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Development of a Novel, Rapid Multiplex Polymerase Chain Reaction Assay for the Detection and Differentiation of *Salmonella enterica* Serovars Enteritidis and Typhimurium Using Ultra-Fast Convection Polymerase Chain Reaction

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Abstract

Salmonella enterica serovars Enteritidis and Typhimurium are the most common causative agents of human nontyphoidal salmonellosis. The rapid detection and timely treatment of salmonellosis are important to increase the curative ratio and prevent spreading of the disease. In this study, we developed a rapid multiplex convection polymerase chain reaction (PCR) method to detect *Salmonella* spp. and differentiate *Salmonella* Enteritidis and *Salmonella* Typhimurium. We used the *invA* gene for *Salmonella* spp. detection. *Salmonella* Enteritidis-specific primers and *Salmonella* Typhimurium-specific primers were designed using the insertion element (*IE*) and *spy* genes, respectively. The primer set for *Salmonella* spp. detection clearly detected both *Salmonella* Enteritidis and *Salmonella* Typhimurium after a 21-min amplification reaction. Serovar-specific primer sets for *Salmonella* Enteritidis and *Salmonella* Typhimurium specifically detected each target species in a 21-min amplification reaction. We were able to detect *Salmonella* spp. at a single copy level in the singleplex mode. The limits of detection for *Salmonella* Enteritidis and *Salmonella* Typhimurium were 30 copies in both the singleplex and multiplex modes. The PCR run time could be reduced to 10.5 min/15 cycles. The multiplex convection PCR method developed in this study could detect the *Salmonella* spp. *Salmonella* Enteritidis and *Salmonella* Typhimurium in artificially contaminated milk with as few as 10^0 colony-forming unit/mL after 4-h enrichment. The PCR assay developed in this study provides a rapid, specific, and sensitive method for the detection of *Salmonella* spp. and the differentiation of *Salmonella* Enteritidis and *Salmonella* Typhimurium.

Keywords: *Salmonella*, molecular diagnostics, ultra-fast convection PCR

Introduction

Salmonella enterica is a common foodborne pathogen that causes gastroenteritis and septicemia in humans. *Salmonella enterica* is divided into six subspecies (I, II, IIIa, IIIb, IV, and VI), and *Salmonella* is classified into over 2600 serovars (Issenhuth-Jeanjean *et al.*, 2014). However, only a small number of these serovars cause human infection. The most common are *Salmonella enterica* serovars Enteritidis and Typhimurium. Both of these serovars are frequently found in contaminated food stuffs, particularly in meats (Liu *et al.*, 2012). Detection of *Salmonella* spp. using serological technology is based on the differences in surface antigens, the O antigen (somatic) and the H antigen (flagella). This technology is labor-intensive, expensive, complicated, and time-consuming (Kim *et al.*, 2006; Jarvik *et al.*, 2010). To overcome these issues, various molecular and genetic-based approaches have been attempted. These include polymerase chain reaction (PCR) assay (Park *et al.*, 2009; de Freitas, 2010; Liu *et al.*, 2012; He *et al.*, 2016), subtractive hybridization, loop-mediated isothermal amplification (Agron *et al.*, 2001; Fan *et al.*, 2015), sequence-based serotyping, and DNA microarray hybridization (Guard *et al.*, 2012; Li, 2016) methods.

Recently, PCR methods have become important in microbial diagnostics because of their speed and higher accuracy compared to traditional serology-based microbial serotyping (Liu *et al.*, 2012). Several multiplex PCR methods have been developed for the detection and identification of *Salmonella* spp. (Park *et al.*, 2009; Liu *et al.*, 2012; He *et al.*, 2016). Rapid molecular detection of infectious diseases, including salmonellosis, has recently attracted attention (Fan *et al.*, 2015; Stamm, 2015; Chin *et al.*, 2017; Hyeon and Deng, 2017). In

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this study, we employed a new technology: convection PCR that uses three heating plates for denaturation, annealing, and polymerization to generate convection in the PCR tube. This method does not require ramping between temperatures as in conventional thermocyclers. Hence, the PCR run time is therefore dramatically reduced in convection PCR (Hwang *et al.*, 2009; Hwang, 2011). In this study, we introduce a rapid, simple, and economical method for the detection of *Salmonella* spp. and the differentiation of *Salmonella* Enteritidis and *Salmonella* Typhimurium in singleplex and multiplex modes using convection PCR.

Materials and Methods

Bacterial strains and growth conditions

The bacterial strains used in this study were *Salmonella* Enteritidis (NCCP Nos. 14554 and 14771), *Salmonella* Typhimurium (NCCP Nos. 12219 and 14760), and *Escherichia coli* O157:H7 (NCCP No. 15739) from the National Culture Collection for Pathogens at Korea Centers for Disease Control and Prevention (Cheongju, Korea). The bacteria were treated as instructed by the provider. They were streaked on Luria Bertani (LB; Duchefa, Netherland) agar plates and incubated overnight at 37°C. For DNA purification, the bacteria were grown in LB broth (Duchefa) at 37°C with shaking overnight.

DNA samples for PCR analysis

Genomic DNA from all strains was extracted using a commercial DNeasy Blood and Tissue Kit (Qiagen, Germany) according to the manufacturer's instructions. After extraction, the DNA concentration was measured using a NanoDrop spectrophotometer (Thermo Scientific). DNA samples were serially diluted to prepare samples with designated DNA concentrations. Mixed DNA samples were prepared by combining equal amounts of the individually prepared genomic DNA samples from each *Salmonella* strain. Copy numbers of the genomic DNA in the samples were calculated from 1 ng of DNA based on the molecular weight of double-stranded DNA and chromosomal DNA size (http://scienceprimer.com/copy-number-calculator-for-realtime-pcr), using 1.9×10^5 copies/ng for *Salmonella* chromosomal DNA.

Serovar-specific primer design and PCR

The primers used in this study are listed in Table 1. The specific invasion protein A (*inv*A) of *Salmonella* was used to design *Salmonella* spp.-specific primers (Spp). To design serovar-specific primers, the insertion element (*IE*) gene (GenBank accession number Z83734) and the periplasmic protein (*spy*) gene (GenBank accession number AE008757.1) were used for *Salmonella* Enteritidis and *Salmonella* Typhimurium, respectively.

The PCR reaction mixture (20 μL) contained $1 \times$ *PalmTaq* HS buffer (including 1.5 mM $MgCl_2$), 0.2 mM dNTPs, 0.4 U *PalmTaq* High-speed DNA polymerase (Ahram Biosystems, Inc., Korea), and primers for either single or multiple PCR detection. For singleplex detection, 10 μM of primers were used. For multiplex detection, 10 μM of Spps, 8 μM of *Salmonella* Enteritidis-specific E primers, and/or 10 μM of *Salmonella* Typhimurium-specific T primers were used. Any deviations are stated in the text. Generally, 1.6 ng of genomic DNA was used as a template.

PCR was performed with a convection thermal cycler Palm PCR device (G2-12; Ahram Biosystems, Inc., Korea). The speed level was set to T1, and the annealing temperature was set to 56°C. PCR reactions were run for 30 cycles in 21 min unless stated otherwise. For conventional PCR, PCR amplification was performed at Verti 96 well thermal cycler (Applied Biosystems) with an initial denaturation of 95°C for 5 min, followed by 30 cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 30 s, and then a final extension at 72°C for 5 min. Upon completion, an aliquot of the PCR mixture was analyzed by 1.5% agarose gel electrophoresis for 30 min at 100 V. PCR products were visualized with an imaging system by fluorescence after ethidium bromide staining (Ultra-Lum Imaging System). All experiments were performed at least in triplicate.

Preparation of artificially contaminated milk and PCR

Fresh milk was purchased from the local market. Ten milliliters of milk was artificially inoculated with 1 mL of different concentrations of *Salmonella* Enteritidis or *Salmonella* Typhimurium viable cells (4.5×10^4–4.5×10^0 colony-forming unit [CFU]/mL). The culture was diluted with 9 mL of buffered peptone water (Oxoid, United Kingdom) and incubated at 37°C for 4 h in a shaking incubator (Vision Scientific, Korea). One milliliter of culture was taken for DNA purification, which was performed as described above. One microliter of DNA eluate was used as a template, and convection PCR was performed as described above. As an internal control, primers for beef Cyt b gene, which generate 274 bp amplicons, were used (Song *et al.*, 2017).

Table 1. Primers Used in This Study

Name	*Sequence (5′ → 3′)*	*Tm (°C)*	*Amplicon size (bp)*	*Specific species*
Spp	Forward: CAC GTT CGG GCA ATT CGT	56.2	241	*Salmonella* spp.
	Reverse: GCT TTC CCT TTC CAG TAC GC	56.3		
E	Forward: GTC AGT GCC ATA CTT TTA ATG ACT GC	56.3	321	*Salmonella* Enteritidis
	Reverse: GTA CTA TGT CGA TAC GGT GGG T	55.7		
T	Forward: GCT GTA TTT GTT CAC TTT TTA CCC CT	55.8	409	*Salmonella* Typhimurium
	Reverse: ACC CTG ACA GCC GTT AGA TAT TC	56.3		

Results

Specificity and the limits of detection for ultra-fast convection PCR in singleplex mode

The primers used in this study were designed to detect *Salmonella* spp. (Spps, forward and reverse), which were designed to detect both *Salmonella* Enteritidis and Typhimurium. Serovar-specific primers, *Salmonella* Enteritidis-specific primers (E primers, forward and reverse), and *Salmonella* Typhimurium-specific primers (T primers, forward and reverse) were designed to specifically detect *Salmonella* Enteritidis and *Salmonella* Typhimurium, respectively. Among various primers we designed for specific detections of designated targets, the primers that showed best amplification with the ultra-fast convection PCR were selected and used in this study. The primer information is shown in Table 1.

Convection PCR reactions were performed for 30 cycles (21 min) with genomic DNA isolated from *Salmonella* Enteritidis and *Salmonella* Typhimurium. As shown in Figure 1A, strong DNA amplification was detected with *Salmonella* spp. detection primers (Spp) and the tested genomic DNA purified from both *Salmonella* Enteritidis and *Salmonella* Typhimurium (241 bp bands in lanes 1 and 4, respectively, in Fig. 1A). Clear DNA amplification was detected with serovar-specific primers and corresponding genomic DNA purified from either *Salmonella* Enteritidis or *Salmonella* Typhimurium (321 bp band in lane 2 and 409 band in lane 6, respectively, in Fig. 1A). These serovar-specific primers did not amplify DNA from the other *Salmonella* serovars (lanes 3 and 5 in Fig. 1A). These results suggested that the convection PCR method used is serovar specific. No DNA amplification was evident in the no template control sample *Salmonella*. One of the members of Enterobacteriaceae, *E. coli* O157:H7, showed no amplification with the primers used in this study (Fig. 1A, lanes 7–9). With the primers used for *Salmonella* detection, clear amplifications were detected by using the conventional ramping temperature PCR. The time period taken for the conventional PCR amplification was ~2 h (Fig. 1B). The primers were also quantified using two different strains in the same serotype (Fig. 1C). These data demonstrate that the convection PCR method effectively amplified DNA from each *Salmonella* species tested and serovar-specific primers can be used to specifically detect specific *Salmonella* serovars using ultra-fast 21-min convection PCR amplification.

To determine the *Salmonella* genomic DNA detection limit for the developed PCR method, genomic DNA from the *Salmonella* species tested was diluted from 3×10^5 copies (1.6 ng) to 3×10^0 (1.6×10^{-5} ng) and singleplex (i.e., with one pair of serovar-Spps) convection PCR was performed (Fig. 2). For *Salmonella* Enteritidis with the *Salmonella* spp. primers, Spp, a single copy level could be detected (Fig. 2A). The *Salmonella* Enteritidis-specific primers could detect *Salmonella* Enteritidis a copy level of 10 (Fig. 2B). For *Salmonella* Typhimurium, the Spps could detect a copy level of 10 (Fig. 2C). The *Salmonella* Typhimurium-specific primers could also detect *Salmonella* Typhimurium a copy level of 10 (Fig. 2D). These data were obtained with 30 cycles in a 21-min operation mode.

Specificity and the limits of detection for ultra-fast convection PCR in multiplex mode

Next, multiplex detection of *Salmonella* species by convection PCR was tested. The *Salmonella* spp. primers, Spp, and the serovar-specific primers for *Salmonella* Enteritidis and *Salmonella* Typhimurium were mixed together and used for rapid PCR identification of *Salmonella* species. In this

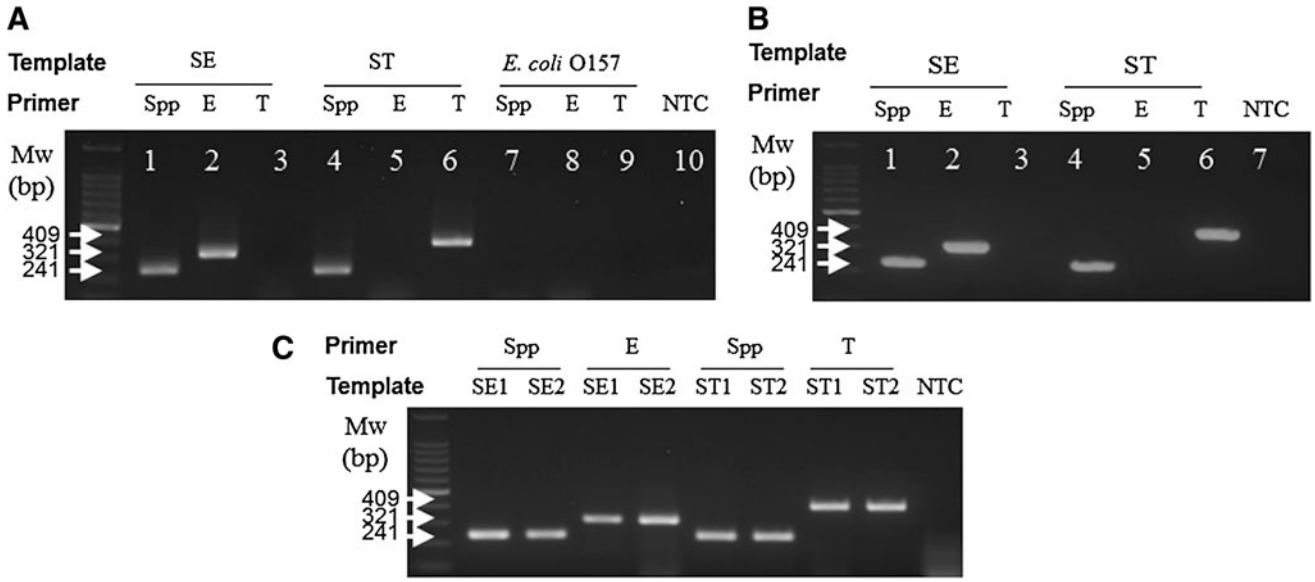

FIG. 1. Detection of *Salmonella* species with Spps. **(A)** Genomic DNA samples isolated from SE and ST were used as templates for ultra-fast convection PCR reactions with the *Salmonella* spp.-specific primer set and each SE- and ST-specific primer set (E or T). DNA amplicons of expected sizes were detected only from DNAs of *Salmonella* strains, not from *Escherichia coli* O157:H7. The PCR operation time was 21 min (equivalent to 30 cycles). **(B)** The same PCR amplification reactions were prepared and used for amplifications with a conventional thermal cycler. The PCR operation time was ~2 h. **(C)** Two strains of SE and ST was tested for primer specificity. SE, *Salmonella* Enteritidis; ST, *Salmonella* Typhimurium; Mw, molecular weight marker; NTC, no template control.

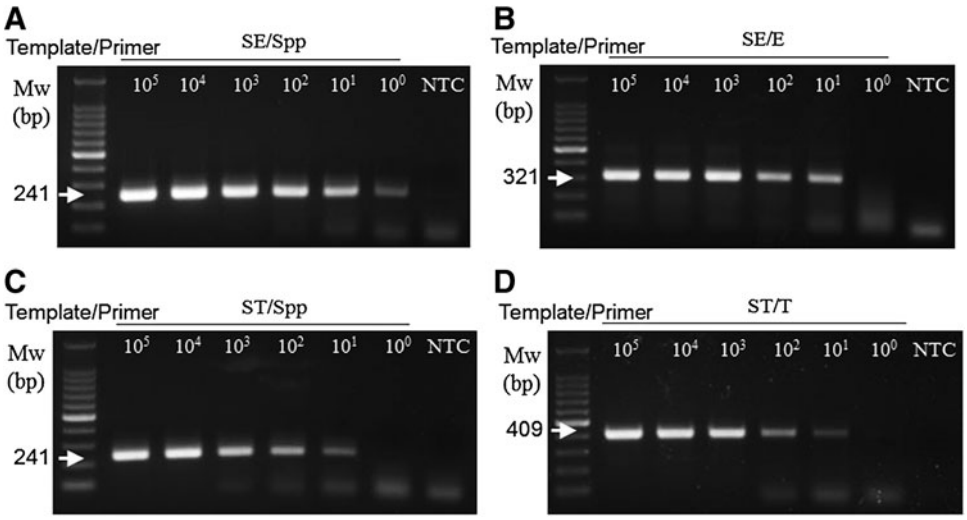

FIG. 2. Determination of the genomic DNA detection limits of *Salmonella* in singleplex convection PCR. Template and primer sets used were SE and Spp primer set **(A)**, SE and E primer set **(B)**, ST and Spp primer set **(C)**, and ST and T primer set **(D)**, respectively. Genomic DNA samples isolated from SE and ST were serially diluted and used as templates. The Spp set and the SE- and ST-specific primer sets (E or T) were used in the singleplex convection PCR reactions. SE, *Salmonella* Enteritidis; ST, *Salmonella* Typhimurium; Mw, molecular weight marker; NTC, no template control.

experiment, mixtures of the same amounts of the two genomic DNA samples were prepared. Each individual genomic DNA sample (Lanes 1–8 in Fig. 3) and a combination of genomic DNA from the two different species (lanes 9–12 in Fig. 3) were used as templates for convection PCR amplification. As anticipated, amplified DNA bands for *Salmonella* spp. detection at 241 bp and *Salmonella* Enteritidis detection at 321 bp were observed when *Salmonella* Enteritidis genomic DNA was used as a template. No amplification of the DNA band at 409 bp, which is specific for *Salmonella* Typhimurium, was observed (Fig. 3, lanes 1–4). Similar results were obtained for DNA amplification of *Salmonella* Typhimurium genomic DNA with the primer mixtures (Fig. 3, lanes 5–8). Amplified DNA bands for *Salmonella* spp. detection at 241 bp and *Salmonella* Typhimurium detection at 409 bp were observed when *Salmonella* Typhimurium genomic DNA was used as a template. No amplification of the DNA band at 321 bp, which is specific for *Salmonella* Enteritidis, was observed. When convection PCR was performed with a mixture of template (genomic DNA of both *Salmonella* Enteritidis and *Salmonella* Typhimurium) with the primer mixtures, duplicate and triplicate DNA amplification bands with the expected sizes, depending on the mixture of primers used, were successfully amplified (Fig. 3, lanes 9–12).

The limits of detection were determined for multiplex convection PCR. Genomic DNAs were mixed and diluted from 3×10^5 copies (1.6 ng) to 3×10^0 (1.6×10^{-5} ng) copies, and multiplex convection PCR was performed (Fig. 4). DNA amplification was clearly visible up to 3×10^2 copies and faintly visible at 3×10^1 copies (1.6×10^{-4} ng). We would like to emphasize that $\sim$30 genome equivalents, subpicogram quantities, were detected in the multiplex mode using the convection PCR method. These data were obtained with 30 cycles in a 21-min operation mode.

Rapid detection and differentiation of Salmonella *species with convection PCR*

Next, we tested the minimal time required for the convection PCR to proceed without losing detection sensitivity (Fig. 5). First, we changed the speed setting of convection PCR from T1 (30 cycles in 21 min) to T2 (30 cycles in 18 min). In both speed settings, clear detection of the three multiplex amplification DNA bands was observed (Fig. 5A). Second, convection PCR speed was set to T1, and the PCR operation time (or the number of PCR cycles) was gradually reduced. As shown in Figure 5B, each individual target in the multiplex approach, *Salmonella* spp. detection and the *Salmonella* Enteritidis-specific and *Salmonella* Typhimurium-specific bands, was clearly detected after 10.5-min PCR operation time, which is equivalent to 15 PCR cycles.

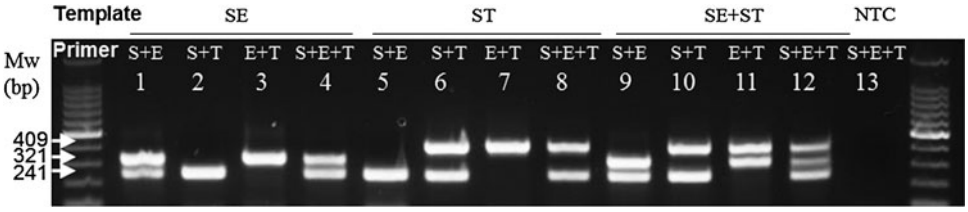

FIG. 3. Multiplex identification and differentiation of *Salmonella* spp., SE and ST. Convection PCR was performed with genomic DNA samples from SE (lanes 1–4) and ST (lanes 5–8), and a 1:1 mixture of the two genomic DNA samples (lanes 9–12). The Spp set and the SE- and ST-specific primer sets (S, E or T) were mixed and used in multiplex convection PCR. DNA amplicons of expected sizes were detected.

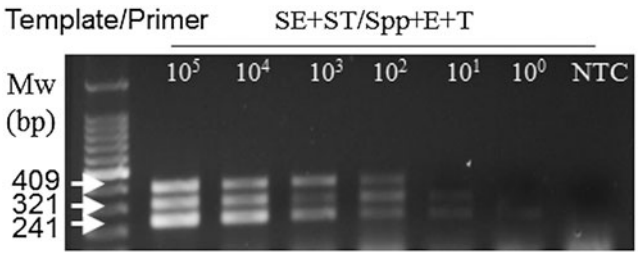

FIG. 4. Determination of the genomic DNA detection limits of *Salmonella* in multiplex convection PCR. Genomic DNA samples isolated from SE and ST were mixed in a 1:1 ratio, serially diluted, and used as template. A mixture of the Spp set and the SE- and ST-specific primer sets (E or T) was used in multiplex convection PCR.

Convection PCR with artificially contaminated milk samples

To validate the application of the assay developed in this study, we attempted to detect *Salmonella* spp., *Salmonella* Enteritidis and *Salmonella* Typhimurium, in artificially contaminated milk samples. Fresh milk was inoculated with both *Salmonella* Enteritidis and *Salmonella* Typhimurium at an inoculum level of 4.5×10^4–4.5×10^0 CFU/mL and enriched for 4 h. As shown in Figure 6A, three bands for *Salmonella* spp., *Salmonella* Enteritidis and *Salmonella* Typhimurium, were clearly detected. No amplification was observed in the samples with no inoculum. The *Salmonella* detection limit for contaminated milk was determined to be 4.5×10^0 CFU/mL after a 4-h enrichment. As an internal control, we used beef cytochrome B gene (Song *et al.*, 2017). The internal control bands of 274 bp were clearly detected in the samples tested, including no-inoculated sample (Fig. 6B).

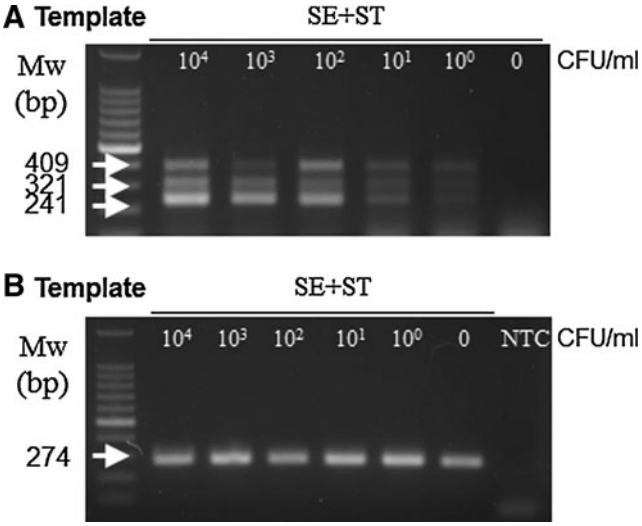

FIG. 6. Detection of *Salmonella* spp., SE and ST, in artificially contaminated milk. **(A)** Fresh milk was contaminated with 4.5×10^4–10^0 colony-forming unit/mL of SE and ST (SE + ST). The sample with no inoculum is marked as 0. Samples were pre-enriched for 4 h. Genomic DNA samples were isolated and used as template for convection PCR. **(B)** As an internal control, primers for beef Cyt b gene were used.

Discussion

Molecular-based detection methods using PCR technology have become an important tool in microbial diagnostics because of their high specificity and sensitivity. To detect and differentiate *Salmonella* Enteritidis and *Salmonella* Typhimurium, various gene loci have been used as targets, including *fliC*, *IE, sdf, sefA, spy*, and STM4495 (de Freitas *et al.*, 2010; Zhang *et al.*, 2010; Liu *et al.*, 2012; Paião *et al.*, 2013; He *et al.*, 2016; Chin *et al.*, 2017). For *Salmonella* spp. detection, *invA*, *ompC*, and *oriC* genes are most commonly used (Germini *et al.*, 2009; McCarthy *et al.*, 2009; de Freitas *et al.*, 2010; Saeki *et al.*, 2013). In this study, we used *Salmonella* spp. detection primer sets targeting the *invA* gene, which is widely spread in *Salmonella* spp. The *IE* and *spy* genes were used for the detection and differentiation of *Salmonella* Enteritidis and *Salmonella* Typhimurium, respectively. We found that these genes were not present in Heidelberg and Newport serovars by Blast search. These serobars are reported to cover 7% and 10% of outbreaks from 2007 to 2011 in the United States (Andino and Hanning, 2015). However, *IE* and *spy* genes are found in Gallinarum and Saintpaul serovars, respectively. To detect other *Salmonella* serotypes with the method developed in this study, each *Salmonella* serotype specific primer set needed to be developed. To make the gel patterns distinguishable with the current gel electrophoresis technology, the number of *Salmonella* serotypes to be tested may be limited. In this case, the method developed in this study can be used with the combination of the sequence-based technology.

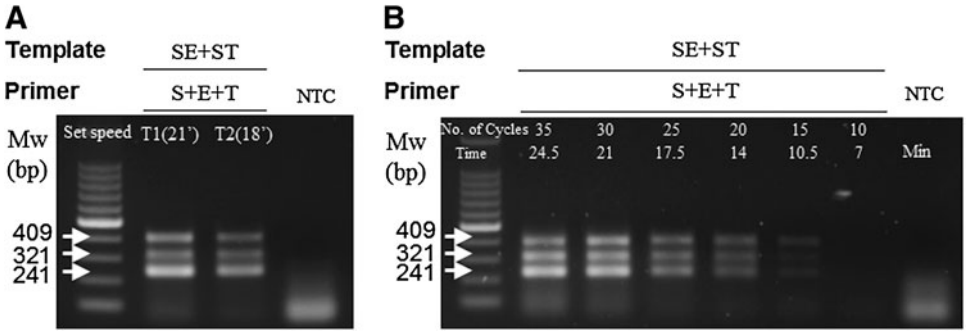

FIG. 5. Rapid detection of *Salmonella* spp., SE and ST, with convection PCR. The reaction speed was changed **(A)** or the operation time of the convection PCR was gradually reduced **(B)**, and the generated DNA amplicons were analyzed by agarose gel electrophoresis. The concentration of genomic DNA was 1.6 ng of both SE and ST. A mixture of all three primer sets was used.

Our multiplex PCR reaction was completed in 21 min (30 cycles), making it an ultra-fast method for the detection of *Salmonella*. The PCR running time could be reduced to 10.5 min without losing sensitivity. This method is the fastest time period reported to date for *Salmonella* detection and differentiation using PCR technology. In most cases, between 1.5 and 2.5 h of PCR operation time is required.

Our data suggest that the ultra-fast convection PCR method is highly sensitive. We could detect 3–30 copies (equivalent to 16–160 fg) of *Salmonella* Enteritidis and *Salmonella* Typhimurium in singleplex mode and 30 copies (equivalent to 160 fg) in multiplex mode. This sensitivity is comparable with and/or higher than other reported methods. The detection limits of previously reported endpoint PCR methods are $\sim$1–2 pg of DNA (Shanmugasundaram *et al.*, 2009; He *et al.*, 2016) or 20–30 copies (Liu *et al.*, 2012). It is generally accepted that real-time PCR is more sensitive than endpoint PCR. However, our data revealed method sensitivity comparable with real-time PCR analyses, where the sensitivity was reported to be $\sim$10 genome equivalents (Suo *et al.*, 2010; Zhou *et al.*, 2014).

We could detect 4.5×10^0 CFU/mL of either *Salmonella* Enteritidis or *Salmonella* Typhimurium with artificially contaminated milk. After 4 h of enrichment, the limit of detection was maintained with the contaminated milk. The milk has indigenous microbes and biomolecules such as DNA and casein and fat, and these can be PCR inhibitors. DNA from dead bacteria may give false positive results. In our data, no band appeared in no-*Salmonella* contaminated milk, therefore there was no false positive result detected. It was suggested that more than 8 h of pre-enrichment was required for the detection of *Salmonella* (Liu *et al.*, 2012). We employed the pre-enrichment time of 4 h and the sensitivity acquired in this study is better than or comparable with other previously reported data, with sensitivities from $\sim 6 \times 10^2$–10^3 (McCarthy *et al.*, 2009; Zhai *et al.*, 2014) to 10^0 CFU/mL (Germini *et al.*, 2009; de Freitas, 2010).

Conclusions

In this study, we demonstrated that the detection of *Salmonella* spp. is achievable using convection PCR at ultra-fast speed of 21 min in both singleplex and multiplex modes. The detection sensitivity was as low as 1.6 fg of gDNA for each *Salmonella* spp., *Salmonella* Enteritidis and *Salmonella* Typhimurium. The PCR running time could be reduced to 15 min without losing the detection sensitivity. This method could detect 4.5×10^0 CFU/mL of *Salmonella* spp., *Salmonella* Enteritidis and *Salmonella* Typhimurium, in artificially contaminated milk after 4 h enrichment. We believe that the ultra-fast speed, specificity, and sensitivity of the molecular detection method presented in this study offer a reliable strategy for *Salmonella* spp. detection and *Salmonella* Enteritidis and *Salmonella* Typhimurium differentiation. By designing additional specific primer, this method can easily be extended to detect and differentiate other bacterial species.

Acknowledgments

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Disclosure Statement

No competing financial interests exist.

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