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Simultaneous Detection of Food-borne Pathogenic Bacteria in Ready-to-eat *Kimbab* Using Multiplex PCR Method

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Abstract *Kimbab* is the most popular ready-to-eat (RTE) food in Korea. A rapid detection method based on multiplex PCR technique was developed for detection of major food-borne pathogens like *Salmonella* spp., *Shigella* spp., *Bacillus cereus*, *Listeria monocytongenes*, and *Staphylococcus aureus*. Specific bands were obtained as 108 bp (Sau, *S. aureus*), 284 bp (Sal, *S. enterica*, *S. enteritids*, and *S. typhmurium*), 404 bp (Lmo, *L. monocytogenes*), 475 bp (Bce, *B. cereus*), and 600 bp (Shi, *S. flexineri* and *S. sonnei*). Visible cell numbers varied from 4.14-5.03, 3.61-4.47, and 4.10-5.11 log CFU/g in randomly collected June, July, and August samples, respectively. Among the 30 *kimbab* samples obtained 83.3% samples were contaminated and 16.7% samples were free from contamination. The highest rate of contamination was with *S. aureus* (56.7%) followed by *B. cereus* (43.3%), *Salmonella* spp. (36.7%), *Shigella* spp. (13.3%), and *L. monocytogenes* (6.7%). The identification of the pathogenic species could be faster using one polymerase chain reaction (PCR) and the ability to test for food-borne pathogenic species in *kimbab* will save time and increase the ability to assure its quality.

Keywords: ready-to-eat food, *kimbab*, food-borne pathogenic bacteria, multiplex polymerase chain reaction (PCR), food safety

Introduction

Microbial safety of food is a significant concern of consumers and industries today. The rapid and accurate identification of food-borne pathogenic bacteria in food is important, both for quality assurance and to trace pathogens within the food supply (1,2). *Salmonella* spp., *Shigella* spp., *Bacillus cereus*, *Listeria monocytongenes*, and *Staphylococcus aureus* are the most important organisms spreading through various foods, afflicting people worldwide (1). *Kimbab* is a ready-to-eat (RTE) food in Korea and has many ingredients which make it delicious. It consists of ham, eggs, cooked vegetables, etc, surrounded by steam rice and seaweed. *Kimbab* is usually prepared by hand after all the ingredients have been cooked and cut, it can be contaminated with food-borne pathogens (3,4). The Korean Food Code stipulates that pathogens should not be detected in *kimbab*. Unfortunately, food-borne pathogens often are found in this product and the Korea Food & Drug Administration (KFDA) reports that *kimbab* has been implicated in many food-borne disease outbreaks. In 2005, 12.0% of food-borne illness in Korea was caused by *kimbab*, and about 70% of these cases are caused by products prepared at retail stores (5). The consumption of *kimbab* could present a risk of a potential increase of exposure to food-borne pathogens like *Salmonella* spp., *B. cereus*, *L. monocytongenes*, and *S. aureus* (3). However, most studies of pathogenic bacteria in *kimbab* have used conventional, culture-based methods (3,4,6-8).

Recently, polymerase chain reaction (PCR) technology has proven to be valuable for the detection of bacteria in foods. With its high levels of sensitivity and specificity, PCR can be used for the rapid detection of the food-borne pathogenic bacteria contaminating various foods (1,2). Since the first description of multiplex PCR (9) that it has been successfully applied to many areas such as genetically modified organisms (GMO), fermentation food, and food-borne pathogens in food samples (10-14). Multiplex PCR allows the simultaneous amplification of more than one target sequence in a single PCR reaction, saving considerable time and effort, and decreasing the number of reactions to be performed in order to assess the possible presence of food-borne pathogens in food samples (15). However, some components of food and chemicals required for selective enrichment of cells may influence the effectiveness of the PCR and cause inhibitory effects (16), Thus, the implementation of PCR based assays as routine microbiology diagnostic tools, especially in testing laboratories with quality assurance programs, requires proper controls to verify the accuracy of the results obtained. An adequate strategy to asses the validity of the PCR results is the use of an internal amplification control (IAC), a non-target DNA sequence which is co-amplified with the target sequence by the same set of primers (17-20). Many primer sets have been used for the PCR detection of *Salmonella* spp., *Shigella* spp., *B. cereus*, *L. monocytongenes*, and *S. aureus* (21-25). The aim of this work was to develop a multiplex PCR based method for the simultaneous direct detection of *Salmonella* spp., *Shigella* spp., *B. cereus*, *L. monocytongenes*, and *S. aureus* in *kimbab* samples.

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Table 1. List of bacterial strains and medium used in this study

Microorganism	Strain	Medium[1)]
Gram-negative		
Salmonella enterica	KCTC 12400	TSB/TSA
Salmonella enteritidis	KCTC 12456	TSB/TSA
Salmonella typhimurium	KCTC 1925	TSB/TSA
Shigella flexneri	KCTC 2008	TSB/TSA
Shigella sonnei	KCTC 2518	TSB/TSA
Gram-positive		
Bacillus cereus	KCTC 1012	TSB/TSA
Listeria monocytogenes	KCTC 3569	TSB/TSA
Staphylococcus aureus	KCTC 1916	TSB/TSA

[1)]Bacterial strains were grown overnight at 37°C in tryptic soy broth/agar (TSB/TSA).

Materials and Methods

Bacterial strains and growth conditions Eight bacterial strains tested in this study are shown in Table 1. Reference strains were collected from the Korea Type Culture Collection (KTCC). The bacterial strains were grown overnight at 37°C in tryptic soy broth (TSB) or agar (TSA, Difico Lab., Detroit, MI, USA).

Sample collection Thirty *kimbab* samples were purchased randomly from local supermarkets and convenience stores in the Jinju area of Korea and transferred to a sterile bag for analysis on the same day. Ten samples were collected in June, July, and August on 2006 accounting to a total of 30 samples. Thirty samples were labeled from KB01 to KB30.

Total visible cell number Ten g of each sample was homogenized for 5 min in 90 mL of TSB. Each homogenate was transferred to a sterile 250-mL Erlenmeyer flask and enriched at 37°C for 24 hr with agitation (160 rpm). An aliquot (1 mL) of each homogenate was diluted 10-fold in 0.85%(w/v) NaCl. To determine the accurate number of the visible cells, 100 μL of appropriate dilutions of each culture were spread-plated on TSA and incubated at 37°C for 24 hr.

Extraction of total DNA from pure cultural bacteria and *kimbab* samples The collected 8 pure cultural bacteria and enriched *kimbab* samples at 24 hr incubation were centrifuged at 13,000×g for 5 min at 4°C. The obtained pellet (approximately 3 mL) was subjected to total DNA extraction G-spin™ Genomic DNA extraction kit (iNtRON Biotechnol., Suwon, Korea), as recommended by the supplier. The extracted DNA was used as a template for the uniplex and multiplex PCR.

Multiplex PCR reaction The primers chosen for the multiplex PCR were determined after first evaluating several previously described primers. The species-specific primers for identification of *Salmonella* spp., *Shigella* spp., *B. cereus*, *L. monocytongenes*, and *S. aureus* were designed based on *inv*A (21), *ipa*H (22), *nhe*A (emetic type, 23), *prf*A (24), and *dna*A (25) genes, respectively (Table 2). Five primer sets were selected to simultaneously detect different types of food-borne pathogenic bacteria using the multiplex PCR method. All primers were synthesized by Bioneer Inc. (Daejeon, Korea). Multiplex PCR was performed with a thermal cycler (MJ Mini™; Gradient Thermal Cycler, Foster, CA, USA). Based on the manufacturer's instruction, the PCR reaction mixture (50 μL) contained 1 μL of Super *Taq* polymerase (2.5 U/μL), 2 mM of each primers (15 pmol), 5 μL of reaction buffer with 2.5 mM $MgCl_2$, 5 μL of 2 mM deoxynucleosied triphophate (dNTP), 5 μL of template DNA, and 30 μL of sterile distilled water. Multiplex PCR was carried out through 35 cycles following a pre-heat step at 94°C for 5 min. Each cycle consisted of denaturation at 94°C for 1 min, annealing at 53°C for 1.5 min, and extension at 72°C for 1 min. The last cycle was followed by a final extension at 72°C for 10 min. The same PCR condition was used for single PCR analysis for reference strains. The total time required for the PCR amplification was 4.5 hr.

Agarose gel electrophoresis The PCR amplicons were analyzed by agarose gel electrophoresis. Ten μL of PCR products was loaded onto a 2.0%(w/v) agarose gel and subjected to electrophoresis for 4 hr at 60 V in 1×TAE buffer. The gel was stained with 1 μg/mL ethidium bromide (EtBr) solution. Gels were observed and documented in a

Table 2. Oligonucelotide primers used for detection of food-borne pathogenic bacteria in multiplex PCR

Target strain	Primer name	Primer sequences	PCR size (bp)	Ref.
S. aureus	SalF	5'-AATCTTTGTCGGTACACGATATTCTTCACG-3'	108	(25)
	SalR	5'-CGTAATGAGATTTCAGTAGATAATACAACA-3'		
Salmonella sp.	InvAF	5'-GTGAAATTATCGCCACGTTCGGGCAA-3'	284	(21)
	InvAR	5'-TCATCGCACCGTCAAAGGAACC-3'		
L. monocytogenes	Lm404F	5'-ATCATCGACGGCAACCTCGGAGAC-3'	404	(24)
	Lm404R	5'-CACCATTCCCAAGCTAAACCAGTGC-3'		
B. cereus	NheF	5'-ATTACAGGGTTATTGGTTACAGCAGT-3'	475	(23)
	NheR	5'-AATCTTGCTCCATACTCTCTTGGATGCT-3'		
Shigella sp.	IpaHF	5'-GTTCCTTGACCGCCTTTCCGATACC-3'	600	(22)
	IpaHR	5'-GCCGGTCAGCCACCCTCTGA-3'		

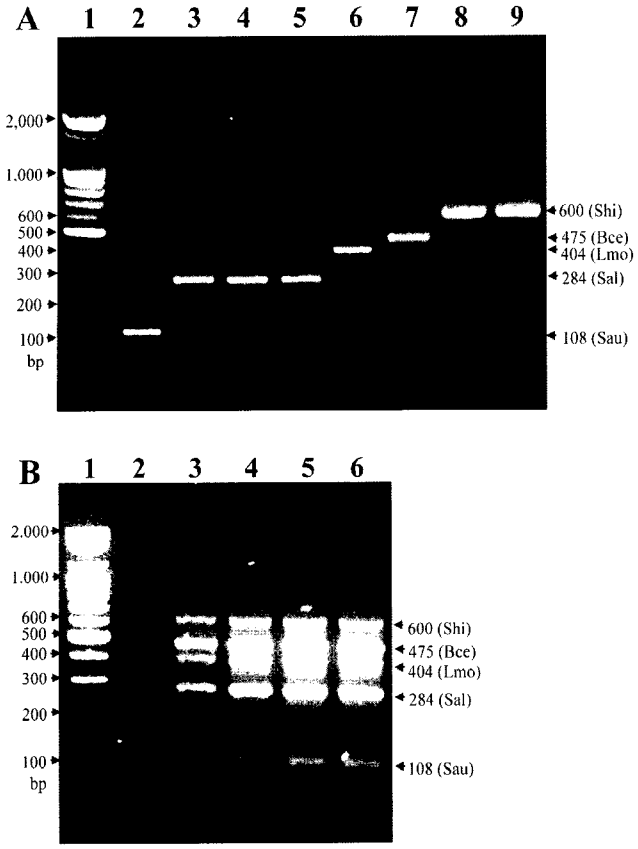

Fig. 1. Agarose gel electrophoresis of the uniplex PCR (A) and multiplex PCR products (B) obtained for the food-borne pathogenic bacteria. A. Lane 1, Marker, 100 bp DNA ladder; lane 2, *S. aureus*; lane 3, *S. enterica*; lane 4, *S. enteritidis*; lane 5, *S. typhimurium*; lane 6, *L. monocytogenes*; lane 7, *B. cereus*; lane 8, *S. flexneri*; lane 9, *S. sonnei*. B. Lane 1, Marker, 100 bp DNA ladder; lane 2, 0 hr incubation of 0.32 log CFU/mL; lane 3, 4 hr incubation of 3.87 log CFU/mL; lane 4, 8 hr incubation of 8.70 log CFU/mL; lane 5, 12 hr incubation of 10.2 log CFU/mL; lane 6, 24 hr incubation of 10.5 log CFU/mL.

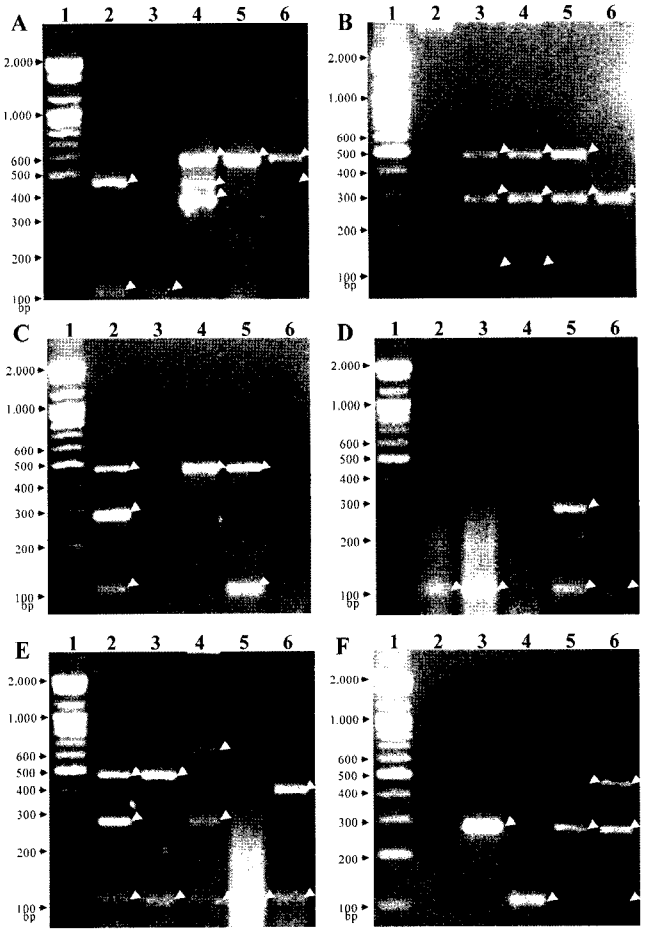

Fig. 2. Agarose gel electrophoresis of the multiplex PCR products obtained for the enrichment culture (24 hr incubation) of 30 *kimbab* samples at the different sample periods (June, July, and August). Lane A1 to F1: Marker, 100 bp DNA ladder (Bioneer Inc., Daejeon, Korea); lane A2 to F2: KB01, KB06, KB11, KB16, KB21, and KB26; lane A3 to F3: KB02, KB07, KB12, KB17, KB22, and KB27; lane A4 to F4: KB03, KB08, KB13, KB28, KB23, and KB28; lane A5 to F5: KB04, KB09, KB14, KB19, KB24, and KB29; lane A6 to F6: KB05, KB10, KB15, KB20, KB25, and KB30.

CCD-camera based gel documentation system (GelDoc 1000; Bio-Rad, Hercules, CA, USA). An 100 bp DNA ladder (Bioneer Inc., Daejeon, Korea) was included in each gel to determine the molecular size of the PCR products.

Results and Discussion

Multiplex PCR assay and detection of food-borne pathogenic bacteria in *kimbab* samples PCR amplification was carried out as described in previous section. Primers sets obtained were initially tested for amplification of specific products against DNA of reference strains. Specific bands were obtained as 108 bp (Sau, *S. aureus* KCTC 1916), 284 bp (Sal, *S. enterica* KCTC 12400, *S. enteritidis* KCTC 12456, and *S. typhimurium* KCTC 1925), 404 bp (Lmo, *L. monocytogenes* KCTC 3569), 475 bp (Bce, *B. cereus* KCTC1012), 600 bp (Shi, *S. flexneri* KCTC 2008, and *S. sonnei* KCTC 2518) (Fig. 1A). Multiplex PCR was performed with samples incubated at increasing time in TSB. Bacterial count was rapidly increased from 0.32 (0 hr incubation) to 10.5 log CFU/mL (24 hr incubation) (Fig. 2A). The PCR result showed that each primer pair in the mixture was sensitive and specific enough to detect its target DNA sequence.

Multiplex PCR was done to detect the presence of pathogenic organisms in different *kimbab* samples to obtain a conclusion of the level of contamination. Samples collected in June (KB01-KB 10) (Fig. 2A and 2B), in July (KB11-KB20) (Fig. 2C and 2D), and August (KB21-KB 22) (Fig. 2E and 2F) were analyzed by multiplex PCR for detection of pathogenic bacteria. Multiplex PCR in *kimbab* samples KB06, KB12, KB18, and KB 26 did not detect any organisms. Detection of organisms can be visualized by multiplex PCR in all the 24 samples which shows the contamination rate of different microorganisms (Fig. 2).

To rapidly detect multiple microorganisms in a single reaction, simultaneous amplification of more than one locus is required; a methodology referred to as multiplex PCR in which several specific primer sets are combined into a single PCR assay. Multiplex PCR might also be useful to define the structure of certain microbial communities, e.g., food-borne pathogens in food samples (1,2,14,26). Like the majority of molecular techniques for the detection,

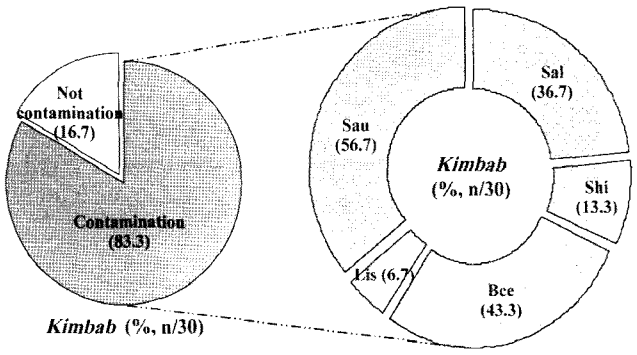

Fig. 3. Contamination rate of food-borne pathogenic bacteria in the 30 *kimbab* samples.

identification, and classification of bacteria (27-29), multiplex PCR commonly targets the 16S rRNA gene, the gene most widely used to infer phylogenetic relationships among bacteria (30). This gene is sometimes insufficient to distinguish closely related species. Thus, in order to ensure high specificity of multiplex PCR, other genes need to be taken into account for primer design (31).

This multiplex PCR assay could not detect any pathogens in the pre-enrichment stage at 0 hr (0.32 log CFU/mL). Detection of the 5 food-borne pathogens was possible after 4 hr (3.87 log CFU/mL) enrichment culture. Taking into account that a 24 hr enrichment culture reaches counts of 4.5 log CFU/mL *S. enterica*, 3.8 log CFU/mL *S. enteritidis*, 4.2 log CFU/mL *S. typhimurium*, 3.6 log CFU/mL *S. flexneri*, 3.9 log CFU/mL *S. sonnei*, 3.2 log CFU/mL *B. cereus*, 3.5 log CFU/mL *L. monocytogenes*, and 3.8 log CFU/mL *S. aureus*, respectively (data not shown), this assay can be used for the simultaneous detection of both equal and unequal amounts of all food borne pathogens. The detection limit of food borne pathogens after pre-enrichment is as low as sample (>0.35 log CFU/g) tested. Previously, the multiplex assay could concurrently detect in the pork ground inoculate with 3.2 log CFU/g *Escherichia coli* O157:H7, 3.15 log CFU/g *S. typhimurium*, and 3.2 log CFU/g *S. flexneri* (13). Recently, the multiplex assay was evaluated using lettuce artificially contaminated with *Yersina enterocolitica*, *S. aureus*, and *S. flexneri*. After 24 hr enrichment cultures, all 3 kinds of bacteria were detected the levels of 5.2, 5.4, and 5.1 log CFU/mL, respectively (32). Also, Kim *et al.* (2) reported that the multiplex PCR procedure was established to detect *E. coli*, *L. monocytogenes*, and *S. typhimurium* in artificially inoculated wheat grain. After 24 hr enrichment, these bacteria were detected the levels of 6.1, 7.3, and 6.9 log CFU/mL, respectively.

It is worth mentioning here that it is almost difficult to recover small number cells from such complex sample matrices and these samples often contribute large number of undefined PCR inhibitors thereby drastically reducing the overall sensitivity of any PCR based assay. Therefore under such circumstances pre-detection enrichment of microbial contaminants under suitable growth condition is invariably an essential prerequisite for a successful PCR amplification. However, some components (organic and phenolic compounds, color agents, glycogen, fats, and calcium ion etc.) of various foods and chemicals required for selective enrichment of cells may influence the effectiveness of the PCR and cause inhibitory effects (19,33-36). Taking into account the enrichment culture this assay can be used for the simultaneous detection of both equal and unequal amounts of food-borne pathogens in *kimbab*.

Distribution of food-borne pathogenic bacteria in *kimbab* samples Colony counts using visible plate method was performed for 30 *kimbab* samples purchased randomly from Korean supermarket at 3 different sampling periods. Ten *kimbab* samples were randomly collected from each season. Visible cell numbers varied from 4.14-5.03, 3.61-4.47, and 4.10-5.11 log CFU/g in June, July, and August seasons, respectively (Table 3).

Among the 30 *kimbab* samples, obtained 83.3% samples were contaminated and 16.7% samples were free from contamination. The highest rate of contamination was with the *S. aureus* (56.7%) followed by *B. cereus* (43.3%), *Salmonella* spp. (36.7%), *Shigella* spp. (13.3%), and *L.*

Table 3. Distributions of the total visible cell number in the 30 *kimbab* samples at the different sample periods (June, July, and August)

Sample periods					
June		July		August	
Sample name	Cell number (log CFU/g)	Sample name	Cell number (log CFU/g)	Sample name	Cell number (log CFU/g)
KB01	4.43	KB11	4.34	KB21	5.11
KB02	4.16	KB12	4.28	KB22	4.94
KB03	5.02	KB13	4.13	KB23	4.69
KB04	4.89	KB14	4.31	KB24	4.44
KB05	4.52	KB15	3.61	KB25	4.29
KB06	4.14	KB16	4.24	KB26	4.10
KB07	4.37	KB17	4.34	KB27	4.50
KB08	4.77	KB18	4.02	KB28	4.32
KB09	4.56	KB19	4.47	KB29	4.37
KB10	4.31	KB20	4.26	KB30	4.82
Average	4.52	Average	4.20	Average	4.56

monocytogenes (6.7%) (Fig. 3).

In previous report, the contamination rate of total aerobic bacteria and coliforms that includes *E. coli* and *Salmonella* spp. in *kimbab* is 10^4 to 10^6 CFU/g. *E. coli* (including *Salmonella* spp.) counts were significantly higher in *kimbab* (65%: 0.7-2.6 log CFU/g) whereas *S. aureus* (45%: 2.61-3.50 log CFU/g) and *B. cereus* (20%: 070-3.18 log CFU/g) were also not significantly different and *L. monocytogenes* were not detected from general restaurants (3). In this study, we could detect *L. monocytogenes* since we used enrichment culture for our multiplex PCR analysis. Recently, Kim *et al.* (37) reported that out of 258 samples tested 20 samples were contaminated with *S. aureus* and 12 samples were contaminated with *B. cereus*.

According to the KFDA (38) limitations of *S. aureus* is 100 CFU/g of RTE food, *B. cerrus* is 1,000 CFU/g, *Salmonella* spp., *Shigella* spp., and *L. monocytogenes* is 0 CFU/g of RTE food. Potential sources of contamination with *S. aureus*, such as the throat, hands and nails of food handling personnel have been discussed previously (39). Food contact surfaces such as grinders, knives, storage utensils, cutting blocks, and saw blades may also be sources of contamination. Several outbreaks of food-borne listeriosis have occurred previously in a variety of foods including RTE foods have been found contaminated with *L. monocytogenes* (40,41). *B. cereus* is increasingly recognized as the etiological agent of gastrointestinal and nongastrointestinal diseases. Due to the increasing number of reports of food-borne disease, especially of severe cases, fast detection methods are required for diagnostic purposes as well as for the prevention of food contamination and food-borne outbreaks. For example, the symptoms caused by emetic *B. cereus* resemble those caused by *S. aureus* (42). The developed assays could contribute substantially to determining the true incidence of the emetic type of food poisoning caused by *B. cereus*, especially by reducing the rate of misdiagnosis.

Salmonella spp. is one of the most important pathogenic genera implicated in food-borne bacterial outbreaks and diseases (43). Traditional detection methods for *Salmonella* were based on cultures using selective media and followed by a series of biochemical and/or serological tests. As long as 5-7 day are generally required to confirm the presence of *Salmonella* species. *S. enteritidis* internally contaminated eggs have been implicated as leading sources of transmission of *S. enteritidis* to humans (44). This could one of the reasons for *S. enteritidis* contamination in *kimbab* samples.

Shigella spp. mainly constitutes 4 species namely *S. boydii*, *S. dysenteriae*, *S. flexneri*, and *S. sonnei* (45). *Shigella* can contaminate several kinds of foods, including raw vegetables, milk, poultry, and some dairy products (46). Contamination of the *kimbab* samples with *Salmonella* spp., *Shigella* spp., *B. cereus*, *L. monocytogenes*, and *S. aureus* could have potential risk involving the above listed symptoms with possible infection rate among *kimbab* consumers (3,4). According to KFDA, a *kimbab* should contain 0 CFU/g of *Salmonella* spp., *L. monocytogenes*, and *Shigella* spp. but our finding suggests that *kimbab* contains potential amount of *Salmonella* spp., *L. monocytogenes*, *Shigella* spp., *B. cereus*, and *S. aureus* accounting to a total of 3.61-5.11 CFU/g in 30 *kimbab* samples collected from 3 months.

In conclusion, we describe a rapid detection method for food-borne pathogenic microorganisms in *kimbab* samples by multiplex PCR. The PCR assay described in this study is a quick and reliable method to detect the presence of 5 bacterial strains in *kimbab* samples. Use of this sensitive methodology will allow a better assessment of the frequency of which *kimbab* is contaminated with these food born pathogens then does the traditional spread plate method. Unfortunately, hazard analysis critical control point (HACCP) systems have not yet been implemented since it is prepared in retail stores. In light of the results presented in this study, there is a new emphasis on good manufacturing practice (GMP) and on retail HACCP, which should be implemented to enhance food safety in *kimbab* preparation.

Acknowledgments

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