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Studies on PCR-Based Rapid Detection Systems for *Salmonella* spp.

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1. Introduction

1.1 Using molecular biological methods to identified *Salmonella* spp.

As the industrialization had made food supply to exceed demand, more and more consumers were interested in ‘Delicious food’ than ‘Good quality food’ today and they ask for not only taste but also safety. Microbiological safety is one of the most critical factors for ensuring safe food supply. Fig 1. is a flow chart of traditional detection procedure for *Salmonella* spp., showing that it takes about one week. Therefore, a rapid screening method using PCR is the basis of most molecular diagnostic laboratories. As the field of molecular pathology becomes more accessible to practicing pathologists, a working knowledge of PCR techniques is necessary related to biological safety of food is very essential in food industry. *Salmonella* spp. are an important cause of food-borne infections throughout the world, and the availability of rapid and simple detection techniques is critical for the food industry.

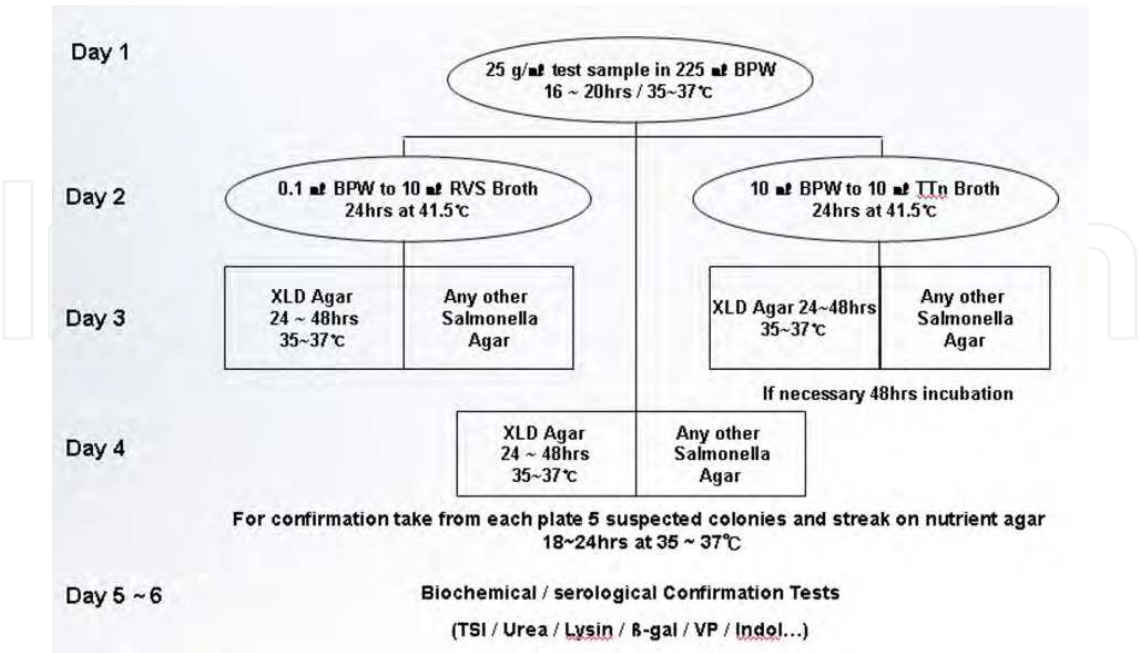


Fig. 1. Traditional procedure for the detection of *Salmonella* spp. (ISO/CD draft standard 6575 revision 2000)

Present commercial detection system for *Salmonella* spp. can be classified into four categories. The first, traditional method which uses culture medium and observe colony morphology formed on it. This requires at least four days and experienced skill to perform biological tests, but it is the only common method authorized throughout the world for now.

The second, Enzyme-Linked Immuno-Sorbent Assay (ELISA) detects certain bacteria using immune reaction between antibody and antigen specific for them. This method is easy to use because it makes color change or forms lines but it can be applied only for those which has specific toxin protein and requires more than 10^6 CFU / ml for detection which needs 16 hours of incubation. The third, Adenosine triphosphate (ATP) detection kit detects level of bacterial contamination by the amount of ATP in sample. This method can not be used for identification of bacteria because it can only tell including the total amount of ATP from food. This is usually used for comparing hygiene level before and after washing. The fourth, genetic method which is based on PCR is highly specific and sensitive enough to detect 100 CFU / ml of bacteria, but at the same time it can detect even the dead cells after processing or cooking food because of the high sensitivity.

1.2 Advanced PCR technologies

1.2.1 Multiplex PCR

Multiplex PCR can amplify two or more amplicons in a single PCR reaction. For multiplex PCR, each primer set is designed to amplify its target gene and make a PCR product of certain size to the target gene. To perform a multiplex PCR, the concentration of primers, Mg^{2+} , free dNTPs and polymerase must be optimized to allow synthesis of the genes of interest, And also the PCR reaction temperature parameters must be optimized to the best average for amplicon production for all primer sets. This technique saves time and labor more than one target DNA sequence can be detected in each reaction, It might not be optimal if the PCR products are limited in certain sizes and agarose gel staining with ethidium bromide (John Maurer, 2006). Therefore, it is possible to detect multiple pathogens in a sample with a single PCR test (Panicker *et al.*, 2004)

1.2.2 Real-Time PCR

Real-Time PCR technology is based on the ability of detection and quantification of PCR products, or amplicons, as the reaction cycles progress. Higuchi and colleagues introduced this technology (Higuchi *et al.*, 1993) and it became possible by including of a fluorescent dye that binds to the amplicon as it is made (Fig. 2. A).

Initially, a fluorescent dye, SYBR green I (A), was used to detect the amplicons. SYBR green I binds the double stranded, DNA amplicon and fluorescences upon illumination with UV light. In TaqMan PCR (B), the oligoprobe contains a fluorescent marker and chemical group that quenches fluorescent of oligoprobe until the dye is liberated by 3' exonuclease activity of the Taq DNA polymerase (Source http://cafe.naver.com/solgent.cafe?iframe_url=/ArticleRead.nhn%3Farticleid=38&)

In TanMan PCR, an intact, "internal" fluorogenic oligoprobe binds to target DNA sequence, internal to the PCR primer binding sites. This oligoprobe possesses a reporter dye that will fluorescence and a suppressor dye known as quencher that prevent fluorescent activity via Fluorescence Resonance Energy Transfer (FRET). After each PCR cycle, when the double-stranded DNA products are made, a measure of fluorescence is taken after the fluorogenic probe is hydrolytically cleaved from the DNA structure by exonuclease activity of the *Thermus aquaticus* DNA polymerase (Heid *et al.*, 1996; Holland *et al.*, 1991). Once cleaved, the probe's fluorescent activity is no longer suppressed (Fig. 2. B). FAM (6-Carboxyfluorescein)

and TAMRA(6-Carboxy-Tetramethyl-Rhodamin) are most frequently used as reporter and quencher, respectively. This PCR is often referred to as 5' exonuclease-based, real-time PCR or TaqMan PCR (Mullah *et al.*, 1998).

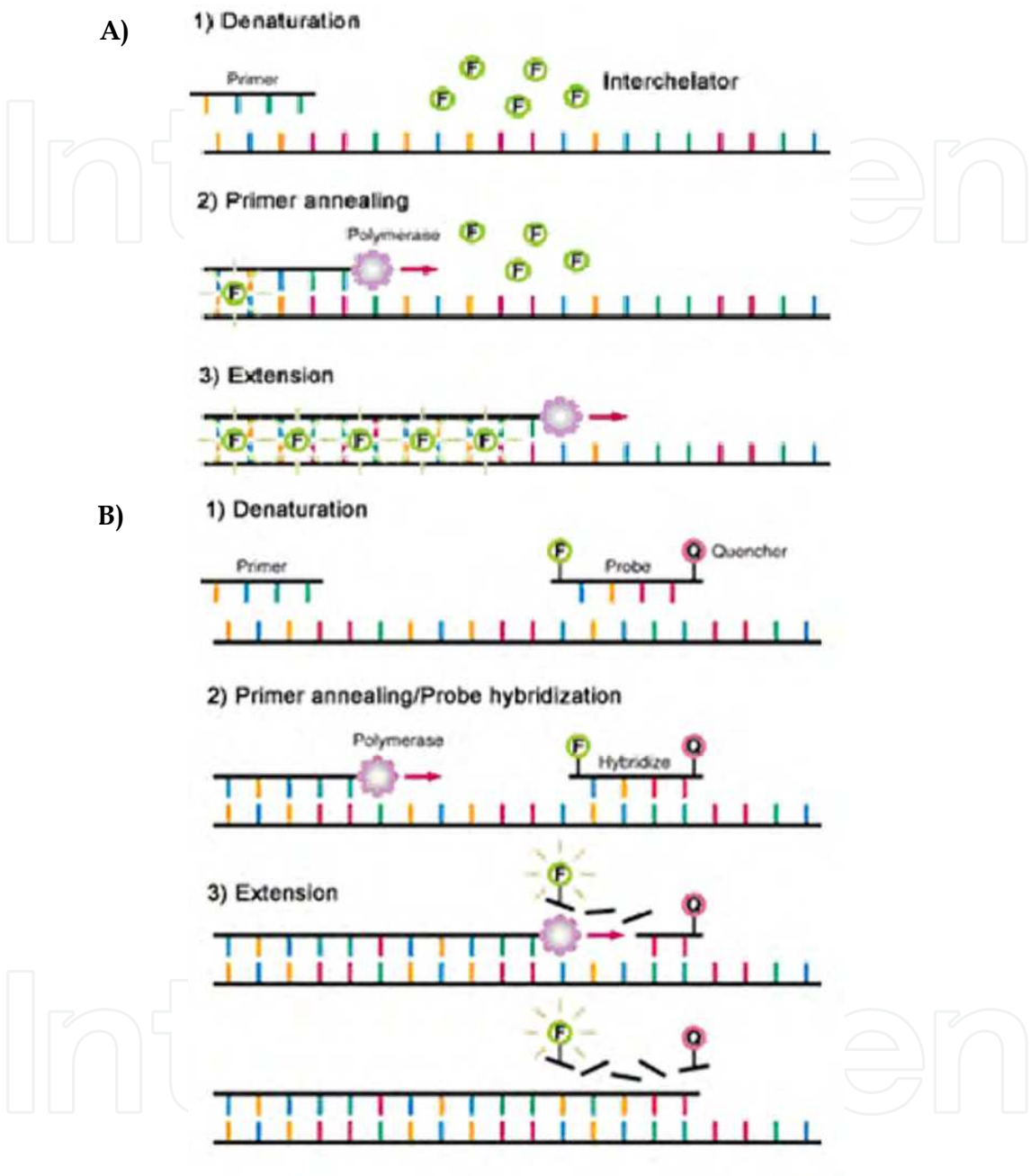


Fig. 2. Real-Time PCR detection of amplicons

1.2.3 Isothermal PCR

Recently, Jung *et al.* (2010) developed a new highly sensitive and specific isothermal amplification and detection system called isothermal target and probe amplification (iTPA) by employing DNA-RNA-DNA chimeric primers and a FRET (fluorescence Resonance Energy Transfer) probe. The iTPA method is based on a combination of novel isothermal chain amplification (ICA) and FRET cycling probe technology (CPT) (Fig. 3).

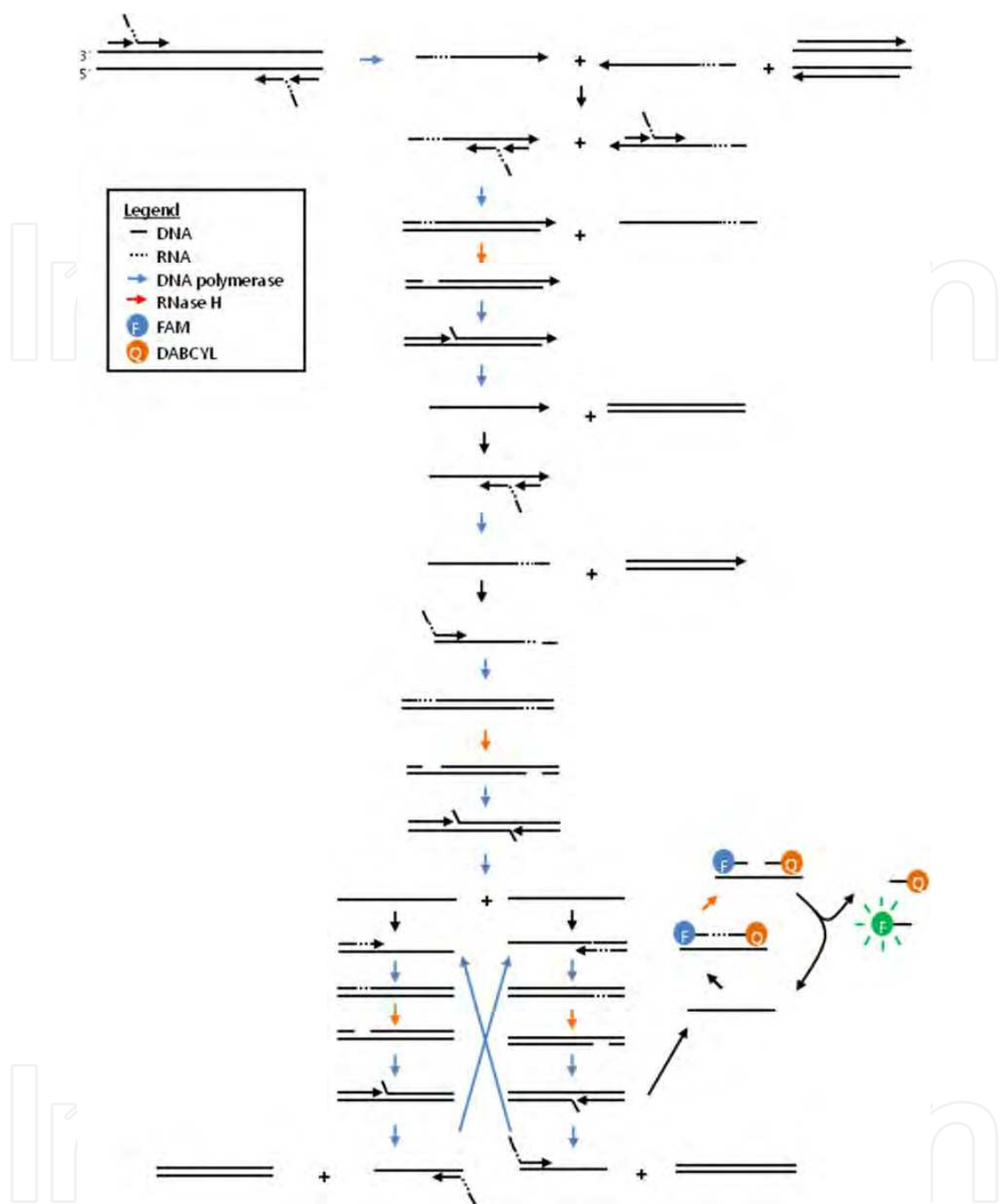


Fig. 3. Scheme of the Isothermal Target and Probe Amplification (iTPA) system

In the ICA method, which relies on the strand displacement activity of DNA polymerase and the RNA-degrading activity of RNase H, two displacement events occur in the presence of four specially designed primers that lead to high specificity for the target sequence. In the CPT method, a DNA-RNA-DNA chimeric probe is hybridized with the target DNA, and the RNA region of the duplex is specifically cleaved by RNase H. The cleaved probe fragment is disassociated from the target DNA and another intact probe is again hybridized and then cleaved. In this cycling event, a single target DNA molecule results in a large number of cleaved probe fragments, which can be designed to generate fluorescence signals (Kim *et al.* 2011).

2. Various PCR methods approaches for the detection of *Salmonella* spp.

2.1 Rapid and simultaneous detection of five pathogenic bacteria by a novel multiplex PCR assay: *Salmonella* spp., *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Staphylococcus aureus* and *Vibrio parahaemolyticus*

According to Centers for Disease Control (CDC), about 5 millions food mediated diseases are killing 4,000 people every year. *Salmonella* was the most frequently found pathogenic bacteria in food poisoning : 1 ~ 4 millions of people were infected, 2,000 (0.1%) of them were dead. *Salmonella* is an important pathogen associated with bacterial foodborne outbreaks in the United states, accounting for 24% of all food outbreaks and 18% of produce-related outbreaks between 1990 and 2009 (Center for Science in the Public Interest, 2009). An outbreak in 2009 associated with *Salmonella*-contaminated peanut butter and peanut containing products caused nine deaths in 46 states as of 17 March 2009. This outbreak led to the largest recall of food items in the United States resulting in over 2100 products being voluntarily recalled by more than 200 companies (FDA, 2009). Recently, more than 500 million eggs were recalled after dangerous levels of *Salmonella* were detected in the eggs from two Iowa producers, who distributed the eggs in 14 US states. Nearly 2000 illnesses were reported between May and July 2010 (CDC, 2010). Food poisoning by *E. coli* O157:H7 broke out in 10000 people, 300 of them were dead. As for *Listeria monocytogenes*, 1500 people were infected and 400 were dead. This shows that stock farm products which were contaminated by these four bacteria (*E. coli* O157:H7, *Salmonella* spp., *Listeria monocytogene* and *Staphylococcus aureus*) is seriously threatening consumer's health. In Korea, 50% of food poisoning are caused by meat or processed meat products, and *Salmonella* strains (50%), *S. aureus* (20%) are two major sources. Different molecular targets have been used to characterize the presence of food-borne pathogenic bacteria. In this study, genes encoding the virulence determinants and their expression regulator have been used to characterize numerous bacteria. A molecular test based on the detection of shiga-like toxin (verotoxin type II), *femA* (cytoplasmic protein), *toxR* (trans-membrane DNA binding protein), *iap* (invasive associative protein), and *invA* (invasion protein A) genes has been applied for identification of *E. coli* O157:H7 (Jinneman *et al.*, 2003; Kaneko *et al.*, 2001; Karpman *et al.*, 1998; Schmidt *et al.*, 1995; Wang *et al.*, 2002), *Staphylococcus aureus* (Mehrotra *et al.*, 2000), *Vibrio parahaemolyticus* (Karpman *et al.*, 1998; Cabrera-Garcia *et al.*, 2004), *Listeria monocytogenes* (Bubert *et al.*, 1992; Bubert *et al.*, 1999; Volokhov *et al.*, 2002), and *Salmonella* spp. (Chiu *et al.*, 1996).

To our knowledge, there is not a single acceptable method which is available to detect these five food-borne pathogenic bacteria simultaneously in food samples. The objective of the present work, therefore, was to establish a multiplex PCR assay method to detect the specific bacterial genus simultaneously and to analyze their distribution in contaminated foods. Our results indicate, that this method is rapid and specific for the simultaneous detection of *E. coli* O157:H7, *Staphylococcus aureus*, *Vibrio parahaemolyticus*, *Listeria monocytogenes* and *Salmonella* spp.

2.1.1 Materials & methods [bacterial strains]

Bacterial strains were obtained from the American Type Culture Collection (ATCC; Manassas, Va.), the Korean Collection for Type Culture (KCTC; Daejeon, South Korea), and the Korean Culture Center of Microorganisms (KCCM; Seoul, South Korea). Also the strains isolated from various food samples were used in this study (Table 1).

All bacterial strains were grown on Luria-Bertani broth (LB; Bactopeptone 10 g, Yeast extract 5 g, and NaCl 10 g, each per Liter) at 37°C. All *Vibrio* species were grown in LB broth with supplementary 2% sodium chloride. Cultures were grown in LB, and a population of visible microorganisms was obtained by plating 10-fold serial dilutions of broth cultures on to plate count agar (Difco, Sparks, USA) and incubating the plates at 37°C for 16 hours. At each sampling dilution ratio, all bacterial cultures were mixed, and 100 µl (approximately 10⁷ CFU) of the suspension was used as DNA templates for PCR.

Strain	Source ^a	Cultural medium
<i>Vibrio</i> spp.		
<i>V. alginus</i>	KCCM41677	Trypticase Soy Broth with 2.5% NaCl
<i>V. carchariae</i>	KCCM40865	Marine Broth
<i>V. cholerae</i>	KCCM41626	Nutrient Broth
<i>V. cincinnatiensis</i>	KCCM41683	Marine Broth
<i>V. diazotrophicus</i>	KCCM41666	Trypticase Soy Broth with 1% NaCl
<i>V. fischeri</i>	KCCM41685	Marine Broth
<i>V. fluvialis</i>	KCCM40827	Marine Broth
<i>V. furnissii</i>	KCCM41679	Trypticase Soy Broth with 1% NaCl
<i>V. hollisae</i>	KCCM41680	Marine Broth
<i>V. marinagilis</i>	KCCM41673	Marine Broth
<i>V. marinofulvus</i>	KCCM41674	Marine Broth
<i>V. marinovulgaris</i>	KCCM41675	Marine Broth
<i>V. mediterranei</i>	KCCM40867	Marine Broth
<i>V. metschnikovii</i>	KCCM41681	Trypticase Soy Broth with 1% NaCl
<i>V. natriegens</i>	KCCM40868	Nutrient Broth with 1.5% NaCl
<i>V. navarrensis</i>	KCCM41682	Marine Broth
<i>V. penaeicida</i>	KCCM40869	Marine Broth
<i>V. proteolyticus</i>	KCCM11992	Nutrient Broth with 3% NaCl
<i>V. salmonicida</i>	KCCM41663	Trypticase Soy Broth with 1% NaCl
<i>V. vulnificus</i>	KCCM41665	Trypticase Soy Broth with 1% NaCl
<i>V. parahaemolyticus</i>	KCCM11965	LB Broth with 1% NaCl
<i>V. parahaemolyticus</i>	KCCM41664	LB Broth with 1% NaCl
<i>V. parahaemolyticus</i>	Inha university	LB Broth with 1% NaCl
Other bacteria		
<i>Staphylococcus xylosus</i>	KCCM41465	LB Broth
<i>Bacillus licheniformis</i>	KCTC1831	LB Broth
<i>Yersinia enterocolitica</i>	KCCM41657	LB Broth
<i>Staphylococcus aureus</i>	KCCM11764	LB Broth
<i>Staphylococcus cohnii</i>	KCTC3574	LB Broth
<i>Bacillus subtilis</i>	KCTC2213	LB Broth

Strain	Source ^a	Cultural medium
<i>Bacillus cereus</i>	KCTC1661	LB Broth
<i>Bacillus cereus</i>	KCTC 3624	LB Broth
<i>Salmonella typhimurium</i>	KCTC 2421	LB Broth
<i>Bacillus subtilis</i>	KCTC 3013	LB Broth
<i>Staphylococcus arlettae</i>	KCTC 3588	LB Broth
<i>Citrobacter freundii</i>	KCCM 11931	LB Broth
<i>Bacillus licheniformis</i>	KCTC 3006	LB Broth
<i>Salmonella choleraesuis</i>	KCCM 41575	LB Broth
<i>Shigella sonnei</i>	KCTC 2009	LB Broth
<i>Staphylococcus aureus</i>	KCTC 1916	LB Broth
<i>Salmonella typhimurium</i>	KCTC 2515	LB Broth
<i>Shigella bongori</i>	KCCM 41758	LB Broth
<i>Staphylococcus caprae</i>	KCTC 3583	LB Broth
<i>Salmonella typhimurium</i>	ATCC 14028	LB Broth
<i>Staphylococcus warneri</i>	KCTC 3340	LB Broth
<i>Salmonella enterica</i>	KCTC 2929	LB Broth
<i>Staphylococcus aureus</i>	KCTC 1927	LB Broth
<i>Listeria grayi</i>	ATCC 700545	LB Broth
<i>Listeria ivanovii</i>	ATCC 49953	LB Broth
<i>Listeria grayi</i>	ATCC 25400	LB Broth
<i>Listeria innocua</i>	ATCC 33091	LB Broth
<i>Listeria murrayi</i>	ATCC 25403	LB Broth
<i>Listeria ivanovii</i>	ATCC 49954	LB Broth
<i>Escherichia coli</i> O157:H7	NVRQ	LB Broth
<i>Listeria innocua</i>	ATCC 33090	LB Broth
<i>Staphylococcus aureus</i>	KCTC 1928	LB Broth

^a KCCM, Korean Culture Center of Microorganisms
KCTC, Korean Collection for Type Culture
ATCC, American Type Culture Collection
NVRQS, National Veterinary Research and Quarantine Service
KACC, Korean Agricultural Culture Collection

Table 1. Bacterial strains used in this study

[Enrichment procedures for detection of food-borne microorganisms]

All food-borne pathogens were grown for 16 hours in LB broth at 37°C in a shaking water bath. Cells were diluted from 1:10 to 1:10⁸ in 10 ml of Luria-Bertani broth and manipulated as described above to make approximate cell count from 10 to 10⁸ CFU / ml. In each dilution ratio, single enrichment broth samples (1 ml) were collected into 1.5 ml micro-centrifuge tubes and used for DNA extraction (Fig. 1).

[Extraction and preparation of DNA templates for PCR assay]

Individual samples (1 ml) were centrifuged at 10,000 X g for 3 min. The cell pellets were resuspended in RNase free water (100 µl) and placed in a 100°C heating block for 20 min. The samples were cooled for 2 min at room temperature and centrifuged at 16,000 X g for 5 min. The supernatant fluids (5 µl) were used to make 25 µl of a multiplex PCR reaction mixture, which included 5 µl of 5 X reaction buffer (2.5 mM MgCl₂ and 0.8 mM concentration of each dNTP), 4 µl of the primer mixtures of the five food-borne bacteria, 1 µl of Super Taq plus polymerase (Rexgene Biotech., Cheongwon, South Korea), and 10 µl of DNase free water in a single tube. The multiplex PCR was run for 35 cycles on a Tpersonal cyclor (Whatman Biometra, Goettingen, Germany) under the following conditions : denaturation at 94°C for 30 sec, primer annealing at 60°C for 30 sec, and extension at 72°C for 30 sec. The final cycle included an additional 5 min of extension time at 72°C. A 5 µl aliquot of the reaction mixture was then electrophoresis on a 2% agarose gel electrophoresis in 0.5 X Tris-borate buffer at 100 V for 25 min. The amplification products were stained with ethidium bromide and visualized by UV trans-illumination.

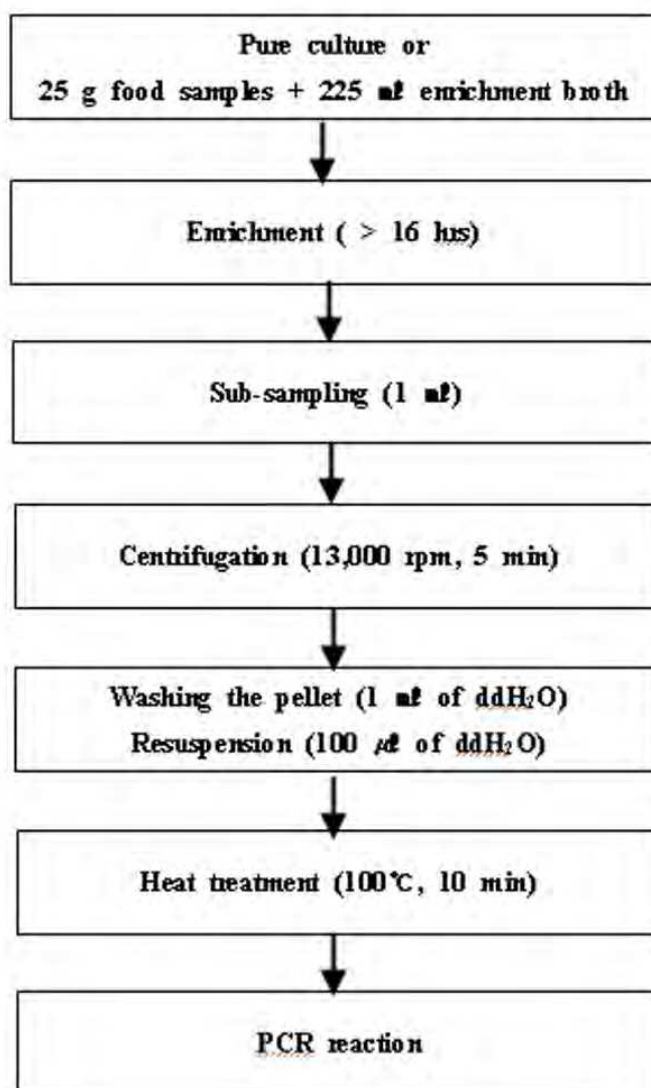


Fig. 4. Flow diagram of experimental protocols for PCR template preparation

[Oligonucleotides]

The oligonucleotide primers designed with Primer 3.0 software (Whitehead Institute, Cambridge, Mass.) were based on sequences obtained from Genbank and were used to amplify chromosomal DNA for the five food-borne pathogens (Table 2). The oligonucleotides and all reagents for PCRs were synthesized and purchased from Incorporation Bioneer (Daejeon, South Korea) and KoGene BioTech. (Seoul, South Korea).

Strains	Primer name	Primer direction	Sequences (5'→3')	Target gene	PCR product (bp)
<i>Vibrio parahaemolyticus</i>	VP	Forward Reverse	CTCATTTGTTACTGTTGAAC GCCTAAATAGA AGGCAACCAGTTGTTGAT GAATCCTCAGTTTTTCAAC	<i>toxR</i>	219 bp
<i>Salmonella</i> spp.	SAL	Forward Reverse	GTTTC TAGCCGTAACAACCAATAC AAATG AATTTAACAGCTAAAGAGT	<i>invA</i>	678 bp
<i>Staphylococcus aureus</i>	SA	Forward Reverse	TTGGT TTCATTAAAGAAAAAGTGT ACGAG GATAGACTTTTCGACCCAA	<i>femA</i>	264 bp
<i>E. coli</i> O157:H7	EC	Forward Reverse	CAAAG TTGCTCAATAATCAGACGA AGATG CTGGCACAAAATTACTTAC	shiga-like toxin	208 bp
<i>Listeria monocytogenes</i>	LM	Forward Reverse	AACGA AACTACTGGAGCTGCTTGT TTTTC	p60 protein	454 bp

Table 2. Oligonucleotide primers used in this study

[Specificity of the primer pairs and the multiplex PCR]

To evaluate the specificity of each oligonucleotide primer pair for its target gene, a PCR assay was carried out by testing all the reference strains reported in Table 2.1. The multiplex PCR was developed specifically and efficiently using amplified reactions and the same PCR program. The reaction was performed in a total volume of 25 µl that contained 5 to 15 µl (50 ng) of template.

[Food sample processing and multiplex PCR assay]

A sample of ham (CJ, Seoul, South Korea) from the Korea Food & Drug Administration was used for all tests. Equal concentration of the bacteria were used for inoculation of the ham. *E. coli* O157:H7, *Staphylococcus aureus*, *Listeria monocytogenes*, *V. parahaemolyticus* and *Salmonella typhimurium* were inoculated either single or as two or three species simultaneously. Media bottles (500 ml) containing 25 g of crushed ham were inoculated with bacteria at 100 CFU of each species alone or with 2 X 10³ CFU for inoculation of the three species together. inoculated ham was vigorously mixed by shaking for about 30sec to

distribute the bacteria. After inoculation, 225 ml of freshly made LB broth was added to each bottle containing ham. To suspend the bacteria, the bottles were shaken for 10 min at 200 rpm and then incubated at 37°C for 16 hours (Kim *et al.*, 2006). Raw pork was also processed as described method above.

The five bacterial species were inoculated simultaneously in raw pork. Water and milk were directly inoculated with five strains; 1 ml of medium containing each strain was added to 9 ml of water and milk and diluted 10 times from 1:10 to 1:10⁸.

2.1.2 Results and discussion

[Multiplex PCR assay]

Five PCR products of different sizes were amplified simultaneously from five food-borne pathogenic bacteria with the multiplex PCR assay used in this study (Fig. 2). For all of the bacteria tested, the optical density (absorbance value) at 600 nm was 0.010 and 0.080. The different sizes of the amplification products allowed rapid and specific discrimination of *Vibrio parahaemolyticus*, *Salmonella* spp., *Staphylococcus aureus*, *E. coli* O157:H7 and *L. monocytogenes*. The annealing temperature, extension time, and primer concentrations used in this multiplex PCR assay were optimized. The PCR products were separated by agarose gel electrophoresis, and the negative controls used with the multiplex PCR produced negative results. Using the multiplex primers, another single amplification was conducted to confirm the chromosomal DNA from samples contaminated with single specific pathogenic bacteria. In the multiplex PCR with mixed DNA samples, five different bands of specific sizes corresponding to the target genes (Table 2) were detected simultaneously after amplification of the contents of a single tube (Fig. 2).

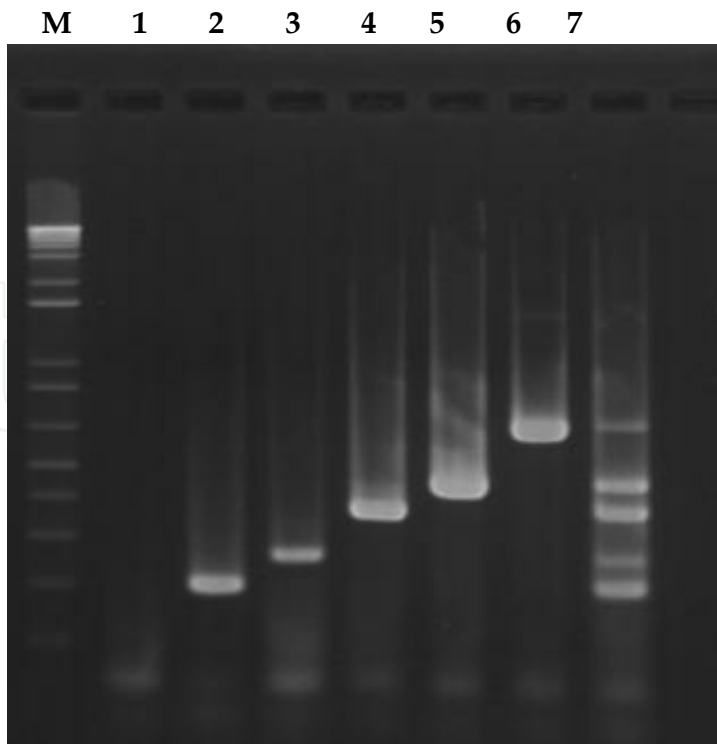


Fig. 5. Agarose gel electrophoresis showing the result of multiplex PCR amplification of five target gene segments from purified DNA of the five microbial pathogens

M, 100 bp size marker; lane 1, negative control (no template); lane 2, *E. coli* O157:H7 NVRQS; lane 3, *Staphylococcus aureus* KCTC1927; lane 4, *Vibrio parahaemolyticus* KCCM41654; lane 5, *Listeria monocytogenes* ATCC15313; lane 6, *Salmonella enteritidis* ATCC10376; lane 7, Multiplex PCR amplification of all five target genes.

[Specificity and sensitivity for selected primer sets]

The sensitivity and specificity of the PCR assay were evaluated with 67 food-borne pathogenic bacteria (Table 1). Fig. 3 shows the result of amplification from a representative sample of *Salmonella* spp. The multiplex primer is highly specific for the five pathogenic bacteria target sequence; all *Salmonella* serovars tested produced amplicons of the expected size (678 bp) without spurious priming and without cross-reactivity with non-*Salmonella* species. Results for the other four bacterial species also highly specific (data not shown). Fig. 4 illustrates the detection sensitivities of the multiplex PCR assay, which were evaluated using whole cell cultures of *S. choleraesuis* KCCM41035 and *S. bongori* KCCM41758, cell cultures diluted 10-fold from 1:10 to 1:10⁸ were tested. Based on these results, the multiplex PCR assay detection limits were approximately 10⁵ CFU / ml. Detection results for the other four bacteria with this assay were similar (data not shown).

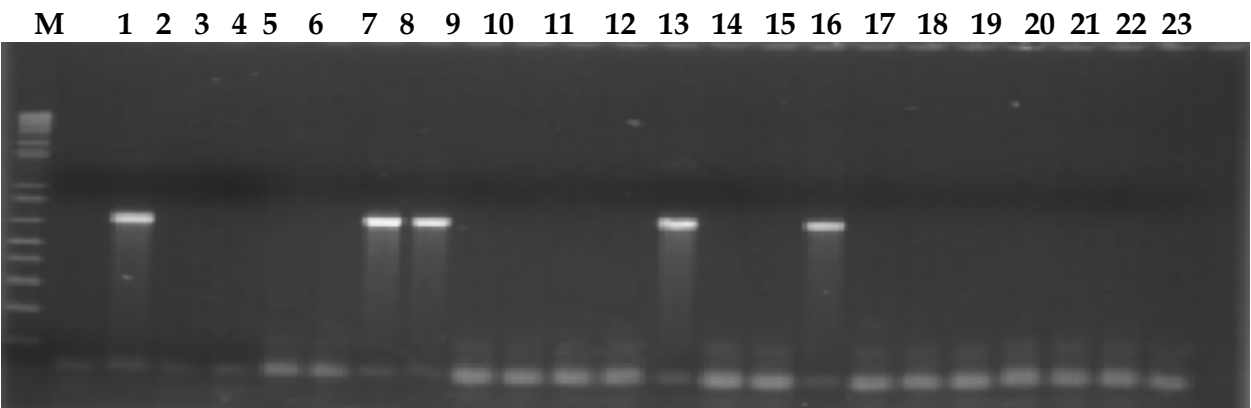


Fig. 6. Specificity for five food pathogenic bacteria using the multiplex primer sets for the detection of *Salmonella* spp.

M,100 bp size marker; lane 1, Negative control(no template); lane 2, *S. bongori* KCCM41757; lane 3, *B. subtilis* KCTC2213; lane 4, *B. cereus* KCTC1526; lane 5, *Listeria monocytogenes* ATCC15313; lane 6, *L. innocua* ATCC3091; lane 7, *S. enteritidis* ATCC13076; lane 8, *S. typhimurium* KCTC2421; lane 9, *Shigella boydii* ATCC12034; lane 10, *Shigella flexneri* ATCC12022; lane 11, *Shigella flexneri* KCTC2517; lane 12, *Shigella sonnei* KCTC2009; lane 13, *S. enteritidis* KCCM12021; lane 14, *Shigella sonnei* KCTC2518; lane 15, *Shigella sonnei* KCCM41282; lane 16, *S. choleraesuis* KCCM41035; lane 17, *Shigella sonnei* KCCM41282; lane 18, *Y. enterocolitica* KCCM41657; lane 19, *B. cereus* KCTC1661; lane 20, *B. lichniformis* KCTC3006; lane 21, *B. thuringiensis* KCTC1510; lane 22, *Citrobacter freundii* KCCM11931; lane 23, *Listeria murray* ATCC25402.

The non-autoclaved ham samples were representative of samples that would be collected from a commercial food processing environment. The detection limit for the five pathogens inoculated individually onto non-autoclaved ham was 2 CFU / ml after enrichment. For

non-autoclaved ham incubated with two or three organisms together, the sensitivity was the same as that achieved when the pathogens were evaluated singly (Fig. 5). Without enrichment of the bacterial culture, the detection limits after inoculation of non-autoclaved ham with *E. coli* O157:H7, *S. aureus* and *L. monocytogenes* together were 20,000 cells, respectively (data not shown).

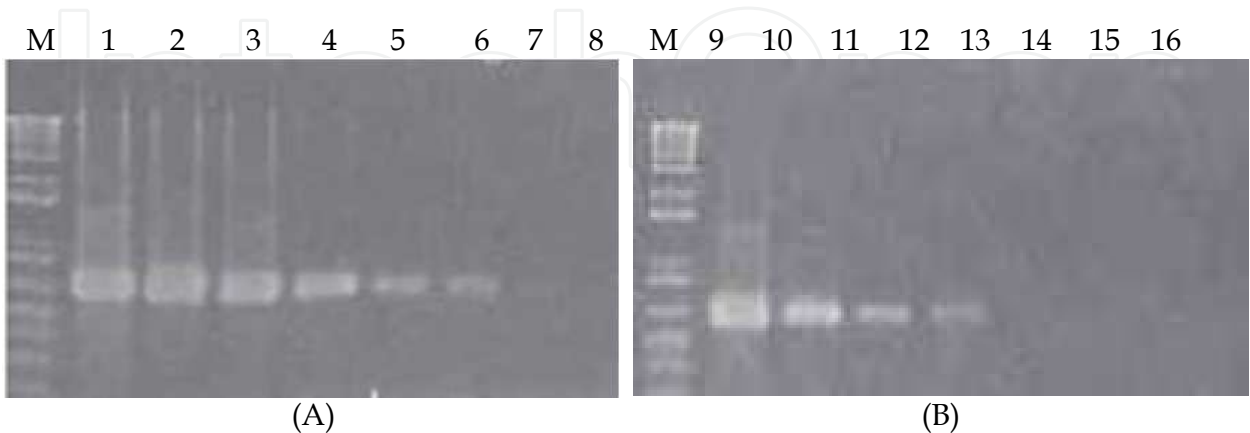


Fig. 7. Sensitivity of the multiplex PCR assay for *Salmonella choleraesuis* KCCM41035 (A) and *Salmonella bongori* KCCM41757 (B)

M, 100 bp size marker; lane 1, 1.2×10^8 CFU / ml; lane 2, 1.2×10^7 CFU / ml; lane 3, 1.2×10^6 CFU / ml; lane 4, 1.2×10^5 CFU / ml; lane 5, 1.2×10^4 CFU / ml; lane 6, 1.2×10^3 CFU / ml; lane 7, 1.2×10^2 CFU / ml; lane 8, 1.2×10 CFU / ml; lane 9, 1.2×10^8 CFU / ml; lane 10, 1.2×10^7 CFU / ml; lane 11, 1.2×10^6 CFU / ml; lane 12, 1.2×10^5 CFU / ml; lane 13, 1.2×10^4 CFU / ml; lane 14, 1.2×10^3 CFU / ml; lane 15, 1.2×10^2 CFU / ml; lane 16, 1.2×10 CFU / ml

[Validity of the multiplex PCR assay for food samples]

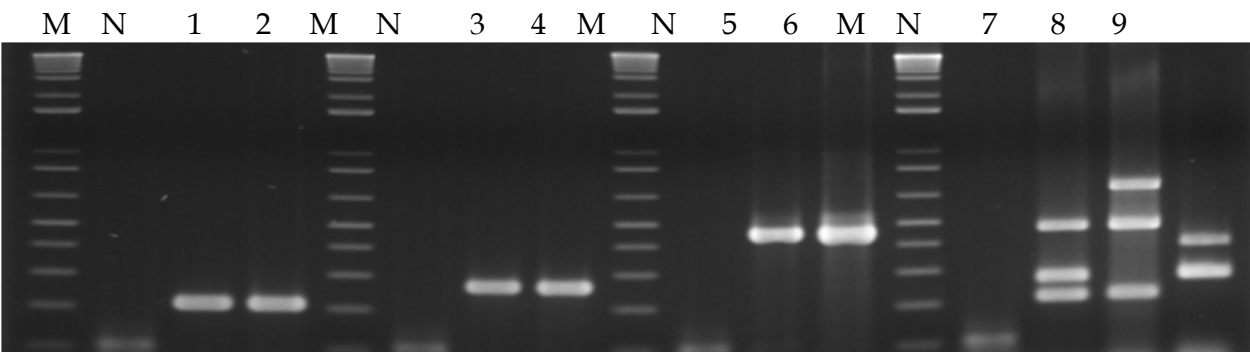


Fig. 8. Amplification products obtained with the multiplex PCR assay

M, 100 bp size marker; N, negative control; lane 1, PCR with *E. coli* O157:H7; lane 2, PCR with *E. coli* O157:H7 DNA (100 pg); lane 3, PCR with *S. aureus*; lane 4, PCR with *S. aureus* DNA (100 pg); lane 5, PCR with *L. monocytogenes*; lane 6, PCR with *L. monocytogenes* DNA (100 pg); lane 7, PCR with 100 pg DNA each from *E. coli* O157:H7, *S. aureus* and *L. monocytogenes*; lane 8, PCR with 100 pg DNA each from *E. coli* O157:H7, *L. monocytogenes* and *Salmonella typhimurium*; lane 9, PCR with 100 pg DNA each from *S. aureus* and *V. parahaemolyticus*

This multiplex PCR assay offers the advantages of significantly short processing time and saving cost. Only one composite DNA sample is required rather than separate samples for each target gene to be analyzed (Kim *et al.*, 2006). To test the efficacy of this PCR assay for detecting pathogenic bacteria in food, *Salmonella typhimurium* ATCC19585 (10 CFU / g of food) was inoculated into samples of selected foods (milk, raw pork and raw chicken) that had been previously screened for detectable pathogenic microbial contamination. The inoculated samples were then incubated aerobically at 37°C for 8 hours (enrichment culture step). The PCR assay detected bacterial cells in all inoculated samples. However, when a 5 hours instead of 8 hours enrichment culture step was used, no bacteria were detected (data not shown).

Thus, our PCR assay requires at least 8 hours of enrichment to detect the added pathogenic bacteria in these foods with a detection sensitivity ranging from 10 to 100 CFU / g. Therefore, the enrichment step is required in this PCR protocol for detection of food-borne pathogenic bacteria. The five specific primer sets tested for *Vibrio parahaemolyticus*, *Salmonella* spp., *Staphylococcus aureus*, *E. coli* O157:H7 and *L. monocytogenes* can be used specifically and simultaneously. These five food pathogens were clearly detected from both culture medium artificially inoculated water, milk and raw pork. Thus, the protocol developed in this study could have important application for the rapid and simultaneous detection and identification of up to five food-borne pathogenic bacteria in many foods. This simple method is expected to enable rapid risk assessment of pathogen contamination of foods at a low cost. The cost of detection could be reduced from the \$ 50 (approximately \$ 10 per pathogen) for the traditional method to less than \$ 1 for this multiplex PCR method.

3. The development of rapid real-time PCR detection system for *Salmonella* spp.

Previously, we developed multiplex conventional PCR assay from the conventional PCR methods (Kim *et al.*, 2007). Conventional PCR methods for the detection of food-borne bacterial pathogens are time consuming and insensitive that it can not provide adequate screening of samples for the presence of potential pathogens. With the advent of automated real-time PCR suspected food-borne contaminants can be detected in less than an hour. This technique, using TaqMan PCR, has been successfully adapted for the detection of pathogenic bacteria, including *Salmonella enterica*, *Listeria monocytogenes*, *Escherichia coli* O157:H7, and *Yersinia pestis* (Bassler *et al.*, 1995; Bellin *et al.*, 2001; Higgins *et al.*, 1998; Hoorfar *et al.*, 2000; Jothikumar *et al.*, 2002; Knutsson *et al.*, 2002; Oberst *et al.*, 1998; Sharma *et al.*, 1999).

Can there be a better method which has the same sensitivity with nested PCR and can be performed with one PCR reaction? It will be more effective if there is more sensitive optical instrument and staining dye which can detect very small amount of product than naked eyes and EtBr. Micro PCR, which was developed for this purpose, uses real-time PCR machine as a detector and SYBR Green reagent as a staining dye. Real-Time PCR is currently used for the diagnosis of *Escherichia coli* strain O157:H7 (Ibekwe *et al.*, 2002) and *Plesiomonas shigelloides* (Loh *et al.*, 2001) in stool specimens. To develop micro PCR, following factors were studied. First, selection of specific primers; primer size (17~25 mer), hybridization ability, secondary structure within primer, GC content (40~60%), melting temperature (T_m) (55~65°C). Second, factors affecting T_m ; product size, GC contents of product. Third, effect of commercial SYBR Green reagent; Takara, A&B, Qiagen and in house reagent. Forth,

product size; 60, 100, 200, 300, 400, 500, 600 and 700bp Fifth, condition of PCR. To enable simultaneous detection, each PCR products were designed to have different melting temperature, at least 2°C apart from each other (Fig. 1).

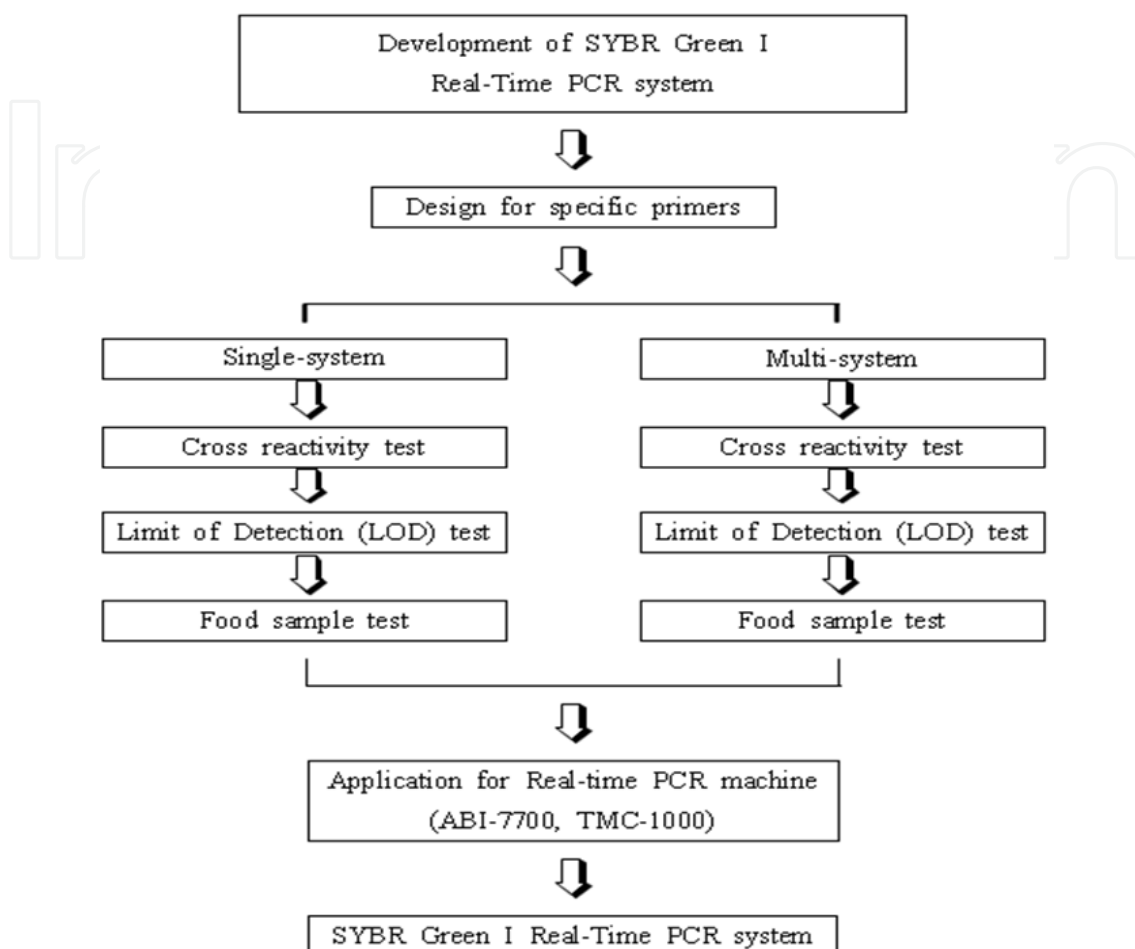


Fig. 9. Strategy for the development of SYBR Green I real-time PCR detection system.

3.1 Materials & methods

[Oligonucleotides]

The oligonucleotide primers were designed by Primer version 3.0 software (Whitehead Institute), referring to Genbank in order to amplify a chromosomal DNA of *Salmonella* spp. (Table 1). The oligonucleotides and all reagents for PCR used in this study were synthesized and purchased from Bioneer (Daejeon, Korea) and Kogene Biotech Inc. (Seoul, Korea).

50 ng of template DNA was used in a 20 µl reaction mixture that included 2X SYBR Green I premix Ex Taq (Takara, Japan), 1X ROX Dye (Takara, Japan), 20 pmol of forward and reverse specific primer (Bioneer, Korea). Cycling conditions began with an initial hold at 94°C for 5 min, followed by 35 cycles consisting of 94°C for 30 s, 60°C for 30 s and 72°C for 30 s. and final extension time carry out 72°C for 5 min. Following amplification, melting curves were acquired on the SYBR channel using a ramping rate of 1°C / 30 s for 60 ~ 94°C. The differentiated data were analysed by 7500 software V1.3.0. with the digital filter set as none.

Strains	Direction (5' → 3')	Primer sequence	Size (bp)
Salmonella spp.	Forward	GAA TCC TCA GTT TTT CAA CGT TTC	
	Reverse	CCA GAC GAA AGA GCG TGG TAA	60
		GAA GCC CGA ACG TGG CGA	137
		GTA TGC CCG GTA AAC AGA TGA GT	284
		AAA GGA ACC GTA AAG CTG GCT	330
		GGG TCA TCC CCA CCG AAA TAC	424

Table 3. Oligonucleotide primers for *Salmonella* spp. used in this study

[SYBR Green I PCR assay using ABI 7500]

[Detection studies with diversity food samples]

Before inoculating into food, five bacterial strains were incubated in 5 ml LB broth for overnight. 100 µl of each culture broth was inoculated in 25 g of each food material. These food samples were then mixed with 225 ml of LB broth and incubated for overnight. Food segments in sample solution must be removed before assay because PCR can be inhibited by them. Among the 10 samples, water contaminated with *Salmonella* spp. were analyzed without any pre-treatment, other samples were filtered through gauze before assay. 1 ml of each prepared sample solution was transferred to 1.5 ml e-tube and centrifuged at 12,000 rpm for 10 min. The supernatant was removed and the pellet was re-suspended in 500 µl deionized water. Centrifugation and re-suspension in deionized water was performed one more time for exact assay. 150 µl of Deionized water and 50 µl of 10% chelex resin was added to the pellet and mixed thoroughly. The solution was heated at 100°C for 10 min, and centrifuged at 12,000 rpm for 10 min. 5 µl aliquot of this solution was taken for SYBR Green I assay. PCR using ABI 7500 (Perkin-elmer, USA) was cycled 35 times as follows: 30 sec denaturation at 94°C, 30 seconds annealing at 60°C, and 30 seconds polymerization at 72°C. The products of real-time PCR were run on 2% agarose gel electrophoresis and melting curves were acquired on the SYBR channel using a ramping rate of 1°C / 30 seconds for 60 ~ 94°C.

3.2 Results and discussion

[Primers design and specificity]

Fig. 2 is the result of PCR reaction performed with various primer sets which are designed for *Salmonella* spp. Specific PCR reaction was observed with primer sets of which product sizes are 60 bp, 284 bp and 678 bp, respectively. However, primer sets of 137 bp, 330 bp and 551 bp showed non-specific products in the place of negative control, meaning that these primers are not available. As for the primers of 424 bp, bacterial DNA was not amplified. Therefore, primer sets of 60 bp, 284 bp, and 678 bp were selected.

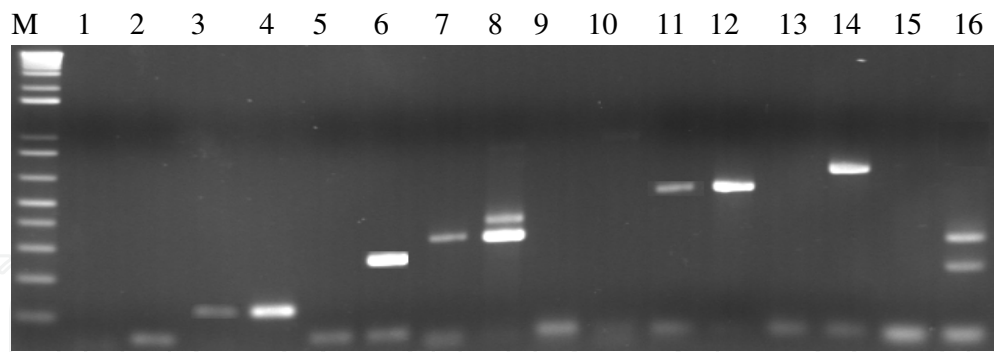


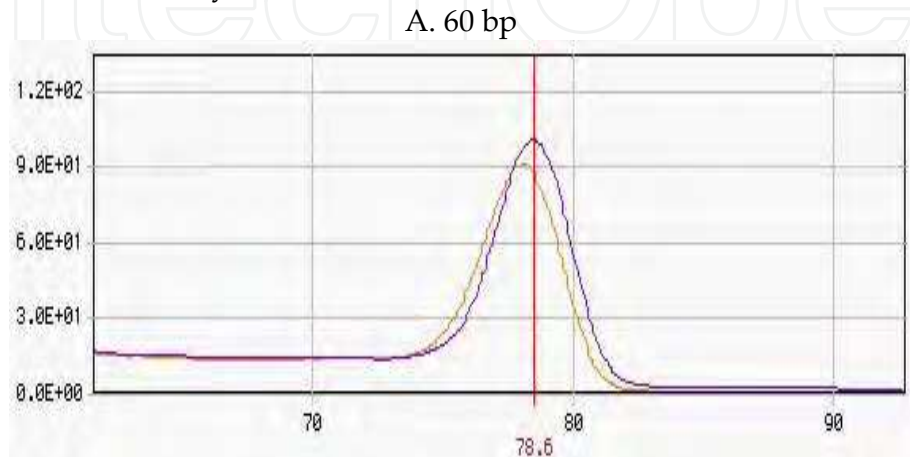
Fig. 10. PCR amplification of *Salmonella* spp. using each primer set

M, 100 bp DNA ladder; lane 1, 2, 60 bp primer; lane 3, 4, 137 bp primer; lane 5, 6, 284 bp primer; lane 7, 8, 330 bp primer; lane 9, 10, 424 bp primer; lane 11, 12, 551 bp primer; lane 13, 14, 678 bp primer; lane 15, 16, 787 bp primer; odd lane number, negative control; even lane number, positive control.

[Real-time PCR system]

It will be more effective if there is more sensitive optical instrument and staining dye which can detect very small amount of product than naked eyes and EtBr. Micro PCR, which was developed for this purpose, uses real-time ABI 7500 PCR machine as a detector and SYBR Green I reagent as a staining dye. First, selection of specific primers; primer size (17~25 mer), hybridization ability, secondary structure within primer, GC contents (40~60%), melting temperature (T_m) (55~65°C). Second, factors affecting T_m ; product size, GC content of product. Third, effect of commercial SYBR green reagent; Takara, A&B, Qiagen and in house reagent. Fourth, products size; approximately 60, 100, 200, 300, 400, 500, 600 and 700 bp. Fifth, running conditions of real-time PCR. to enable simultaneous detection, each PCR products were designed to have different melting temperature, at least 2°C apart from each other.

As shown in Fig. 3 primer of 60 bp showed two peaks of positive and negative control at the same position. Primer of 284 bp did not showed non-specific products at all but it was also unavailable because the peak of positive control was too weak. The T_m value was 86.8°C. Only the primer of 678 bp was proved to be available. Although a weak undesired peak was appeared beneath 75°C, it is ignorable because its temperature is sufficiently low. The T_m value was measured as 86.7°C. Therefore, the primer of 678 bp is finally selected for SYBR Green I system.



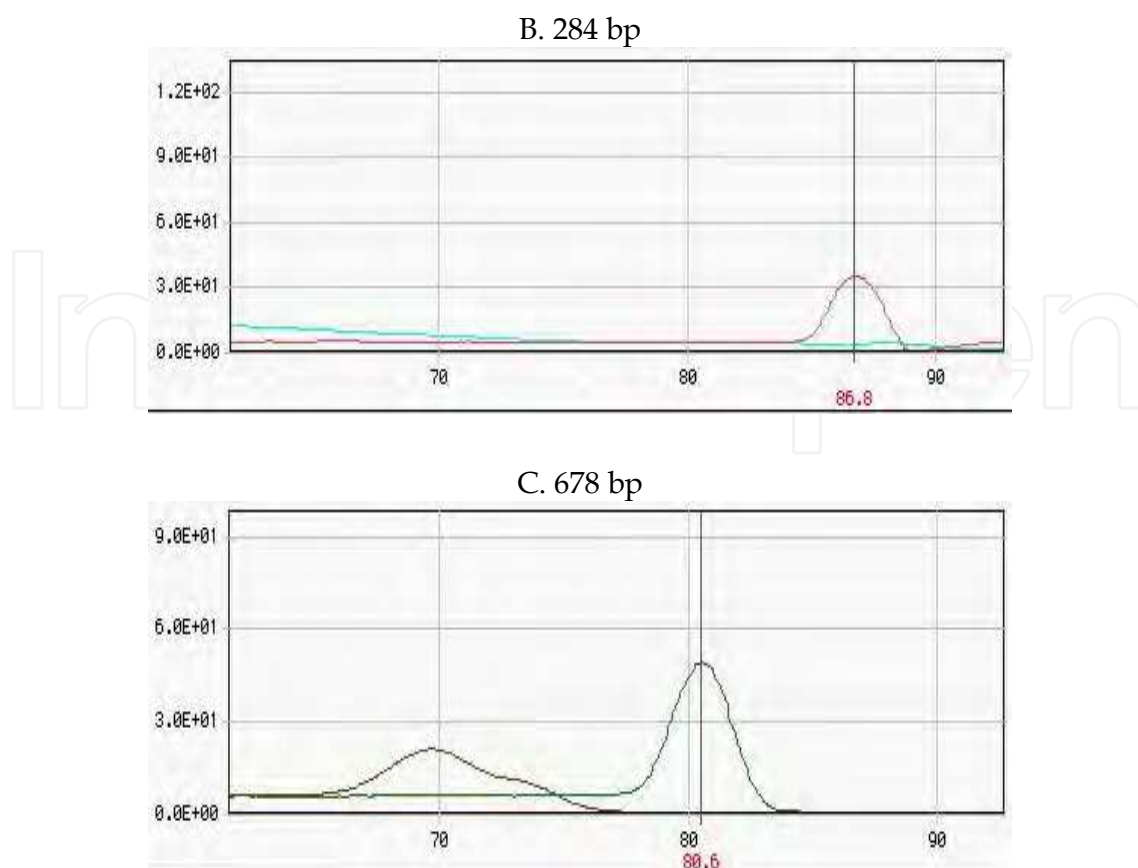


Fig. 11. Melting curve analysis for *Salmonella* spp. A ~ C : melting curve of 60, 284, 678 bp primer sets

[Application of Real-time SYBR Green I PCR system to food]

To apply real-time SYBR Green I system to food, various food samples were artificially contaminated by the *Salmonella* and incubated for enrichment. After overnight enrichment, *Salmonella* spp. gave positive PCR reaction. No signals were observed in negative (un-inoculated) controls. As shown in this result, no other strains but the inoculated *Salmonella enteritidis* was detected (Fig. 4). In conclusion, the SYBR Green I PCR assay combined with DNA extraction using boiling method offers rapid and non-sequence-specific detection of amplicons.

M, 100 bp ladder; N, negative control; lane 1, hamburger patty contaminated with *Escherichia coli* O157:H7; lane 2, ground poultry with *Escherichia coli* O157:H7; lane 3, soondae (a sausage made of bean curd and green-bean sprouts stuffed in pig intestine) with *Staphylococcus aureus*; lane 4, kimbob (rice rolled in dried laver) with *Staphylococcus aureus*; lane 5, sea water with *Vibrio parahaemolyticus*; lane 6, shrimp with *Vibrio parahaemolyticus*; lane 7, salad with *Listeria monocytogenes*; lane 8, ice-cream with *Listeria monocytogenes*; lane 9, frozen chicken with *Salmonella enteritidis*; lane 10, salad with *Salmonella enteritidis*; lane 11, Soybean paste with *B. cereus*; lane 12, korean red pepper paste with *B. cereus*; lane 13, bottled water with *Yersinia enterocolitica*; lane 14, milk with *Yersinia enterocolitica*; lane 15, spring water with *Shigella* spp.; lane 16, oyster with *Shigella* spp.; P, Positive control (100 pg)

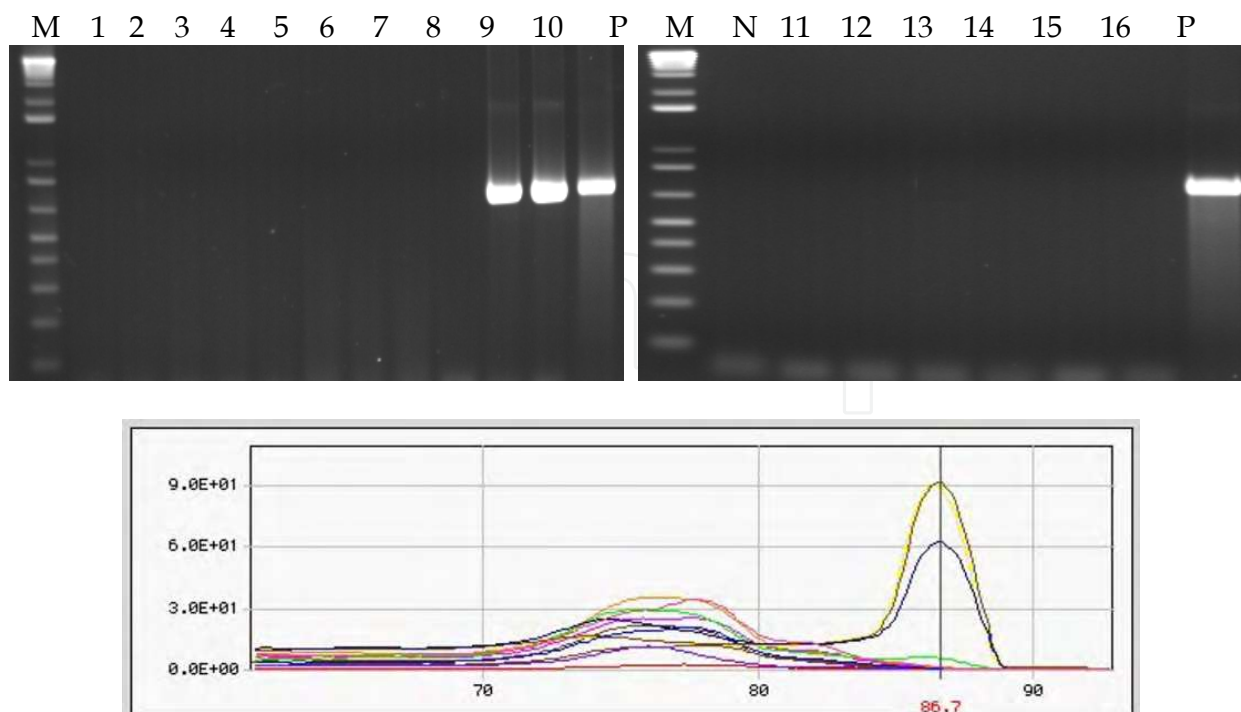


Fig. 12. Detection specificity using the *Salmonella* spp. 674 bp primer in contaminated variety food samples

The minimum detection limit was 10 cells / ml with pure culture, which is far more sensitive than conventional PCR which has detection limit of 10,000 cells / ml. In conclusion, we developed a highly sensitive and specific real-time PCR assay for detection of the five food-borne pathogenic bacteria in food samples. This newly developed assay was successfully used to monitor the dynamics of this novel bacterium in food (Abu *et al*, 2005).

4. Rapid and simple detection of *invA* gene in *Salmonella* spp. by isothermal target probe amplification (iTPA)

Nucleic acid amplification methods are widely used for detection of food-borne pathogens and the PCR is the most popular and useful method, requires a high precision thermal cycling instrument, which often prevents PCR from being used in routine food pathogen detection by the food industry. Recently, Jung *et al.* (Jung *et al.* 2010) developed a new highly sensitive and specific isothermal amplification and detection system called isothermal target and probe amplification (iTPA) by employing DNA-RNA-DNA chimeric primers and a FRET probe. The iTPA reaction is done under isothermal conditions between 55 and 65°C using a simple incubator such as a water bath or block heater which is sufficient for amplification. The detection of the fluorescence signal is acquired directly from the amplification reaction tube without any post-amplification handling that reduces the risk of any amplicon-carryover cross-contamination. The iTPA assay is highly specific for the target sequence because the primers and probe recognize five distinct regions on the targeted DNA. The iTPA method is based on a combination of novel ICA (isothermal chain amplification) and FRET cycling probe technology (CPT). In the ICA method, which relies on the strand displacement activity of DNA polymerase and the RNA degrading activity of

RNase H, two displacement events occur in the presence of four specially designed primers that lead to powerful amplification of target DNA. Since the amplification is initiated only after hybridization of the four primers, the ICA method leads to high specificity for the target sequence. In the CPT method, a DNA-RNA-DNA chimeric probe is hybridized with the target DNA, and the RNA region of the duplex is specifically cleaved by RNase H. The cleaved probe fragments are disassociated from the target DNA and another intact probe is again hybridized and then cleaved. In the cycling events, a single target DNA molecule results in a large number of cleaved probe fragments, which can be designed to generate fluorescent signals (Fig. 1). In the present study, a sensitive and specific iTPA assay for detecting *Salmonella* spp. in experimentally inoculated food samples was developed.

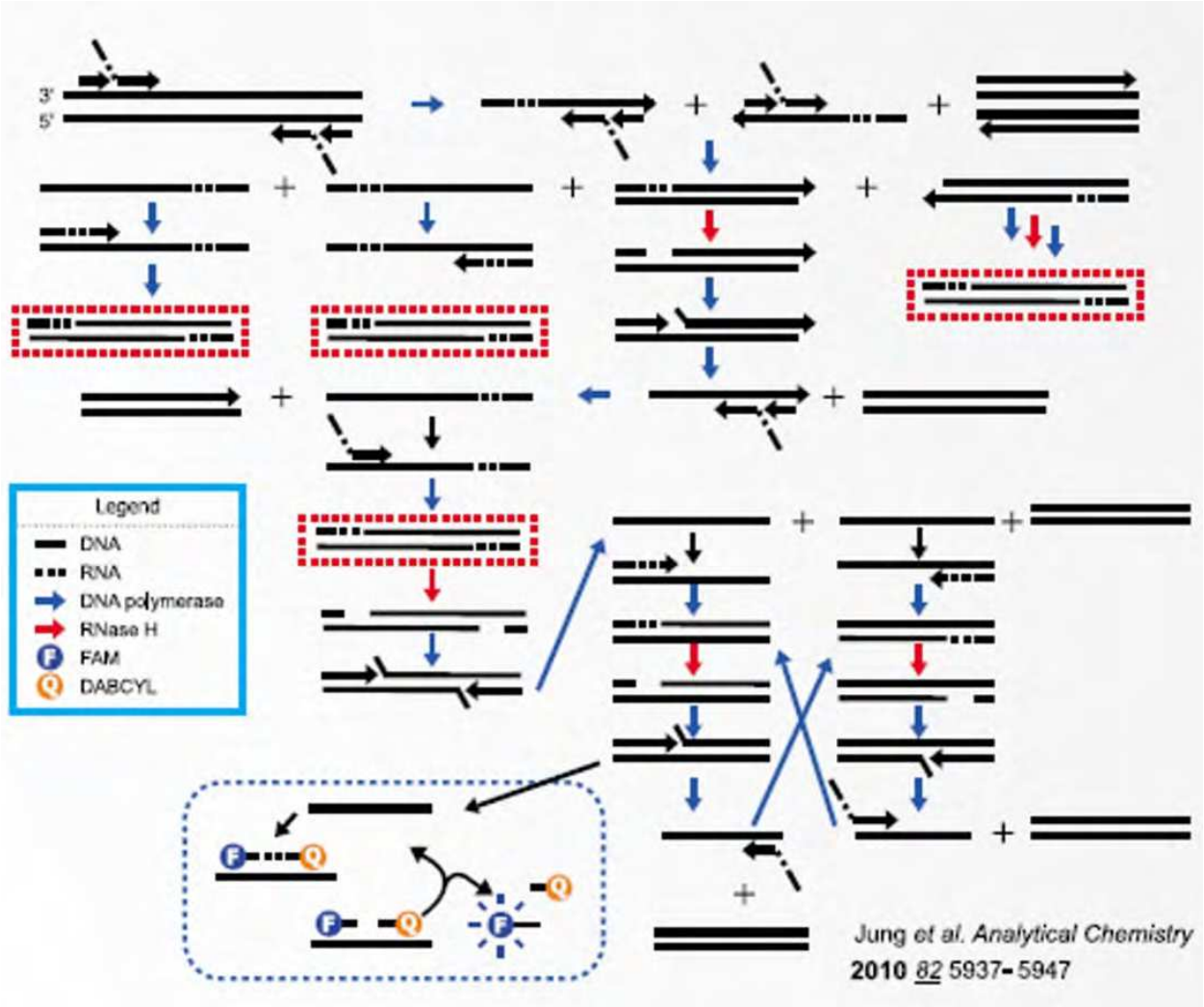


Fig. 13. The process for the Isothermal target & Probe amplification

4.1 Materials & methods

[iTPA primers, FRET probe, and reaction conditions]

The *Salmonella invA* gene (GenBank: EU348369) was used as the target for iTPA primer and probe design. Four primers, two outer and two inner, and one FRET probe which

recognized five distinct regions of the target sequence were designed using the DNASTAR software (Maison, WI). Oligonucleotide sequences and locations of the primers and the probe are shown in Table 1. The DNA primers were synthesized by Genotech (Daejeon, South Korea). The chimeric primers and a FRET probe were synthesized by IDT (San Diego, CA). The iTPA reaction mix in a 20 μ l volume consisted of the following: 10 mmol / L of Tris-HCl (pH 8.5), 22 mmol / L of MgSO₄, 10 mmol / L of KCl, 10 mmol / L of (NH₄)₂SO₄, 0.05 mg ml / L of acetylated BSA, 3 mmol / L of DTT, 0.4 mmol / L of deoxynucleotide triphosphate (dNTP), 0.22 mol / L of each outer primers, 2.2 mol / L of each inner primers, 100 nmol / L of the FRET probe, 5 units of *bst* polymerase (NEB, Ipswich, MA), 5 units of RNase H (Epicentre, Madison, WI), 6 units of RNase inhibitor (Solgent, South Korea), and 2 μ l of DNA template (2 μ l of sterilized water was used for a negative control). The iTPA reaction mix was incubated at 58°C for 60 min in a water-bath and then cooled to room temperature. After a quick spin-down, the reaction tube was inserted into a RF-1000 fluorescent reader (Raplegene, Inc., Sungnam, South Korea) to read the relative RFU (fluorescence relative unit) signal. The RF-1000 fluorescent reader calculates the F-score and it is display on the LCD window. The result was interpreted as a *Salmonella* spp. positive if the F-score was ≥ 35 or a *Salmonella* spp. negative if the F-score was < 35 . This cut-off value was determined using uninoculated food samples that had also undergone cultural pre-enrichment. F-scores of uninoculated egg yolk samples and chicken meat samples were 20 ± 9.5 and 20 ± 7.2 respectively ($p\leq 0.001$). The equation used to calculate the F-score is the following:
F-score = [(fluorescence of the sample – fluorescence of the negative control) / fluorescence of the negative control] $\times 100$. Sterilized water was used for the negative control reaction instead of the extracted nucleic acid from inoculated food samples. Since uninoculated food samples may be contaminated, we used sterile water as the negative control.

Name	Sequence (5'-3')	Position#
Outer forward	CCT GAT CGC ACT GAA TAT C	99-117
Outer reverse	CGA AAG AGC GTG GTA ATT AAC	195-215
Inner forward	CGA TGA CTG ACT ATA CAA GrUrA rCrGC TGG CGA TAT TGG TGT TTA TG	121-131
Inner reverse	CTA GTA CAT GAA GCT rArArA rGAC CGC AGG	174-191
FRET probe	AAA CGT TGA A FAM-CGT TCT ACA TTrG rArCrA rGrAA TCC TCA G- DABCYL	146-170

Table 4. iTPA primers and FRET probe used in this study to detect Salmonella spp.

[iTPA specificity and detection limits]

Fifty bacterial strains were used to determine the iTPA specificity. DNA templates were prepared from bacterial cultures and aliquots were subjected to the iTPA assay. For *Salmonella* strains, the genomic DNA was isolated from the overnight culture grown in LB

medium and then quantified. One picogram of genomic DNA was used as the template. For non-*Salmonella* strains, the genomic DNA was isolated from the overnight culture grown in LB medium and then 2 L of the DNA extraction TE buffer solution was used. Specificity tests were repeated 10 times. To determine iTPA detection limits, serial 10-fold dilutions of a mid-log phase *S. typhimurium* KCTC2515 culture (ca. 10^8 CFU) grown in LB broth were prepared in PBS and quantified using the standard plating method. The detection limit tests were repeated 10 times and the lower limits of detection (CFU per assay) were reported.

[iTPA testing in experimentally inoculated food samples]

Three kinds of foods were used for the study: peanut butters, egg yolk and chicken breasts. Food samples were processed as described in a previous study (Kim *et al.* 2007) with slight modifications. Briefly, A 500 ml Erlenmeyer flask (LB broth 225 ml) containing 25 g of chicken breast was incubated at 37°C overnight and then 9 ml of this solution was transferred into a 10 ml conical tube followed by adding 1 ml of inoculated buffered peptone water of *Salmonella* spp. (1.0×10^9 CFU / ml) to prepare a stock solution. Plastic food bags containing 25 g of chicken breast were inoculated with 1 ml of serial dilutions (1:10 to 1:10⁸) of the stock solution and vigorously mixed using a homogenizer (Pro-media SH-001, ELMEX Ltd., Tokyo, Japan) for about 30 sec to distribute the bacteria followed by adding 225 ml of freshly made LB broth to prepare pre-enriched solutions. The sample preparations for peanut butters and other food samples were the same except that for peanut butters which required an additional washing with a washing solution (0.05% NaOH, 0.5% Tween 20 in PBS buffer solution) due to the high viscosity. 100 L of the pre-enriched solution was mixed with the washing solution and centrifuged at $10,770 \times g$ for 5 min followed by discarding the supernatant. The pellet was washed with 100% ethanol and then with TE buffer solution twice. The washed pellet was suspended in 200 L of TE buffer solution and heated at 100°C for 10 min in a dry heating block. The crude cell lysate was centrifuged at $10,770 \times g$ for 5 min and an aliquot (2 L) of the supernatant was used for the iTPA assay. For negative samples, the same amount of aliquot (2 L) of uninoculated food samples that had also undergone cultural pre-enrichment was used. The inoculated food sample tests were repeated 10 times and the lower limits of detection (CFU per assay) were reported.

4.2 Results and discussion

[Inclusivity and exclusivity of the iTPA assay]

The *Salmonella* spp. *invA*-based iTPA assay, which required only a water bath and the RF-1000 fluorescent reader successfully detected 10 *Salmonella* spp. strains while showing negative results for 40 non-*Salmonella* spp. strains (Table 1), indicating that the *invA*-based iTPA assay was specific for *Salmonella* spp.. The PCR assay using iTPA outer primers yielded amplicons of the expected size (117 bp) for all 10 *Salmonella* spp. strains. (data not shown) Two sample t-tests were performed for negatives and positives in pure culture. The mean F-score of the negatives was 3.97 ± 0.44 and the mean F-score of the positives was 82.9 ± 6.1 ($p \leq 0.001$, data not shown). For a rigorous exclusivity comparison, the positive strains were used at a low concentration of the genomic DNA (1pg, ca. 10^2 CFU) as the template while the negative strains were used at very high concentration of the genomic DNA (ca. 10^5 CFU). Neither false positive nor false negative results for the 50 bacterial

strains were observed by the iTPA assay using two primer sets and a FRET probe, indicating good specificity. (Table 1)

[Detection limits of the iTPA assay]

The detection limits of the iTPA assay using serial in *S. Typhimurium* strain were determined. KCTC2515 were determined and the lowest number of cells detected was 4 x 10¹ CFU per iTPA reaction (Table 2).

	Dilution	No. of Bacteria (CFU)	iTPA reaction (F-score#)
<i>S. Typhimurium</i> KCTC2515	10 ⁻⁴	4 x 10 ⁴	142±9
	10 ⁻⁵	4 x 10 ³	96±30
	10 ⁻⁶	4 x 10 ²	82±35
	10 ⁻⁷	4 x 10 ¹	69±18
	10 ⁻⁸	4	0

F-score = [(fluorescence of the sample – fluorescence of the negative control) / fluorescence of the negative control] x 100

Table 5. Sensitivity of the iTPA assays

[Detection of *Salmonella* spp. cells in inoculated food samples]

The detection limits of *Salmonella* spp. inoculated in three food samples are shown in Table 3. In inoculation experiments, the *invA*-based iTPA assay using the serial dilution platforms consistently detected at an initial inoculums level of less than 10 CFU in the pre-enriched food samples (egg yolk, chicken breast, and peanut butter). In Table 4 the results of the F-score measurement for the iTPA reaction for 60 min at 58°C in the artificially contaminated samples are shown. For statistics, one-way analysis of variance (ANOVA) test was performed (Clarke and Cooke 1998). The mean F-scores for uninoculated peanut butter, egg yolk and chicken breast were 14±4.7, 20±10, and 20±7.2, respectively. The mean F-scores for inoculated peanut butter, egg yolk and chicken breast were 87.34±30.24, 59.09±36.16 and 68.24±26.33(p≤0.001), respectively. The lowest detection limit achieved in this study was a less than 10 CFU per 25 g of food samples.

In this study, we designed a set of DNA-RNA-DNA chimeric primers and a FRET probe to specifically target the *Salmonella* spp. *invA* gene. So, a novel and rapid DNA detection system has been developed which we have termed isothermal target and probe amplification (iTPA). By simultaneously utilizing the dual amplification powers of the target DNA and FRET probe, we have demonstrated that iTPA can be used to rapidly detect less than 10 CFU of *Salmonella* spp. in food samples after pre-enrichment. The four chimeric primers and one FRET probe were designed from five regions of the *Salmonella* spp. *invA* gene coding sequence that are highly specific for *Salmonella* spp. (Table 1). In conclusion, we have developed a DNA detection system which is conveniently performed by requiring only a water bath and a fluorometer and has great potential in applications for hand-held or point-of-care-testing (POCT) diagnostics. The *invA*-based iTPA assay developed in this study is a specific, sensitive, and rapid method for the detection of *Salmonella* spp. in food

samples. This simple method is expected to enable a rapid risk assessment of pathogen contamination of foods at a low cost. (Kim *et al.* 2011)

Food samples	Dilution	No. of Bacteria#(CFU)	F-score
Peanut butter	10 ⁻⁵	1.7±0.4 x 10 ³	87±5.7
	10 ⁻⁶	1.7±0.4 x 10 ²	86±6.6
	10 ⁻⁷	1.7±0.4 x 10 ¹	82±12
	10 ⁻⁸	1.7±0.4 x 10 ⁰	82±13
	10 ⁻⁹	1.7±0.4 x 10 ⁻¹	39±40
		un-inoculated	14±4.7
Egg yolk	10 ⁻⁵	1.7±0.4 x 10 ³	72±4.0
	10 ⁻⁶	1.7±0.4 x 10 ²	73±4.0
	10 ⁻⁷	1.7±0.4 x 10 ¹	69±7.3
	10 ⁻⁸	1.7±0.4 x 10 ⁰	44±3.4
	10 ⁻⁹	1.7±0.4 x 10 ⁻¹	11±26
		un-inoculated	20±10
Chicken meat	10 ⁻⁵	1.7±0.4 x 10 ³	72±3.6
	10 ⁻⁶	1.7±0.4 x 10 ²	72±2.3
	10 ⁻⁷	1.7±0.4 x 10 ¹	71±4.8
	10 ⁻⁸	1.7±0.4 x 10 ⁰	56±19
	10 ⁻⁹	1.7±0.4 x 10 ⁻¹	34±29
		un-inoculated	20±7.2

Seven *Salmonella* spp. strains were tested: *S. typhimurium* (KCTC 2515, KCTC 2412, ATCC 14028), *S. choleraesuis* (KCTC 2929, KCCM 41575), *S. enetritidis* (KCCM 12021), *S. bongori* (KCCM 41758)

Table 6. Detection limits for inoculated food samples by iTPA assay

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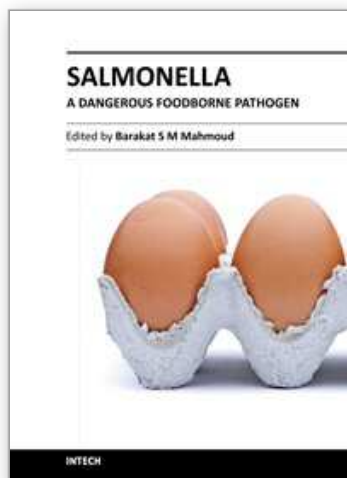
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Salmonella - A Dangerous Foodborne Pathogen

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More than 2,500 serotypes of Salmonella exist. However, only some of these serotypes have been frequently associated with food-borne illnesses. Salmonella is the second most dominant bacterial cause of food-borne gastroenteritis worldwide. Often, most people who suffer from Salmonella infections have temporary gastroenteritis, which usually does not require treatment. However, when infection becomes invasive, antimicrobial treatment is mandatory. Symptoms generally occur 8 to 72 hours after ingestion of the pathogen and can last 3 to 5 days. Children, the elderly, and immunocompromised individuals are the most susceptible to salmonellosis infections. The annual economic cost due to food-borne Salmonella infections in the United States alone is estimated at \$2.4 billion, with an estimated 1.4 million cases of salmonellosis and more than 500 deaths annually. This book contains nineteen chapters which cover a range of different topics, such as the role of foods in Salmonella infections, food-borne outbreaks caused by Salmonella, biofilm formation, antimicrobial drug resistance of Salmonella isolates, methods for controlling Salmonella in food, and Salmonella isolation and identification methods.

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