



Novel multiplex PCR for the detection of the *Staphylococcus aureus* superantigen and its application to raw meat isolates in Korea

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Abstract

A multiplex PCR assay that allows for the rapid screening of the 19 genes that encode staphylococcal enterotoxins (SEs) (*sea* to *see*, and *seg* to *sei*), SE-like (SEL) toxins (*sej* to *ser*, and *seu*), and toxic shock syndrome toxin-1 (TSST-1) (*tst*) was developed in this study. These toxins are included in the pyrogenic toxin superantigen (PTSAg) family and are responsible for many diseases such as staphylococcal food poisoning (SFP) and TSS. The primers were designed based on dual priming oligonucleotide (DPO) technology to detect all of the 19 SAg genes in three sets of PCR. The developed multiplex PCR was applied to 143 *Staphylococcus aureus* strains isolated from pork and chicken meat in Korea. Almost 50% of the strains possessed at least one of the 19 SAg genes. The most frequently found genes were *seg*, *sei*, *sem*, and *sen* (53 isolates, 37%), which were often found simultaneously in the same isolate. In those isolates, the *seo* (39 isolates, 27%) or *seu* (6 isolates, 4%) genes were frequently found together and this combination (*seg*, *sei*, *sem*, *sen*, and *seo* or *seu*) was considered to be a part of the enterotoxin gene cluster (*egc*). The *sea* gene (10 isolates, 7%) was the gene most frequently detected out of all the classical SE genes (*sea* to *see*). Although these classical SEs are considered to be major etiological factors in SFP, newly described SE or SEL genes (*seg* to *ser*, and *seu*) were more frequently detected than the classical SE genes in this study. There was no isolate detected containing the *seb*, *sec*, *sek*, *sel*, or *seq* genes. *S. aureus* possessing mobile genetic elements known to encode these SAg genes, such as *egc*, were presumed to be widely distributed among pork and chicken meats in Korea. The multiplex PCR developed in this study could be applied to the investigation of SAg genes in *S. aureus* strains isolated from various sources. © 2007 Published by Elsevier B.V.

Keywords: Staphylococcal enterotoxin; Superantigen; Multiplex PCR; DPO; *egc*

1. Introduction

Staphylococcus aureus produces staphylococcal enterotoxins (SEs) which are responsible for staphylococcal food poisoning (SFP), a major type of food-borne illness (Balaban and Rasooly, 2000) and the toxic shock syndrome toxin-1 (TSST-1), which is known to cause TSS in humans (Dinges

et al., 2000). Both the SEs and TSST-1 are included in the pyrogenic toxin superantigen (PTSAg) family. SAGs, unlike conventional antigens, bind to the outside of the major histocompatibility complex (MHC) class II molecules and form a complex with the V β chain of a T-cell receptor (TCR). It leads to the stimulation of T-cell proliferation in a nonspecific manner causing the host immune system to be finally suppressed (Dinges et al., 2000; Chang et al., 2005).

SEs are named according to their emetic activities as the etiological factor of SFP, but the exact mechanism is not yet fully understood. Several SEs are described as SE-like (SEL) toxins because they lack the emetic properties or their emetic effects have not been tested in a primate model (Lina et al., 2004). Thus, a

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total of 19 SEs and SEIs have been introduced thus far. Classical SEs, ranging from SEA to SEE, are traditionally known as common causes of SFP (Balaban and Rasooly, 2000), and 14 additional types of SEs (SEG to SEI) or SEIs (SEIJ to SEIR, SEIU, and SEIV) have been newly described (Ren et al., 1994; Su and Wong, 1995; Munson et al., 1998; Zhang et al., 1998; Fitzgerald et al., 2001; Jarraud et al., 2001; Kuroda et al., 2001; Orwin et al., 2001, 2002; Yarwood et al., 2002; Letertre et al., 2003; Omoe et al., 2003; Thomas et al., 2006).

Many of the SAg genes are encoded in the mobile genetic elements, such as plasmids, prophages, and staphylococcal pathogenic islands (SaPIs) (Kuroda et al., 2001; Baba et al., 2002; Novick, 2003; Schmidt and Hensel, 2004), which are responsible for the horizontal transfer of virulence or antibiotic resistance genes between strains of *S. aureus* (Ubeda et al., 2005; Maiques et al., 2006). Novel types of SE genes can be generated during the transfer of these elements. For example, the enterotoxin gene cluster (*egc*) containing several SE or SEI genes (*seg*, *sei*, *sem*, *sen*, *seo*, and *seu*) is a possible source of new SE genes (Jarraud et al., 2001; Thomas et al., 2006). Therefore, the investigation of the distribution of SAg genes in *S. aureus* isolates is needed not only for epidemiological tracing but also for understanding the evolution of virulence factors in *S. aureus*.

Many SFP outbreaks are suspected to be caused by newly described SEs or SEIs (Ikeda et al., 2005; Jorgensen et al., 2005). However, immunological assay kits currently used for the detection of SEs have limitations because they can only detect the classical SEs. Therefore, the DNA-based approach is thought to be an essential tool for investigating these newly identified SE or SEI genes (Omoe et al., 2002). Several multiplex PCR systems have been developed for studying the SAg gene profile and they save time and labor by detecting many SAg genes simultaneously (Becker et al., 1998; Monday and Bohach, 1999; Sharma et al., 2000; Omoe et al., 2005). However, these multiplex PCR methods do not include the newly described SE genes or the number of PCR reactions greater than 4 reactions.

Recently, dual priming oligonucleotide (DPO) has been developed, which contains two separate primer segments joined by a polydeoxyinosine linker (Chun et al., in press). This structure made one primer anneal on the two sites with its two segments which have distinct properties. The primer applying this technology generated high PCR specificity under sub-optimal conditions and improved the specificity of the multiplex PCR. Therefore, in this study, a novel multiplex PCR system for detecting the 19 kinds of SAg genes (*sea* to *see*, *seg* to *ser*, *seu* and *tst*) in three sets of the reactions was developed based on the DPO technology and was applied to *S. aureus* strains isolated from pork and chicken meat in Korea.

2. Materials and methods

2.1. Bacterial strains and isolation of genomic DNA

Eight reference strains whose toxin gene compositions or whole genome sequences were revealed in previous studies were used to establish the multiplex PCR (Table 1). A total of 143 *S. aureus* strains were isolated from pork and chicken meat

obtained from retail stores (20 and 38 isolates, respectively), pork carcasses (36 isolates) and the rinsing water of chicken carcasses (49 isolates) from abattoirs in Korea between 2001 and 2003. *S. aureus* was identified by colony morphology on Baird-Parker agar (Becton Dickinson; BD, Franklin Lakes, NJ, USA), gram staining, catalase, oxidase, DNase, TNase, and VP tests, and was confirmed by PCR targeting the *S. aureus*-specific *nuc* gene according to Brakstad et al. (1992). Genomic DNA was isolated using a DNeasy tissue kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions with a modification of the cell lysis step performed with 50 U/ml lysostaphin (Sigma, St. Louis, MO, USA). The density of the isolated DNA was estimated using Specgene (Techne, Cambridge, UK) at A_{260} and the DNA was eluted to a density of 45 ng/ μ l.

2.2. Primer design

Primers specific to 19 kinds of SAg genes (*sea* to *see*, *seg* to *ser*, *seu* and *tst*) in 3 sets of the reaction were designed based on DPO technology. The *femA* gene encoding the FemA protein, which is involved in the synthesis of the staphylococcal cell wall (Mehrotra et al., 2000), was used as an internal positive control target to confirm the fidelity of the PCR. The primers used to detect the *femA* gene were included in every set of the reaction. The primer sequences, their predicted PCR product sizes, and the type of PCR set (1, 2, or 3) in which they were included are shown in Table 2.

2.3. Single PCR reactions and sequence analysis

Single PCR reactions were performed on reference strains with each primer pair to evaluate the specificity of the primer. Each reaction mixture contained 1 μ l of prepared template DNA, 0.5 μ M of each primer, 5 μ l of 10 \times buffer (Takara, Kyoto, Japan), 2 mM MgCl₂ (Takara), 200 μ M each of dGTP, dATP, dTTP, and dCTP (Takara), and 0.5 U of EX Taq DNA polymerase (Takara). The final volume was adjusted to 50 μ l with distilled water. PCR was performed in a Mastercycle gradient (Eppendorf, Hamburg, Germany) with the following steps: 94 °C for 10 min, 30 cycles of 94 °C for 30 s, 55 °C for

Table 1
S. aureus reference strains used in this study

Strain	Superantigen gene(s)	Reference
FRI137	<i>sec</i> , <i>seg</i> , <i>seh</i> , <i>sei</i> , <i>sel</i> , <i>sem</i> , <i>sen</i> , <i>seu</i>	Bania et al. (2006)
FRI361	<i>sec</i> , <i>sed</i> , <i>seg</i> , <i>sei</i> , <i>sej</i> , <i>sel</i> , <i>sem</i> , <i>sen</i> , <i>seo</i> , <i>ser</i>	Omoe et al. (2002)
FRI472	<i>sed</i> , <i>seg</i> , <i>sei</i> , <i>sej</i> , <i>sem</i> , <i>sen</i> , <i>seo</i> , <i>ser</i>	Monday and Bohach (1999)
FRI569	<i>seh</i>	Monday and Bohach (1999)
FRI913	<i>sea</i> , <i>sec</i> , <i>see</i> , <i>sek</i> , <i>sel</i> , <i>seq</i> , <i>tst-1</i>	Bania et al. (2006)
MNHOCH	<i>seb</i>	Monday and Bohach (1999)
N315	<i>sec</i> , <i>seg</i> , <i>sei</i> , <i>sel</i> , <i>sem</i> , <i>sen</i> , <i>seo</i> , <i>sep</i> , <i>tst-1</i>	Kuroda et al. (2001)
RN4220	None of the toxin genes	Monday and Bohach (1999)

Table 2
Primer sequences and predicted product size

Target gene	Primer	Oligonucleotide sequence (5'–3') ^a	Accession no.	Product size (bp)	PCR set
<i>sea</i>	SEA-f	ATGGTTATCAATGTGCGGGT <u>GHIIII</u> CCAAACAAAAC	M18970	344	2
	SEA-r	TGAATACTGTCTTGAGCACCA <u>IIIIII</u> ATCGTAATTAAC			
<i>seb</i>	SEB-f	TGGTATGACATGATGCCTGCAC <u>IIIIII</u> GATAAAATTTGAC	M11118	196	1
	SEB-r	AGGTACTCTATAAGTGCCTGCCT <u>IIIIII</u> ACTAACTCTT			
<i>sec</i>	SEC-f	GATGAAGTAGTTGATGTGTATGGATC <u>IIIIII</u> ACTATGTAAAC	X05815	399	2
	SEC-r	AGATTGGTCAAACCTATCGCCTGG <u>IIIIII</u> GCATCATATC			
<i>sed</i>	SED-f	CTGAATTAAGTAGTACCGCCT <u>IIIIII</u> ATATGAAAC	M28521	451	1
	SED-r	TCCTTTTGCAAATAGCGCCTT <u>IIIIII</u> GCATCTAATTC			
<i>see</i>	SEE-f	CGGGGGTGTAAACATTACATGAT <u>IIIIII</u> CCGATTGACC	M21319	286	3
	SEE-r	CCCTTGAGCATCAAACAAATCATA <u>IIIIII</u> CGTGGACCCTTC			
<i>seg</i>	SEG-f	ATAGACTGAATAAGTTAGAGGAGGT <u>IIIIII</u> GAAGAAATATC	AF064773	594	3
	SEG-r	TTAGTGAGCCAGTGTCTTGC <u>IIIIII</u> AAATCTAGTTC			
<i>seh</i>	SEH-f	CATTCACATCATATGCGAAAGCAG <u>IIIIII</u> TTACACG	U11702	218	3
	SEH-r	CTTCTGAGCTAAATCAGCAGTTGC <u>IIIIII</u> TTACTCTC			
<i>sei</i>	SEI-f	AGGCGTCACAGATAAAAACCTACC <u>IIIIII</u> CAAATCAACTC	AF064774	154	1
	SEI-r	ACAAGGACCATTATAATCAATGCC <u>IIIIII</u> TATCCAGTTTC			
<i>sej</i>	SEJ-f	TGTATGGTGGAGTAACACTGCATG <u>IIIIII</u> AAATCAACTTTATG	AF053140	102	1
	SEJ-r	CTAGCGGAACAACAGTTCTGATG <u>IIIIII</u> ATCCATAAAT			
<i>sek</i>	SEK-f	GTGTCTTAATAATGCCAGCGCT <u>IIIIII</u> CGATATAGG	U93688	282	2
	SEK-r	CGTTAGTAGCTGTGACTCCACC <u>IIIIII</u> TGTATTAG			
<i>sel</i>	SEL-f	ATTCACCAGAATCACACCGCT <u>IIIIII</u> TACTCGTA	AF217235	469	3
	SEL-r	GTGTAATAAATATACAGAG <u>IIIIII</u> AGAACCATCATT			
<i>sem</i>	SEM-f	CGCAACCGCTGATGCGG <u>IIIIII</u> TGAATCTTAGG	AF285760	572	2
	SEM-r	CAGCTTGTCTGTTCCAGTATC <u>IIIIII</u> AGTCATAAG			
<i>sen</i>	SEN-f	TCATGCTTATACGGAGGAGTTAC <u>IIIIII</u> TGATGGAAATC	AF285760	103	2
	SEN-r	AACCTTCTTGTGGACACCATC <u>IIIIII</u> ATACATTAACGC			
<i>seo</i>	SEO-f	GTGGAATTTAGCTCATCAGCGATTT <u>IIIIII</u> AAATTTCTAGG	AF285760	116	3
	SEO-r	GTACAGGCAGTATCCACTTGATGC <u>IIIIII</u> ATGACAATGTGC			
<i>sep</i>	SEP-f	ATCATAACCAACCGAATCACCAG <u>IIIIII</u> GGGTGAAACTC	BA00018	547	1
	SEP-r	GTCTGAATTGCAGGGAACCTGC <u>IIIIII</u> GCAATCTTAG			
<i>seq</i>	SEQ-f	GGTGAATTACGTTGGCGAATCA <u>IIIIII</u> TAGATAAAACC	U93688	330	1
	SEQ-r	CTCTGCTTGACCAGTTCGGT <u>IIIIII</u> CAAATCGTATG			
<i>ser</i>	SER-f	TTCAGTAAGTGCTAAACCAGATCC <u>IIIIII</u> CTGGAGAATTG	AB075606	368	1
	SER-r	CTGTGGAGTGCATTGTAACGCC <u>IIIIII</u> ATATGCAAACCTCC			
<i>seu</i>	SEU-f	ATGGCTTAAAATTGATGGTTCTA <u>IIIIII</u> TTAAAAACAG	AY205307	410	3
	SEU-r	GCCAGACTCATAAGGCGAACTA <u>IIIIII</u> TTATATAAAA			
<i>tst</i>	TST-f	GTTGCTTGCACAACCTGCTACAG <u>IIIIII</u> ACCCCTGTTC	J02615	209	2
	TST-r	TCAAGCTGATGCTGCCATCTGTG <u>IIIIII</u> TATACGCATAG			
<i>femA</i>	Fem-f	ACAGCTAAAGAGTTTGGTGCCT <u>IIIIII</u> GATAGCATGC	M23918	723	1, 2, 3
	Fem-r	TTCATCAAAGTTGATATACGCTAAAGGT <u>IIIIII</u> CACACGGTC			

^aUnderlined base pairs are polydeoxyinosine bridge region.

30 s, and 72 °C for 30 s with a final extension at 72 °C for 10 min. The amplified PCR products were resolved by electrophoresis in a 0.8% agarose (Sigma) gel at 100 V for 30 min and were purified from the gels using a QIAquick Gel extraction kit (QIAGEN). The sequencing of the extracted PCR product was performed by Bionics Co., Ltd (Seoul, Korea) and the data were analyzed using the DNASTAR software (DNASTAR, Inc., Madison, WI, USA).

Other PCR systems which were described by Omoe et al. (2005) (for *sea* to *ser* and *tst* genes) and Letertre et al. (2003) (for the *seu* gene) were applied to the reference strains to validate the results.

2.4. Multiplex PCR reactions

Multiplex PCR reactions were performed with 1 µl of the prepared template DNA, 10 µl of 5× primer mixture (0.5 µM of

each primer), and 25 µl of 2× Multiplex Master Mix (Seegene) and the final volume was adjusted to 50 µl with distilled water. The PCR was performed using the following steps: 94 °C for 15 min, 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 2 min with a final extension at 72 °C for 10 min. The amplified PCR products were resolved by electrophoresis in a 2% agarose (Sigma) gel at 100 V for 60 min.

The multiplex PCR was performed with serial dilutions of template DNA (24 steps from 45 ng/µl to 5 fg/µl) from reference strains to determine the sensitivity.

2.5. Statistical analysis

The detection rate of the SE and *tst* genes in the *S. aureus* isolates from animal origins (pork and chicken) was statistically compared using Fisher's exact test (Analyse-it Software, Ltd., Leeds, England, UK).

3. Results

3.1. Specificity of the primers

Each primer pair was tested individually on *S. aureus* reference strains to ensure specificity. All of the PCR products showed the expected size without nonspecific reaction and were coordinated with the results of the PCR described by Omoe et al. (2005) and Letertre et al. (2003). The *seo* gene was not detected in the FRI137 (ATCC 19095) strain, and this result was confirmed using the primers of Omoe et al. (2005) although Bania et al. (2006) reported that the *seo* gene was detected in this strain. An additional finding reported the presence of the *sem*, *sen*, *seo*, and *ser* genes in the FRI472 strain, which was previously reported as having the *sed*, *seg*, *sei*, and *sej* genes (Monday and Bohach, 1999; Kwon et al., 2004). The sequencing results confirmed that all of the sequences of the PCR products showed 100% homology with the sequences in GenBank (data not shown).

3.2. Multiplex PCR reactions on reference strains

The primer sets successfully amplified the target genes in the multiplex PCR without nonspecific or additional bands on the reference strains (Fig. 1). The intensities of the bands were different depending on the number of target genes present in the strain. In a strain possessing fewer numbers of genes, the bands showed higher intensities especially in the strains yielding larger amplicons. However, there was no difficulty in discriminating between each band in all of the cases.

The sensitivity of the multiplex PCR was estimated by the minimal concentrations that could produce all of the expected bands. The reaction that had the highest minimal concentration was set 2 when the concentration of template DNA was about 200 fg/μl (data not shown).

3.3. Superantigen genes in pork and chicken meat isolates

Seventy-two (50%) of the 143 *S. aureus* isolates contained at least one toxin gene (Table 3). All of the isolates produced a band corresponding to *femA* (723 bp), an internal positive control, and there was no nonspecific reaction. All of the multiplex PCR results were confirmed by a single PCR reaction with the primers designed in this study. The most frequently detected genes were *seg*, *sei*, *sem*, and *sen* (53 isolates, 37%) and they were always found together in the same isolate. The *seo* (39 isolates, 27%) and *seu* (6 isolates, 4%) genes were frequently detected along with the *seg*, *sei*, *sem*, and *sen* genes, except for one strain that had the *sed*, *sej*, *ser*, and *seo* genes. The *see* gene (9 isolates, 6%) was also found together with the *seg*, *sei*, *sem*, and *sen* genes. The *sea* and *seh* genes, which were almost solely detected without other genes, were found in 10 (7%) and 12 isolates (8%), respectively. The *sep* (3 isolates, 2%) and *tst* (2 isolates, 1%) genes were also found, but the *seb*, *sec*, *sek*, *sel*, and *seq* genes were not detected in this study. The genes that showed significantly different detection rates

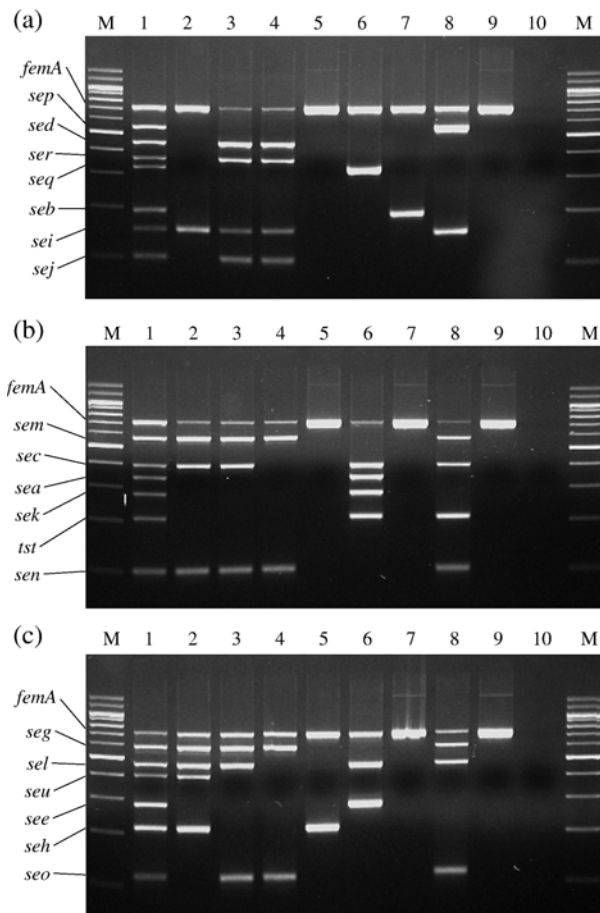


Fig. 1. Multiplex PCR results of each set on *S. aureus* reference strains. (a) Set 1: *sej* (102 bp), *sei* (154 bp), *seb* (196 bp), *seq* (330 bp), *ser* (368 bp), *sed* (451 bp), *sep* (547 bp), and *femA* (723 bp). (b) Set 2: *sen* (103 bp), *tst* (200 bp), *sek* (282 bp), *sea* (344 bp), *sec* (399 bp), *sem* (572 bp), and *femA* (723 bp). (c) Set 3: *seo* (116 bp), *seh* (218 bp), *see* (286 bp), *seu* (410 bp), *sel* (469 bp), *seg* (594 bp), and *femA* (723 bp). M: 100 bp marker, Lane 1: size marker of positive bands, Lane 2: FRI137 (*sei*, *sen*, *sec*, *sem*, *seh*, *seu*, *sel*, *seg*, and *femA*), Lane 3: FRI361 (*sej*, *sei*, *ser*, *sed*, *sen*, *sec*, *sem*, *seo*, *sel*, *seg*, and *femA*), Lane 4: FRI472 (*sej*, *sei*, *ser*, *sed*, *sen*, *sem*, *seg*, *seo*, and *femA*), Lane 5: FRI569 (*seh* and *femA*), Lane 6: FRI913 (*seq*, *tst*, *sek*, *sea*, *sec*, *see*, *sel*, and *femA*), Lane 7: MNHOCH (*seb* and *femA*), Lane 8: N315 (*sei*, *sep*, *sen*, *tst*, *sec*, *sem*, *seo*, *sel*, *seg*, and *femA*), Lane 9: RN4220 (*femA*, control with no toxin), Lane 10: Distilled water (negative control).

between the isolates that originated from pork and chicken were the *see*, *seu* and *seh* genes ($p < 0.05$). The *see* and *seu* genes were detected only in the pork isolates and the *seh* gene was detected only in the chicken isolates in this study.

4. Discussion

Multiplex PCR is an essential tool for a high throughput gene screening of pathogenic bacteria (Markoulatos et al., 2002). In the case of SAg genes in *S. aureus*; however, designing multiplex PCR primers having similar annealing temperatures, distinguishable product sizes, and high specificity without cross reactions is very complicated because there are many kinds of SAg genes with high homology (Balaban and Rasooly,

to amplify the target genes without adversely increasing the annealing temperature.

The *sea* gene was most frequently detected classical SE gene. SEA is considered to be a primary cause of food poisoning (Balaban and Rasooly, 2000; Le Loir et al., 2003) and almost 90% of food poisoning isolates were reported to encode the *sea* gene in Korea (Cha et al., 2006). SEA also is a primary SE detected in food poisoning cases in Japan (Shimizu et al., 2000). The *sea* gene is carried by prophage ϕ Sa3mu (Kuroda et al., 2001) or encoded in ϕ Sa3mw along with other genes (*seg* and *sek*) (Baba et al., 2002). In this study, however, the *sea* gene was mostly frequently detected without other genes and was presumed to be related to ϕ Sa3mu. The *sed* gene is encoded on a plasmid named pIB485 with *sej* and *ser* (Zhang et al., 1998; Omoe et al., 2003) and this composition was found in one isolate in this study.

The *seh* gene was the second most frequently detected gene in the chicken isolates and was detected without other genes. SEH was found to have emetic activity (Su and Wong, 1995) and was considered to be a potential causative agent in food poisoning. The *sep* gene, known to be encoded on ϕ Sa3n (Kuroda et al., 2001), was also found in pork isolates. The SaPIbov carrying the *tst*, *sec*, and *sel* genes was discovered (Fitzgerald et al., 2001) and the co-existence of the *sec* and *tst* genes was observed in many studies (Orden et al., 1992; Becker et al., 2003). However, the *tst* gene was detected without *sec* in this study, and this suggests that another, unknown mobile element may be involved with the *tst* gene. Both the *seb* and *sec* genes were not detected, although *seb* was a prevalent gene in food poisoning cases reported in Taiwan (Chiang et al., 2006) and Japan (Shimamura et al., 2006) and *sec* was a major SE gene type in isolates from bulk milk in Switzerland (Scherrer et al., 2004) and in Korea (Yoon, 1998). The patterns of SE genes seemed to be variable between the different geographical origins and years. Differences in SE patterns according to the origin of the isolates were observed in this study and it was also previously reported (Smyth et al., 2005). This difference may result from host adaptations of *S. aureus* in the different animal species. However, more strains isolated from other animal sources must be studied further in order to prove this hypothesis.

In conclusion, we developed an easy and rapid multiplex PCR system showing high specificity capable of detecting 19 SAG genes of *S. aureus* in three sets of reactions. Toxin typing with this multiplex PCR system showed that the newly described SE or SEI genes were more prevalent than the classical SE genes in pork and chicken meat in Korea. Furthermore, currently known mobile genetic elements, especially variable types of the *egc*, were widely distributed. This multiplex PCR can be used to investigate SAG genes in *S. aureus* isolated from many other sources. In addition, it can provide further information about the distribution of these SAG genes and various mobile genetic elements for the understanding of the evolution of *S. aureus* as a pathogenic microorganism.

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