and PBS solution. After inoculation, the sample was immediately stomached with a stomacher (Stomacher 400 circulator; Seward Laboratories, West Sussex, U.K.) for 30 sec at 230 rpm. One ml of sample was harvested at 10,000 ×*g* for 10 min for plate count and real-time PCR methods. For the real-time PCR method, genomic DNA of the harvested sample was extracted using the DNeasy Tissue Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instruction. Sixty μl of triplicate distilled water (GIBCO, Grand Island, NY, U.S.A.) was loaded in a spin column for elution of extracted genomic DNA and centrifuged after 10 min. For the plate count method, CFUs were determined by decimally diluting the cell suspension with triplicate plates.

Standard Curve of Quantified Genomic DNA of *Salmonella* Typhimurium Using Real-Time PCR

Purified genomic DNA of *S.* Typhimurium ATCC 19585 was used for the standard curve of real-time PCR. Thus, genomic DNA was isolated from an overnight culture of *S.* Typhimurium ATCC 19585 and quantified using a Qubit Fluorometer and Quant-iT dsDNA HS assay kit (Invitrogen, Carlsbad, CA, U.S.A.). Purified genomic DNA was diluted to final concentrations of 4×10^5, 4×10^4, 4×10^3, 4×10^2, 4×10^1, and 0 genome equivalents per 5 μl. DNA genome equivalent was calculated by the following equation: DNA genome equivalent=$(A\times6.022\times10^{23})\ (660\times B)^{-1}$, where A is the DNA concentration and B is the length of genomic DNA.

Primers and Probe for Quantification of *Salmonella* Typhimurium

Primers and probe used for the detection and quantification of *S.* Typhimurium are shown in Table 2. The PCR primers and fluorogenic probe for the TaqMan assay were designed using Primer Express 2.0 software (Applied Biosystems, Forest City, CA, U.S.A.), in order to amplify the specific DNA fragment of *S.* Typhimurium LT2 [12, 17]. The probe was purchased from Applied Biosystems (Forest City, CA, U.S.A.) and primers were purchased from Bionics Corp. (Seoul, Korea). The probe was labeled with a fluorescent reporter dye, 6-carboxyfluorescein (FAM), on the 5' end and minor groove binder (MGB) probe with non-fluorescent quencher (NFQ) at the 3' end of the probe.

Real-Time PCR Condition

The amplification reactions were performed in a total volume of 25 μl and every sample was reacted three times. Each real-time PCR reaction tube contained 12.5 μl of Mastermix (TaqMan Universal PCR Master Mix; Applied Biosystems), 0.4 μM each of primers, 0.2 μM probe, 5 μl of extracted sample DNA, and 5 μl of

Table 2. Oligonucleotide primers and probe used in PCR.

Oligonucleotide	Sequence (5'-3')	Reference
Sal-F	GCG CAC CTC AAC ATC TTT C	This study
Sal-R	CGG TCA AAT AAC CCA CGT TCA	This study
Sal-Probe	FAM[a] ATC ATC GTC GAC ATG C MGB/NFQ	This study

[a]FAM, 6-carboxyfluorescein (the reporter dye).

three times distilled water (GIBCO, Grand Island, NY, U.S.A.). Thermal cycling was as follows using a two-step PCR protocol: 50°C for 2 min, 95°C for 10 min, and 50 cycles of 95°C for 15 sec and 60°C for 1 min, and fluorescence signals were measured once in each cycle at the end of the extension step.

RESULTS

Inclusivity and Exclusivity of Primer Set for *Salmonella* Typhimurium

To verify and evaluate the specificity of the primers and probe used, the primer set and probe in Table 2 were evaluated with DNAs of *S.* Typhimurium and other various bacterial type-strains using real-time PCR. The constructed primers and probe used in this study (Table 1) were amplified to specific PCR products with *S.* Typhimurium.

Real-Time PCR Results of Quantified Genomic DNA of *Salmonella* Typhimurium

Amplification plot and standard curves of real-time PCR were obtained from serially 10-fold diluted genomic DNA of *S.* Typhimurium ATCC 19585, as shown in Figs. 1 and 2, and Table 3. Total 5 independent experiments with three repeats were analyzed (Table 3), and the amplification plot of one experiment is shown in Fig. 1. According to the known size (4.99 Mb) of whole *S.* Typhimurium ATCC 19585, one DNA genome equivalent corresponds to 5.46904 fg. Quantified genomic DNAs of *S.* Typhimurium ATCC 19585 from 4.5×10^5 to 0 genome equivalents were reacted using real-time PCR in each single experiment with 3 repeats. As shown in Fig. 1 and Table 3, no DNA amplification was observed with 0 genome equivalent, and other quantified genomic DNAs showed amplification plots for 4.5×10^5 to 4.5 genome equivalents of *S.* Typhimurium ATCC 19585.

Furthermore, 10-fold diluted genomic DNA samples showed almost identical threshold cycle (Ct) values in 3 repeated experiments, and the mean value of the threshold cycle and standard deviation based on the DNA genome equivalent of *S.* Typhimurium ATCC 19585 are shown in Table 3. The standard curve of real-time PCR with quantified *Salmonella* genomic DNA was plotted with averaged results of 3 repeated experiments and is shown in Fig. 2, demonstrating a linear relationship between the log cell numbers. The Ct values showed a standard curve with a high correlation coefficient (R^2) of 0.9951 and generated a slope of −3.4037.

Quantification of *Salmonella* Typhimurium ATCC 19585 Artificially Inoculated in Chicken Samples by Real-Time PCR and Plate-Count Methods

Quantitative amplification plots of genomic DNA extracted from artificially inoculated chicken samples are shown in Fig. 3. Results showed quantitative amplification from 4.5×10^5 to 4.5 CFU/ml of chicken-PBS solutions. The Ct values of 6 experiments ranged from 20.72±0.23 to 36.25±0.63: Each Ct value was averaged from 3 repeated reactions. From the standard amplification plot in Fig. 2, these Ct values were converted to genome equivalents, as shown in Table 4. Results showed that the number of *S.* Typhimurium quantified with real-time PCR was higher than that of inoculated *S.* Typhimurium in the sample. *S.* Typhimurium from the artificially inoculated

Table 3. Threshold cycle (Ct) values of real-time PCR, quantitatively reacted with genomic DNA of *Salmonella* Typhimurium ATCC 19585.

Genome equivalents[a]	Ct value					Mean	Standard deviation
	1	2	3	4	5		
400,000	17.99	17.65	17.41	17.51	17.31	17.57	0.26
40,000	21.55	21.12	20.99	21.08	20.79	21.11	0.28
4,000	25.05	24.94	24.53	24.61	24.42	24.71	0.27
400	28.64	28.49	28.74	28.62	28.44	28.59	0.12
40	31.81	31.18	32.53	32.11	32.09	31.94	0.50
0	ND	ND	ND	ND	ND		

[a]Genome equivalents of *Salmonella* Typhimurium ATCC 19585.
[b]ND: Not detected.

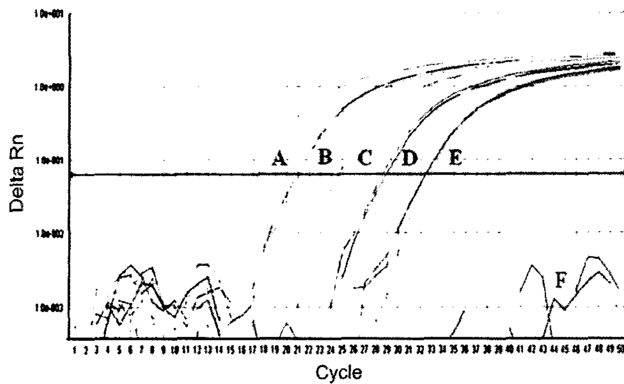

Fig. 1. Amplification plot of a 10-fold serially diluted genomic DNA of *S.* Typhimurium ATCC 19585 ranging from 400,000 to 0 genome equivalents, using real-time PCR. Delta Rn is plotted on the Y-axis, and amplification cycle number is plotted on the X-axis.

A, 400,000; B, 40,000; C, 4,000; D, 400; E, 40; F, 0 DNA genome equivalents of *S.* Typhimurium.

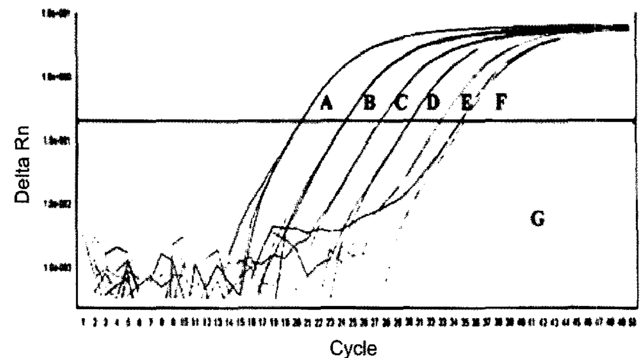

Fig. 3. Amplification plot of genomic DNA extracted from artificially 10 fold serial inoculated chicken samples, ranging from 450,000 to 0 CFU/ml of chicken-PBS solution using real-time PCR.

A, 4.5×10^5; B, 4.5×10^4; C, 4.5×10^3; D, 4.5×10^2; E, 4.5×10^1; F, 4.5×10^0; G, 0; number of inoculated *S.* Typhimurium (CFU/ml of chicken-PBS solution).

chicken samples was also quantified by the traditional plate-count method (Table 4). The number of cells calculated from the plate-count method was similar to that of inoculated *S.* Typhimurium measured by real-time PCR in the sample.

Discussion

Salmonella enterica serovar Typhimurium is a major serovar of foodborne outbreak of *Salmonella*, and rapid detection of *Salmonella* is important for the food industry and public health. Many studies have been carried out for the qualitative and quantitative detection of *Salmonella* in food, using the PCR method. Several target genes of *Salmonella*, including *invA* [6, 8, 16, 19], *ompC* [13, 16], *oriC* [16], *fimA* [4], *iroB* [1], *rfb* O antigen gene cluster [7], and *spaQ* [28], have been reported. However, primer sets used in these studies were specific only to the *Salmonella* genus but not specific to the serovar Typhimurium. In our recent study, primers and probe were designed from the STM4497 gene of *S.* Typhimurium LT2 [17] for the specific detection of *S.* Typhimurium using real-time PCR. The STM4497 gene had been suggested to be specific to *S.* Typhimurium and proved to be highly specific to *S.* Typhimurium in our previous study [12]. *S.* Typhimurium is the major pathogenic serovar isolated mostly from recent food outbreaks with *S.* Enteritidis. The specific detection of *S.* Typhimurium in food is important. In our present study, a novel primer set and probe, based on the STM4497 gene, were applied to real-time PCR for the possibility of quantitative detection of *Salmonella* genomic DNAs and artificially inoculated chicken samples.

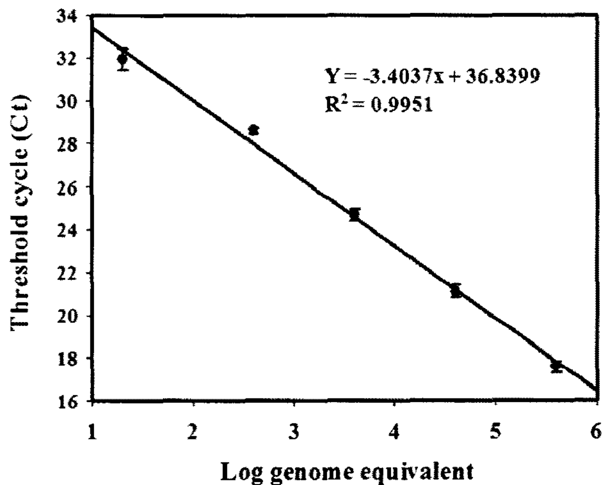

Fig. 2. Standard curve of a 10-fold serially diluted genomic DNA of *S.* Typhimurium ATCC 19585 with averaged results from 3 repeated real-time PCR experiments.

Threshold cycle (Ct) is plotted on the Y-axis and Log genome equivalent is plotted on the X-axis.

Table 4. Quantified results of real-time PCR artificially inoculated in chicken samples by *S.* Typhimurium ATCC 19585.

Number of inoculated *S.* Typhimurium (CFU/ml of chicken-PBS solution)	TaqMan real-time PCR	Plate count
4.5×10^5	$(6.3\pm0.9)\times10^5$	$(4.3\pm0.3)\times10^5$
4.5×10^4	$(6.8\pm0.6)\times10^4$	$(3.3\pm1.6)\times10^4$
4.5×10^3	$(7.2\pm0.9)\times10^3$	$(4.0\pm1.2)\times10^3$
4.5×10^2	$(8.1\pm0.1)\times10^2$	$(5.5\pm1.4)\times10^2$
4.5×10^1	$(1.2\pm0.6)\times10^2$	$(1.2\pm1.0)\times10^2$
4.5×10^0	35 ± 15	15 ± 8
NI[b]	ND[c]	ND

[a]NI: Not inoculated.

[b]ND: Not detected.

Most previous studies with the real-time PCR method contained an enrichment step to increase the detection limit of foodborne pathogens in food [2, 5, 9, 15]. However, this enrichment step could possibly make false quantitative results owing to unexpected change of the initial amount of target pathogens during enrichment time. Recently, the direct detection of pathogens from samples has been shown to reduce detection time [26, 30–32]. This rapid quantification method of foodborne pathogens without an enrichment step is important for food safety. In the present study, genomic DNA was extracted without an enrichment step after a stomach mix step, and the genomic DNA extracted was reacted for rapid quantification of *S.* Typhimurium. The recovery of *S.* Typhimurium inoculated artificially to chicken samples with various cell concentrations was compared by using this real-time PCR and plate-count methods (Table 4). The number of cells quantified by the real-time PCR method was of the same order of magnitude as the numbers inoculated to samples; however, the results quantified were different from the numbers calculated by the plate-count method. Nevertheless, this difference might be explained by a variation of DNA recovery and detection of DNA from injured or dead cells [31].

In our previous study, primer and probe were designed based on the *invA* gene, which has often been used as a target gene of the *Salmonella* genus, and the detection limit was suggested with artificially inoculated milk using real-time PCR with an enrichment step [9]. In this study, a novel specific primer set and probe were designed based on the STM4497 gene, and they were applied to real-time PCR with quantified genomic DNA of *S.* Typhimurium for quantitative detection. Using real-time PCR, direct quantification of *S.* Typhimurium was evaluated with extracted DNA from artificially inoculated chicken sample without the enrichment step.

Finally, this real-time PCR method could be applicable to detection of foodborne pathogens contaminated in food.

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