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Detection of *Salmonella* in Chicken Meat by Insulated Isothermal PCR

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ABSTRACT

Consumption of *Salmonella*-contaminated foods, such as poultry and fresh eggs, is known to be one of the main causes of salmonellosis. Conventional PCR methods, including real-time PCR for rapid detection of *Salmonella,* in general require skilled technicians and costly instruments. A recently developed novel convective PCR, insulated isothermal PCR (iiPCR), is carried out in polycarbonate capillary tubes. In this study, we designed TaqMan probes and PCR primers based on the *yrfH* gene encoding a heat shock protein for the iiPCR detection of *Salmonella* in chicken meat samples. The TaqMan probe was labeled with 6-carboxyfluorescein and 6-carboxytetramethylrhodamine at the 5′ and 3′ ends, respectively. The PCR amplicon was 133 bp. A typical run of this iiPCR assay was completed within 1 h. Specific PCR products were obtained for 148 strains representing 49 serotypes of *Salmonella* tested. Under the same conditions, false-positive results were not obtained for 98 non-*Salmonella* strains tested, including strains of *Enterobacteriaceae* closely related to *Salmonella*. For chicken meat samples, with a 5-h enrichment step *Salmonella* at as low as 10^0 CFU/g of poultry meat could be detected. Because the amplification signals from the probes are detectable at 520 nm, identification of the PCR products by gel electrophoresis is not required. Compared with conventional PCR, the iiPCR system requires less expertise and provides an economical, reliable, and rapid tool for result interpretation. Detection results can be obtained within 8 h, including the enrichment and DNA extraction steps.

Salmonella is a gram-negative facultative anaerobic pathogen that can cause serious gastrointestinal illness in humans and animals *(15)*. In many countries, *Salmonella* is one of the leading causative agents of foodborne infections. In the European Union, salmonellosis is one of the most common foodborne diseases *(13)*. Consumption of *Salmonella* from contaminated meat, eggs, and poultry has long been recognized as the primary cause of salmonellosis *(27)*. In 2011, an outbreak of human *Salmonella* infection in the United States was linked to ground turkey and ultimately led to the recall of 3.6×10^7 lb (1.6×10^7 kg) of ground turkey products *(2)*.

Because of the health risks and potential economic losses posed by *Salmonella* infection, detection of this pathogen in food products remains an important issue for the food industry and for public health. Conventional methods currently used for the detection of *Salmonella* are based on nonselective preenrichment culture followed by selective enrichment and subsequent culture on differential media and are usually labor intensive and time-consuming. In recent years, molecular techniques based on DNA analysis have been developed as rapid, sensitive, and highly specific tools for the detection of food pathogens. Various molecular methods, including PCR, real-time PCR, DNA probes, biochips, and loop-mediated isothermal amplification (LAMP), have been developed for the detection of *Salmonella (5, 11, 31)*. However, these methods often require qualified laboratory personnel and costly instruments, which may limit their practical application in the food industry. An affordable, user-friendly, and rapid diagnostic method is needed.

In this study, we attempted to develop such a method based on the principle of Rayleigh-Bénard convection, which is a common, naturally occurring physical phenomenon that occurs as a fluid flow is driven by buoyancy when a fluid layer is heated from below *(14)*. In Rayleigh-Bénard convective PCR, heating of the reaction vessel from the bottom results in temperature gradients due to the fluid convection of the solution. Consequently, reaction ingredients are circulated through zones of temperature gradients. Krishnan et al. *(17)* developed the Rayleigh-Bénard convective PCR using a simple design that can circulate the reaction components through sequential temperature zones to carry out each stage of the PCR. To improve performance of the Rayleigh-Bénard convective PCR, a

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capillary tube has been used as the reaction chamber *(9),* and a thermally baffled device has been designed to offer highly stabilized convective PCR conditions *(3).* Recently, a low-cost convective PCR performed in capillary tubes under insulated isothermal conditions (insulated isothermal PCR [iiPCR]) has been reported for the detection of marine virus *(29).* iiPCR also was integrated with the TaqMan probe technique to develop a rapid on-site diagnostic method for the detection of white spot syndrome virus *(30).* This iiPCR assay was carried out in a special polycarbonate capillary tube, the R-tube (GeneReach Biotechnology Corp., Taichung, Taiwan), within a thermally baffled device. The R-tube was heated by a copper ring attached to its bottom to generate temperature gradients. Compared with conventional PCR methods, cyclic reactions in the R-tube allow each PCR cycle to be completed in a relatively short period of time. iiPCR assays may require conditions and primers that allow amplification of relatively short DNA segments. In the present study, we attempted to design a novel convective PCR primer set and a TaqMan probe specific for the detection of *Salmonella* with the iiPCR assay.

MATERIALS AND METHODS

Bacterial strains and cell cultivation. Bacterial strains used in this study were *Salmonella* isolates of various serotypes and strains of other bacterial species, such as those of the family *Enterobacteriaceae* (Table 1). These strains were obtained from the American Type Culture Collection (ATCC; Manassas, VA), the Bioresource Collection and Research Center (BCRC; Hsin-Chu, Taiwan, Republic of China), the Culture Collection of the University of Göteborg (CCUG; Göteborg, Sweden), and the Center for Disease Control (CDC; Taichung, Taiwan, Republic of China).

Cells were cultivated in tryptic soy broth (30 g in 1,000 ml) at 37°C for 8 h and then plated on tryptic soy agar or used for DNA preparation. For the detection of *Salmonella* in poultry samples, samples were subjected to enrichment in Rappaport-Vassiliadis R10 broth (RVB; 26.6 g in 1,000 ml) at 42°C for 5 h to allow for growth of *Salmonella*. All media used in this study were purchased from Difco (BD, Sparks, MD).

DNA preparation. Bacterial genomic DNA was prepared using the taco total DNA extraction kit (GeneReach Biotechnology Corp., Taichung, Taiwan, Republic of China). Cells collected by centrifugation (15,700 × *g* for 3 min) from 1 ml of culture broth were washed with 1 ml of double deionized water and pelleted (15,700 × *g* for 3 min). The pellet was resuspended in 500 μl of lysis buffer (GeneReach Biotechnology). The mixture was then transferred to a 96-well extraction plate. The plate was loaded with reagents provided in the kit and installed in the taco nucleic acid automatic extraction system (GeneReach Biotechnology) according to the manufacturer's instructions. The extraction system automatically extracted total DNA from the bacteria, which was stored at −20°C until used.

Designing the primers and the TaqMan probe for the detection of *Salmonella* based on the *yrfH* gene. PCR primers and the TaqMan probe for the specific detection of *Salmonella* were designed from the *yrfH* gene coding for a heat shock protein (GenBank accession no. FQ312003.1, positions 3674582 to 3674983). Sequences of the *yrfH* gene from different *Salmonella* serovars were aligned with the *yrfH* gene sequences of other enterobacteria such as *Escherichia coli, Citrobacter* spp., and *Shigella* spp. available in GenBank using a basic local alignment search tool (NCBI, Bethesda, MD, http://www.ncbi.nlm.nih.gov/BLAST). Primers S15S (5′-AACGGACGGTGATCGTTAAA-3′, positions 3674769 to 3674788) and S15A (5′-AGGGCGTTAAGCTTGCGC-3′, positions 3674901 to 3674884) were designed with Primer Premier 5.0 software (PREMIER Biosoft, Palo Alto, CA) to amplify a 133-bp fragment. The specific probe S15P (5′-CCAGCGAAGCCGTCGCTC-3′, positions 3674814 to 3674831) designed with GenScript Real-time PCR (TaqMan) Primer Design (GenScript, Piscataway, NJ, https://www.genscript.com/ssl-bin/app/primer) was labeled at the 5′ end with 6-carboxyfluorescein and at the 3′ end with 6-carboxytetramethylrhodamine for iiPCR detection.

Specificity of the primers and the TaqMan probe. Specificity of the PCR primers was tested with conventional PCR, and the primers and probe were tested with the iiPCR. For these studies, DNA isolated from bacterial strains listed in Table 1 was used.

For conventional PCR, primers S15S and S15A were used in a single PCR containing 200 μM concentrations of each deoxynucleoside triphosphate, 1× PCR buffer (PROtech Technology, Taipei, Taiwan, Republic of China), 0.4 μM concentrations of each primer, 0.4 U of Prozyme (PROtech Technology), 300 to 400 ng of bacterial DNA, and double deionized water to a final volume of 50 μl. A reference strain, *Salmonella* Enteritidis ATCC 13076, was used as the positive control, and distilled water was used as the blank. PCR conditions in a thermal cycler (Gene Amp PCR system 2720, Applied Biosystems, Carlsbad, CA) were as follows: initial denaturation at 94°C for 5 min, 35 cycles of amplification at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s, and a final extension at 72°C for 5 min. The amplified products were analyzed by 2% agarose gel electrophoresis. The expected molecular size of the amplified product was 133 bp.

For iiPCR detection of *Salmonella* using primers S15S and S15A and probe S15P, a 50-μl iiPCR mixture containing 1× HS Premix buffer (GeneReach Biotechnology), 0.25 μM concentrations of each primer, 0.25 μM concentration of probe, 3.25% glycerol, 15 U of *Taq* DNA polymerase (BioMi, Taichung, Taiwan, Republic of China), and 5 μl of the target DNA (300 to 400 ng of bacterial DNA) was pipetted into the R-tube. Because Rayleigh-Bénard convection is driven by buoyancy when a fluid layer is heated from below *(3),* the bottom of the R-tube was heated at 95°C for the development of thermal convection and kept under insulated isothermal conditions in the iiPCR system (POCKIT Nucleic Acid Analyzer, GeneReach Biotechnology). This system comprises the iiPCR device *(30)* and an optical detection module. The fluorescent amplification signals of the target were filtered through a 520-nm bandpass filter and collected by an integrated circuit controller-regulated complementary metal oxide semiconductor sensor in the POCKIT system. The fluorophore for the TaqMan probe used in this study was 6-carboxyfluorescein with an emission wavelength at 520 nm. Only fluorescent signals at 520 nm were recorded before and after the iiPCR, respectively. The POCKIT system indicates positive amplification when the fluorescent signal after amplification is 20% higher than that before amplification. The iiPCR ingredients as a premix, i.e., the HS Premix buffer, primers, probe, and *Taq* DNA polymerase, were freeze-dried with a vacuum concentrator (Ecospin 3180C, Biotron, Daejeon, Korea). After addition of double deionized water, glycerol (to a final concentration of 3.25%), and 300 to 400 ng of template DNA to the iiPCR premix, the performance of the

TABLE 1. *Bacterial strains used for detection with conventional PCR and iiPCR*[a]

Bacterial strain	Source[b]
Salmonella (148 strains of 49 serotypes)	
Choleraesuis	BCRC 10743
Enteritidis	ATCC 13076
Salamae	BCRC 15450
Typhimurium	BCRC 10747, BCRC12947
Agona ($n = 2$)	CDC
Albany ($n = 2$)	CDC
Anatum ($n = 2$)	CDC
Augustenborg ($n = 2$)	CDC
Bareilly ($n = 2$)	CDC
Blockley ($n = 2$)	CDC
Braenderup ($n = 2$)	CDC
Cerro ($n = 2$)	CDC
Chester ($n = 2$)	CDC
Choleraesuis ($n = 2$)	CDC
Derby ($n = 2$)	CDC
Dublin ($n = 2$)	CDC
Enteritidis ($n = 10$)	CDC
Hadar ($n = 9$)	CDC
Haifa ($n = 2$)	CDC
Havana ($n = 2$)	CDC
Houten ($n = 2$)	CDC
Infantis ($n = 10$)	CDC
Indiana ($n = 2$)	CDC
Isangi ($n = 2$)	CDC
Kedougou ($n = 2$)	CDC
Litchfield ($n = 2$)	CDC
Livingstone var. O14 ($n = 2$)	CDC
London ($n = 2$)	CDC
Massenya ($n = 2$)	CDC
Mbandaka ($n = 2$)	CDC
Montevideo ($n = 2$)	CDC
Muenchen ($n = 2$)	CDC
Newport ($n = 2$)	CDC
Panama ($n = 2$)	CDC
Paratyphi A ($n = 2$)	CDC
Paratyphi B var. Java ($n = 2$)	CDC
Potsdam ($n = 2$)	CDC
Saintpaul ($n = 2$)	CDC
Schwarzengrund ($n = 2$)	CDC
Senftenberg ($n = 2$)	CDC
Seremban ($n = 2$)	CDC
Singapore ($n = 2$)	CDC
Stanley ($n = 2$)	CDC
Tambacounda ($n = 2$)	CDC
Tennessee ($n = 2$)	CDC
Thompson ($n = 2$)	CDC
Typhi ($n = 10$)	CDC
Typhimurium ($n = 10$)	CDC
Uganda ($n = 2$)	CDC
Victoria ($n = 2$)	CDC
Virchow ($n = 10$)	CDC
Weltevreden ($n = 2$)	CDC
Bacteria other than *Salmonella* (98 strains of 52 species)	
Acinetobacter baumannii	BCRC 15318
A. calcoaceticus	BCRC 11562
A. johnsonii	BCRC 14853
A. ursingii	BCRC 17329
Acinetobacter sp.	BCRC 15420, BCRC17454
Alcaligenes faecalis	BCRC 10828
Bacillus cereus	BCRC 10250
Brevibacterium linens	BCRC 10041
Citrobacter freundii	BCRC 12292
Clostridium acetobutylicum	BCRC 10639
C. difficile	BCRC 17678
C. haemolyticum	BCRC 10643
C. perfringens	BCRC 10914
Corynebacterium renale	BCRC 10657
Cronobacter sakazakii	BCRC 13988, BCRC 14122, BCRC 14153
Enterobacter aerogenes	BCRC 10370
E. cloacae	ATCC 23355
Erwinia carotovora	BCRC 12613
Escherichia coli	ATCC 35150, ATCC 45750, ATCC 43890, BCRC 11509, BCRC 13086, BCRC 13093, BCRC 13094, BCRC 13095, BCRC 13096, BCRC 14824, BCRC 14825, BCRC 15370, BCRC 15371, BCRC 15373, BCRC 15375, BCRC 15536, BCRC 41443
Hafnia alvei	BCRC 10906
Listeria innocua	BCRC 14843
L. monocytogenes	BCRC 14845, BCRC 14846, BCRC 14847, BCRC 14848, BCRC 14930, BCRC 15327, BCRC 15329, BCRC 15330, BCRC 15331, BCRC 15332, BCRC 15333, BCRC 15334, BCRC 15387
L. grayi	BCRC 14849, BCRC 15360
Moraxella catarrhalis	BCRC 10629
M. osloensis	BCRC 10705
Proteus vulgaris	ATCC 8427
Pseudomonas aeruginosa	BCRC 10944
P. fluorescens	BCRC 10304, BCRC 11028, BCRC 13902, BCRC 13904, BCRC 16016
P. mendocina	BCRC 10458
P. putida	BCRC 10459
Rahnella aquatilis	BCRC 14814
Saccharomyces cerevisiae	BCRC 20577
Serratia ficaria	BCRC 14809
S. fonticola	BCRC 14810
S. odorifera	BCRC 12223
S. quinivorans	BCRC 14811
Shigella dysenteriae	BCRC 13983
S. sonnei	BCRC 10773
Staphylococcus sp.	BCRC 12660

TABLE 1. *Continued*

Bacterial strain	Source[b]
S. aureus	BCRC 12654, BCRC 12656, BCRC 13824, BCRC 13825, BCRC 13826, BCRC 13828, BCRC 13829, BCRC 13830, ATCC 700699
S. epidermidis	BCRC 10785
S. haemolyticus	BCRC 12923
S. hyicus	BCRC 12925
S. intermedius	BCRC 12157, BCRC 15236
S. saprophyticus	BCRC 10786
S. xylosus	BCRC 15252
Streptococcus agalactiae	BCRC 10787
S. bovis	CCUG 4214
S. uberis	ATCC 700407
Streptomyces filipinensis	BCRC 11472
Vibrio alginolyticus	BCRC 12829
V. parahaemolyticus	BCRC 13023, BCRC 13025

[a] All *Salmonella* strains were positive by both conventional PCR and iiPCR. All other bacterial strains were negative by both methods.

[b] ATCC, American Type Culture Collection; BCRC, Bioresource Collection and Research Center; CCUG, Culture Collection of the University of Göteborgn; CDC: Center for Disease Control, Taiwan.

iiPCR premix was also evaluated with the POCKIT system using the same criteria. All experiments were performed in duplicate.

Detection limit for *Salmonella* cells artificially spiked into poultry meat samples. Refrigerated raw chicken breast fillets were purchased from local supermarkets in Taichung, Taiwan. These chicken meat samples were used to determine the detection limit for *Salmonella* in poultry samples.

Chicken meat (25 g) was mixed with 225 ml of 0.1% peptone water and homogenized with a Stomacher 400 (Seward Medical, London, UK) at high speed for 2 min. An aliquot of this mixture was examined by culture on plate count agar to determine endogenous microflora contamination; the presence or absence of *Salmonella* in these samples was confirmed by the *Salmonella* culture method described in the *Bacteriological Analytical Manual* (BAM) *(32).* Poultry meat samples were then artificially contaminated with *Salmonella* Enteritidis ATCC 13076, which was used as a reference strain. One hundred microliters of the 10-fold serial dilutions (10^{-4} to 10^{-8}) of the overnight culture of *Salmonella* Enteritidis ATCC 13076 (10^9 CFU/ml) was mixed with 1 ml of the meat homogenate, and 8.9 ml of RVB was added. Five tubes were assayed for each serial dilution. A 1-ml aliquot of the sample, with or without 5 h of enrichment at 42°C, was used for DNA extraction followed by the iiPCR detection according to the methods described. Uninoculated poultry samples also were used for the iiPCR detection of *Salmonella*. All experiments were performed in duplicate.

RESULTS

Specificity of the PCR primers and probe. Using the sequences available in GenBank, PCR primers and probe based on the *yrfH* gene were designed for the specific detection of *Salmonella*. Conventional PCR and iiPCR were performed to confirm the detection specificity of these primers and of the TaqMan probe, respectively. When

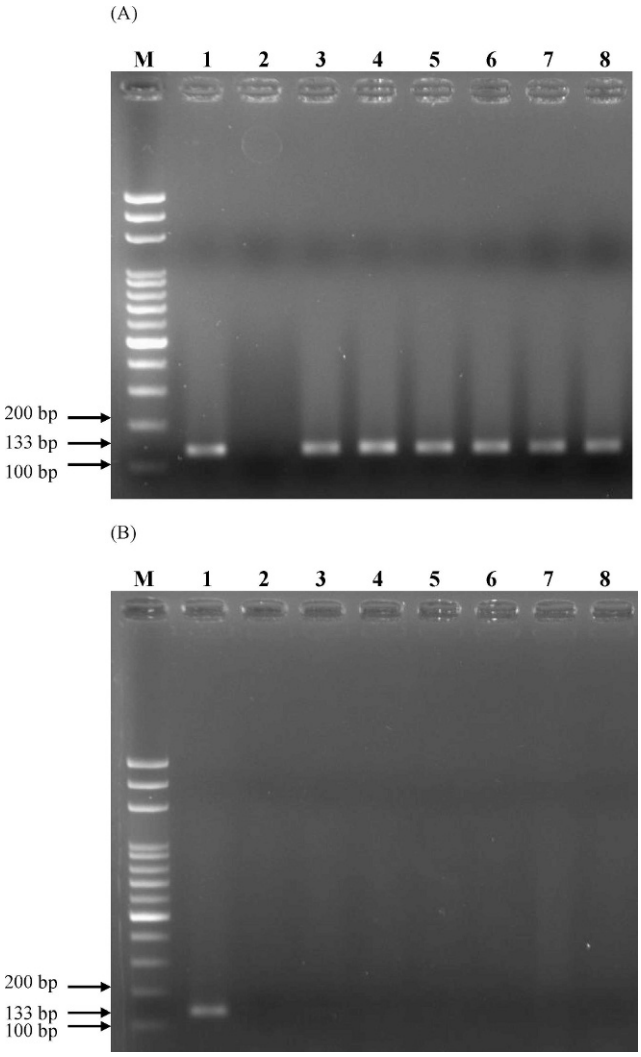

FIGURE 1. *Conventional PCR detection of (A)* Salmonella *isolates and (B) non-*Salmonella *isolates using primers S15S and S15A. Experimental conditions are described in the ''Materials and Methods.'' Bacterial strains shown in Table 1. Only part of the results are shown here. (A) Lane M, 100-bp ladder marker (PROtech Technology); lane 1,* Salmonella *Enteritidis ATCC 13076; lane 2, blank control; lanes 3 through 8,* Salmonella *Typhimurium BCRC 10747,* Salmonella *Typhimurium BCRC 12947,* Salmonella *Salamae BCRC 15450,* Salmonella *Choleraesuis BCRC 10743,* Salmonella *Hadar CA08.102, and* Salmonella *Infantis CC07.003, respectively. (B) Lane M, 100-bp ladder marker; lane 1,* Salmonella *Enteritidis ATCC 13076; lane 2, blank control; lanes 3 through 8,* Escherichia coli *BCRC 11509,* Listeria monocytogenes *BCRC 14848,* Vibrio alginolyticus *BCRC 12829,* Streptococcus agalactiae *BCRC 10787,* Bacillus cereus *BCRC 10250, and* Cronobacter sakazakii *BCRC 13988, respectively.*

Salmonella-specific primers S15S and S15A were used for conventional PCR, all of the 148 *Salmonella* strains (representing 49 serotypes), including reference strains from BCRC and laboratory isolates, generated the expected PCR products of 133 bp (Table 1). A total of 98 strains of bacterial species other than *Salmonella,* including those of the family *Enterobacteriaceae* such as *Shigella, E. coli,* and *Citrobacter,* were also used in this study; none of these generated a false-positive reaction. Some of the PCR results are shown in Figure 1.

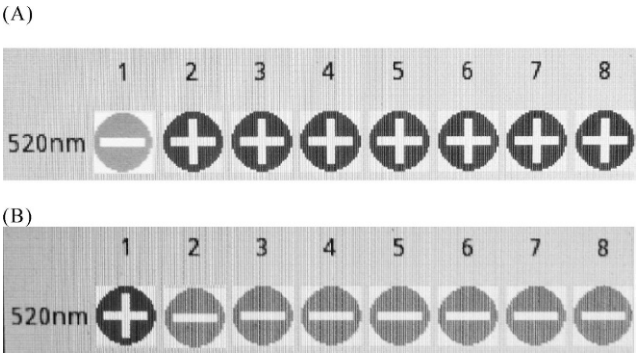

FIGURE 2. *iiPCR detection of (A)* Salmonella *isolates and (B) non-*Salmonella *isolates using the TaqMan probe S15P and primers S15S and S15A. Experimental conditions are described in the ''Materials and Methods.'' Bacterial strains shown in Table 1. Only part of the results are shown here. (A) Lane 1, blank control; lanes 2 through 8:* Salmonella *Enteritidis ATCC 13076 (positive control),* Salmonella *Typhimurium BCRC 10747,* Salmonella *Typhimurium BCRC 12947,* Salmonella *Salamae BCRC 15450,* Salmonella *Choleraesuis BCRC 10743,* Salmonella *Hadar CA08.102, and* Salmonella *Infantis CC07.003, respectively. (B) Lane 1,* Salmonella *Enteritidis ATCC 13076 (positive control); lane 2, blank control; lanes 3 through 8:* Escherichia coli *BCRC 11509,* Listeria monocytogenes *BCRC 14848,* Vibrio alginolyticus *BCRC 12829,* Streptococcus agalactiae *BCRC 10787,* Bacillus cereus *BCRC 10250, and* Cronobacter sakazakii *BCRC 13988, respectively.*

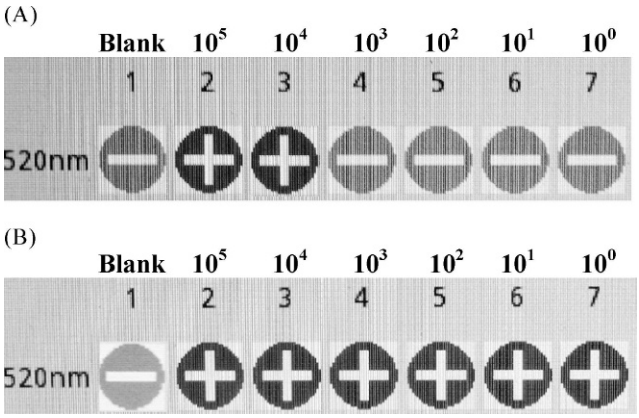

FIGURE 3. *Detection limits for iiPCR detection of* Salmonella *Enteritidis cells spiked into poultry meat samples at 10^5 to 10^0 CFU/g. Experimental conditions are described in the ''Materials and Methods.'' Lane 1, blank control; lanes 2 through 7, chicken meat homogenates without the preenrichment step (A) and after a 5-h preenrichment step (B).*

For the iiPCR detection of *Salmonella,* the primer set S15S-S15A and probe S15P were used for amplification of the DNA from both *Salmonella* and non-*Salmonella* strains. In the POCKIT system, all of the *Salmonella* strains generated positive results, and all of the non-*Salmonella* strains gave negative results (Fig. 2). Results from the iiPCR assay were consistent with those from conventional PCR (Table 1). Thus, the iiPCR assay developed in this study was highly specific for the detection of *Salmonella*. For the iiPCR assay, control of the fluid flow rate inside the tube is important. To slow the flow rate in this study, glycerol was added to increase the viscosity of the iiPCR mixture; thus, unambiguous results of the iiPCR assay could be obtained. The freeze-dried iiPCR premix generated consistent results, indicating that this freeze dried premix can be used for the iiPCR to allow convenient and rapid detection of *Salmonella*.

iiPCR detection of *Salmonella* in poultry meat samples. The raw chicken meat samples used in this study were contaminated by endogenous microflora at 2×10^4 CFU/g. The BAM method was used to confirm that these samples were *Salmonella* free. The detection limit of the iiPCR assay for poultry meat samples was determined by inoculating the samples with known levels of *Salmonella*. Under the conditions described, *Salmonella* at 10^3 CFU/g could be detected in chicken meat without the preenrichment step. To improve the detection limit of the iiPCR assay, a 5-h preenrichment step was added before the iiPCR assay. The preenrichment step, reduced the *Salmonella* detection limit to 10^0 CFU/g (Fig. 3). Poultry meat samples that were not inoculated with *Salmonella* produced negative results both with and without the preenrichment step (data not shown).

DISCUSSION

Molecular methods based on various molecular markers such as 16S rDNA *(18), hilA (22), invA (24),* and *fimY (34)* have been used for the detection of *Salmonella*. Previously, we found that heat shock protein (hsp) family genes can be used for specific detection of pathogenic bacteria in food *(4, 6)*. Wong and Chow *(33)* used primers based on the bacterial hsp60 gene for the specific detection of enteric pathogenic bacteria, including *Salmonella*.

Because some primer sets that could generate expected amplicons in conventional PCR could not produce amplicons efficiently in iiPCR *(29),* new primers may be needed for iiPCR. Circulation in the iiPCR R-tube allows for relatively short (only a few seconds) PCR cycles, which suggests that the optimal parameters, such as primer criteria and buffer compositions for iiPCR, may be different from those used for conventional PCR *(29)*. Our goal was to design new primers that could be used for the iiPCR detection of all serovars of *Salmonella*. We focused on the gene *yrfH,* a heat shock protein family gene coding for HSP15, to design the primers and TaqMan probe for the iiPCR detection of *Salmonella*. HSP15 is a heat shock protein that is abundant in bacterial cells and is able to bind to the free 50S ribosomal subunit instead of acting as a chaperone or protease *(16)*. Malorny et al. *(19)* reported that targeting a genetically stable gene is advantageous in the detection of all *Salmonella* serovars. Natural deletions might occur within the *Salmonella* pathogenicity island 1, which consists of the *inv, spa,* and *hil* loci. The use of such genetically unstable targets as molecular markers could lead to false-negative results. In contrast, HSP15 is highly conserved *(16)*. The possibility of failure to identify any one of the *Salmonella* serovars due to sequence variation among *Salmonella* serotypes in the primer or probe binding sites is low.

The primers and probe used in this study were designed to anneal to only the *Salmonella*-specific regions in the *yrfH* gene. The specificities of primers S15S and S15A and the probe S15P were evaluated by both conventional PCR and iiPCR assays. Results from both assays were comparable. All *Salmonella* strains produced the amplified products with the expected size of 133 bp in the PCR assay or the positive signals in the iiPCR assay. No nonspecific reactions occurred with non-*Salmonella* strains, including those of the family *Enterobacteriaceae* such as *E. coli, Citrobacter,* and *Shigella* spp. This finding indicates that these primers and probe can be used as a specific means to detect *Salmonella*. However, for conventional PCR, the analysis of amplicons was carried out by gel electrophoresis, which is labor intensive, and post-PCR steps may increase the risk of cross-contamination *(29)*. Ethidium bromide, which is commonly used for staining nucleic acids in electrophoresis gels, is mutagenic and may pose health risks to the operators *(26)*. The use of a sequence-specific TaqMan probe further ensures the production of target amplicons; nonspecific products were occasionally found in iiPCR assays *(30)*. The POCKIT system we used includes an optical system for the detection of fluorescent signals at 520 nm, which are generated by hybridization and cleavage of the TaqMan probe. The combination of the TaqMan probe and the POCKIT system thus allowed us to detect *Salmonella* without the electrophoresis step. The integration of amplification hardware with a signal detection system also makes iiPCR efficient and reduces the time required for postamplification analysis.

Using the POCKIT system, positive signals were obtained from all the *Salmonella* test strains. The fluorescent signal is generated only when the probe hybridizes to *Salmonella*-specific regions in the *yrfH* gene. By using a *Salmonella*-specific probe in the iiPCR assay, we ensured that expected PCR products were obtained in the iiPCR assay without the need for further sequencing. Data obtained from the iiPCR assay were automatically processed and analyzed by the POCKIT system; thus, human errors and discrepancies in interpretation of results can be avoided. The system described here is more reliable and user friendly than the conventional PCR assay.

The iiPCR assay was developed based on the principle of Rayleigh-Bénard convection, which is driven by buoyant forces when a fluid layer is heated from below. In Rayleigh-Bénard convection, the physical properties of the fluid, e.g., viscosity, can affect the fluid state in the system *(1)*. Consequently, adjustment of the viscosity of the reaction solution can affect the efficiency of nucleic acid amplification in the iiPCR assay. In this study, a nonreactive viscous liquid material (glycerol) was added to increase the viscosity of the iiPCR mixture. In this way, unambiguous results could be obtained from the iiPCR assay.

When all of the iiPCR ingredients (except the target DNA and glycerol) were freeze dried before the assay, the specificity was not affected. The detection method developed is convenient and ready to use with the addition of a simple premix. The nucleic acid extraction system (taco Nucleic Acid Automatic Extraction System), the iiPCR device (POCKIT Nucleic Acid Analyzer), and the reaction tube (R-tube) used in this study are all commercially available (GeneReach Biotechnology). This iiPCR assay can be easily commercialized into a diagnostic kit for the rapid detection of *Salmonella*.

Several PCR-based methods with high specificity and sensitivity have been used to detect *Salmonella* in food. Chiu et al. *(8)* developed a PCR method for *Salmonella* detection based on internal transcribed space regions. With an 8-h enrichment step, the detection limit was 1 to 9 CFU/g for a chicken meat sample. Patel et al. *(21)* reported that by using molecular beacon real-time PCR after 16 to 18 h of enrichment, 2 ± 1 CFU of *Salmonella* could be detected in 25 g of hydrodynamic pressure-treated chicken meat. Perelle et al. *(23)* compared PCR plus an enzyme-linked immunosorbent assay with the LightCycler real-time PCR assay for the detection of *Salmonella* in milk and meat samples. With both of these PCR methods, less than five *Salmonella* cells could be detected in 25 g of food product after 18 h of enrichment. However, amplification of specific gene(s) in these PCR-based methods requires thermal cyclers, which may be expensive. One advantage of isothermal nucleic acid amplification methods over conventional PCR-based methods is that isothermal nucleic acid amplification methods do not require a thermal cycler. Isothermal nucleic acid amplification methods, including iiPCR, allow the use of a simple heating device, which may reduce the overall cost of pathogen detection.

Other reported isothermal nucleic acid amplification methods, including LAMP and nucleic acid sequence-based amplification (NASBA), have been used for the detection of *Salmonella* in food. Techathuvanan et al. *(28)* used the LAMP assay based on the *invA* gene for the detection of *Salmonella* Typhimurium in pork products. With a 10-h enrichment, the detection limit of the LAMP assay was 10^2 CFU/25 g of pork product. D'Souza and Jaykus *(12)* applied NASBA and electrochemiluminescent hybridization based on the *dnaK* gene for the detection of *Salmonella* Enteritidis in food commodities (fresh meats, poultry, fish, ready-to-eat salads, and bakery products). After an 18-h enrichment, positive signals were achieved at initial inoculum levels of 10^1 CFU/25 g of food sample. In the present study, the detection limit of the iiPCR assay was 10^0 CFU/g of poultry sample with a 5-h enrichment step. The detection limits of these various isothermal methods are comparable to those found for PCR-based methods. However, the enrichment time used in our study is less than those needed for other previously reported isothermal and PCR-based methods, and total processing time of the iiPCR assay for the detection of *Salmonella* in food is thus shortened. In addition, the reagent cost for the iiPCR assay is lower than that of existing isothermal amplification kits, e.g., Loopamp *Salmonella* Detection Kit (Eiken Chemical Co., Tokyo, Japan) and NucliSENS EasyQ Basic Kit (bioMérieux SA, Marcy l'Etoile, France).

For the detection of *Salmonella* with the LAMP assay *(28),* specific primers consisting of two inner primers, two outer primers, and two loop primers are required. In the iiPCR assay described here, only two primers and one

TaqMan probe are required for the specific detection of *Salmonella*. Compared with the LAMP assay, iiPCR methods for bacterial detection are easier to design because the primer system is simpler. In the NASBA assay described by D'Souza and Jaykus *(12),* primer annealing at 65°C was required before isothermal amplification at 41°C, and the enzyme mixture was added after primer annealing, which could be inconvenient. In the iiPCR assay, the experimental procedures are simpler than those of the NASBA assay.

To exclude interference from inhibitors in food sample matrices and from endogenous microflora in poultry meat, samples used for the iiPCR assay were homogenized and then examined for levels of endogenous bacteria. Although some endogenous bacteria were detected in the poultry meat samples (2×10^4 CFU/g of raw chicken), these samples were confirmed by the BAM method to be *Salmonella* free. Uninoculated poultry meat samples produced negative results in the iiPCR assay either with or without the preenrichment step; no matrix-associated false-positive results were obtained. In contrast, samples artificially contaminated with *Salmonella* Enteritidis ($>10^3$ CFU/g), which were used for external positive controls, produced positive results; no false-negative results were obtained. These results confirmed that neither the food components nor the endogenous microflora in the food samples interfered with the iiPCR assay.

Typical procedures of PCR-based methods for the detection of foodborne pathogens include homogenization of food samples, enrichment of target bacteria, extraction of bacterial nucleic acid, amplification of target-specific genes, and detection of amplified products. Generally, enrichment (the most time-consuming step) for 8 h to overnight may be required. Most methods require 18 h or more to obtain results *(7, 10, 20, 25)*. With respect to the time frames in which analysts typically are conducted, most methods usually take two working days to obtain the results. To reduce the overall analysis time, strategies applied in this study include (i) introduction of a nucleic acid automatic extraction system, (ii) reduction of the time required for the enrichment step, and (iii) application of an integrated system for nucleic acid amplification and signal detection.

Manual methods for nucleic acid preparation, such as using spin column–based commercial nucleic acid extraction kits, often require hours of hands-on time and are susceptible to contamination and handling variations. With the taco nucleic acid automatic extraction system, only a few steps require liquid transfer; thus, the potential for cross-contamination is low and the hands-on time is shortened. The extraction system allows extraction of high-quality nucleic acid from solutions in 1 h. To shorten the time required for bacterial enrichment and to maintain an acceptable detection limit for diagnostic needs, various enrichment periods up to 8 h were tested. With an enrichment time of less than 4 h, a minimum *Salmonella* contamination level of 10^2 to 10^3 CFU/g of poultry meat sample was detectable (data not shown). With a 5-h enrichment step, the detection level of *Salmonella* could be as low as 10^0 CFU/g.

A typical run of the POCKIT system takes about 50 min. The time required for nucleic acid amplification and signal detection is greatly shortened compared with the conventional PCR methods with electrophoresis. Thus, our analysts were able to complete all the procedures described in this study within 8 h, which means that the results can be obtained within one working day.

In conclusion, the primers S15S and S15A and the TaqMan probe S15P designed from *yrfH,* a heat shock protein gene, are specific for the iiPCR detection of *Salmonella*. Including homogenization of food samples, enrichment of target bacteria, extraction of bacterial nucleic acid, amplification of target-specific genes, and detection of amplified products, the detection results can be obtained within 8 h. The iiPCR system is accurate, time saving, and user friendly and can be a convenient method for the routine inspection of poultry samples.

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