J. Microbiol. Biotechnol. (2007), **17**(3), 461–467

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

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Expression of Enterotoxin Genes in *Staphylococcus aureus* Isolates Based on mRNA Analysis

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Received: September 26, 2006
Accepted: November 21, 2006

Abstract *Staphylococcus aureus* strains are important foodborne pathogens that produce various toxins. To evaluate the risk of the enterotoxins, four *S. aureus* strains from *kimbap* and two clinical samples were isolated and identified, and their expression of the enterotoxin genes were analyzed using a reverse transcription real-time PCR. Various enterotoxin genes were detected, including *sea, seg, seh, sei, sen, seo*, and *sem*, where each isolate contained one or two. When the mRNA detection of the enterotoxin genes was analyzed using a reverse transcriptase PCR, various levels of expression were found depending on the species and enterotoxin gene. Therefore, it is reasonable to suggest that the poisoning risk of *S. aureus* can be effectively evaluated based on the gene expression at the mRNA level.

Keywords: *S. aureus*, food isolates, enterotoxin expression, real-time PCR

Staphylococcus aureus is recognized worldwide as an important foodborne pathogen, and the resulting diseases are characterized by such symptoms as nausea, vomiting, abdominal cramps, and diarrhea lasting from 24 to 48 h, where complete recovery usually requires 1–3 days. Staphylococcal foodborne diseases due to the consumption of contaminated food are the second most common cause of foodborne illnesses reported in the U.S. [2, 11, 18, 23, 27]. In Canada, an estimated $50 million is spent every year to manage *S. aureus* poisoning in hospitals, and the costs to the dairy industry are even higher [24, 43]. The number of reported cases in France was around 1,600 between 1999 and 2000 [28], and according to a report by the Korea Food and Drug Association, *S. aureus* poisoning increased from 363 patients in 2001 to 863 patients in 2005.

S. aureus strains are very important foodborne pathogen owing to their toxin-mediated virulence, invasiveness, and antibiotic resistance [26, 36]. *S. aureus* strains produce various virulence factors, including staphylococcal enterotoxins and toxic shock syndrome toxin 1 that induces superantigenic activity [29]. The enterotoxins have already been assigned to the pyrogenic toxin superantigen family based on their biological activity and structural similarity [3]. The major antigenic types of staphylococcal enterotoxin have been recognized as SEA to SEJ; however, further staphylococcal enterotoxins have recently been identified as SEK, SEL, SEM, SEN, SEO, and SEU [31, 33, 38, 44]. The following provides a brief overview of SEA to SEE. SEA is the cause of most food poisoning by *S. aureus*, is expressed in the mid-exponential phase, and its gene seems to be transferred by a temperate bacteriophage [6, 10]. Unlike the *seb, sec*, and *sed* genes, the *sea* gene is not regulated by an accessory gene regulator *agr* [45], is composed of 771 base pairs, and encodes an enterotoxin A precursor of 257 amino acid residues [20]. Unlike other staphylococcal enterotoxins, SEB is known to resist heat, is composed of about 267 amino acids and 27 signal peptide amino acids, and is included within the chromosome, plasmid, and transposon [1, 41, 42]. SEC is classified into three sub groups: SEC1, SEC2, and SEC3, plus *S. aureus* isolates from different animal species have been found to produce a unique host-specific SEC, suggesting that the toxin heterogeneity is due to the selection of modified SEC sequences that facilitate the survival of *S. aureus* isolates in

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their respective host [9, 20]. SED is also known to cause food poisoning with a frequency close to SEA, is included in a 27.6 kb penicillinase plasmid, and consists of 228 amino acids and 30 signal peptide amino acids [4, 12]. The SEE gene encodes a 29 kDa protein, which in a mature extracellular form has a molecular mass of 26 kDa, plus the DNA sequence of SEE is similar to that of SEA and SED [16]. Although the individual roles of staphylococcal enterotoxins in food poisoning have not yet been elucidated, they are all heat stable and resistant to most proteolytic enzymes, such as pepsin and trypsin, allowing their activities to continue in the digestive tract after ingestion [5].

However, previous studies of *S. aureus* enterotoxins have only focused on enterotoxin detection within certain countries, without any effective comparison and analysis of individual enterotoxin expression. Accordingly, this study used a reverse transcription real-time PCR to confirm the gene expression. Although this method has already been applied to confirm gene expression in other countries, most Korean studies have been limited to quantitative bacterial analyses.

Therefore, *S. aureus* strains were isolated from *kimbap* and identified. The enterotoxins in the *S. aureus* isolates were then detected using a PCR and TECRA kit-based immunoassay. In addition, the gene expression in the food isolates was compared with that in the clinical isolates using a reverse transcription real-time PCR.

Materials and Methods

Bacterial Strains and Culture Conditions

The *S. aureus* KCCM12103 and clinical isolates were obtained from the Microbiology Laboratory, Yonsei University Medical School, grown in a tryptic soy broth or agar (Difco Laboratory, Detroit, MI, U.S.A.), and incubated at 37°C for 18 h in an aerobic incubator. The wild-type *S. aureus* were isolated from ready-to-eat *kimbap*, rice rolled in laver, purchased from a convenience chain store during winter.

Isolation and Identification of *S. aureus* from Food Sample

The wild-type *S. aureus* were isolated according to the modified *Food Code* of Korea [25], where a 25-g food sample was homogenized in a stomacher and the homogenate was inoculated in a 10% NaCl tryptic soy broth for enrichment. The enriched broth was then incubated at 37°C for 24±2 h under aerobic conditions, diluted with 0.1% sterile peptone water, and spread onto a Baird-Parker agar (BPA) supplemented with egg-yolk tellulite (Difco, Detroit, U.S.A.). Thereafter, the BPA was incubated at 37°C for 24±2 h under the aerobic conditions, then presumptive colonies were picked, placed on new BPA, and further identified by Gram stain, catalase, and coagulase tests. A PCR was carried out for further identification of the *S. aureus* isolates. The primers targeted the staphylococcal *nuc* gene encoding the nuclease [36] were synthesized commercially (Bioneer, Daejon, Korea). For the PCR template, the whole-cell lysis method was used without DNA purification, and performed in a MJ minicycler (Bio-Rad, Hercules, U.S.A.). The PCR products were analyzed using a 1% TAE buffer with 0.5 mg/ml of ethidium bromide, and the gel were visualized and photographed under a UV transilluminator (Seolin, Suwon, Korea) after electrophoresis at 5 V/cm.

Detection of Staphylococcal Enterotoxins Using Commercial Kit

The food and clinical *S. aureus* isolates were incubated under aerobic conditions at 37°C for 18 h in a tryptic soy broth. After centrifugation of the cultured broth for 5 min at 10,000 ×*g*, the supernatant was transferred into a 1.5 ml tube for the detection of staphylococcal enterotoxins A to E using a TECRA staphylococcal enterotoxin VIA kit (TECRA, New South Wales, Australia) according to the manufacturer's instructions.

DNA Extraction and Amplification of Staphylococcal Enterotoxin Genes

One ml of an overnight culture in TSB was centrifuged at 10,000 ×*g* at 4°C for 5 min and washed two times with sterile distilled water. The DNA was then isolated using a DNA tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The detection of the genes encoding enterotoxins SEA to SEO was performed using the method described by Boerema *et al.* [8]. The primers used to detect the *sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, *sei*, *sej*, *sem*, *sen*, and *seo* genes were based on published sequences (Table 1). The PCR was performed using the method already described [37].

RNA Extraction and cDNA Synthesis

One ml of a culture solution (stationary phase state) in TSB was centrifuged at 10,000 ×*g* at 4°C for 5 min and washed two times with DEPC water. The resulting *S. aureus* pellet was then suspended in a 40 mg/ml lysozyme solution. The RNA was extracted using an RNeasy mini kit according to the manufacturer's recommendations, where the column was eluted with 50 µl of DEPC water. The residual DNA was digested using DNase (Sigma-Aldrich, St. Louis, U.S.A.) treatment. The concentration of the eluted total RNA was determined by the optical density (OD_{260}) using a SmartSpec Plus Spectrophotometer (Bio-Rad, Hercules, U.S.A.). The total RNA (~1 µg) was then used in a 20 µl reverse transcription reaction to synthesize cDNA using an AccuPower RT PreMix system and oligomers (Bioneer, Daejon, Korea). The reverse transcription reactions were performed for 1 h at 42°C and the reverse transcriptase

Table 1. Oligonucleotide primer sequences used in the present study.

Gene	Primer	Sequence (5'→3')	PCR product
sea	SEA1	TTGGAAACGGTTAAAACGAA	120 bp
	SEA2	GAACCTTCCCATCAAAAACA	
seb	SEB1	TCGCATCAAACTGACAAACG	478 bp
	SEB2	GCAGGTACTCTATAAGTGCC	
sec	SEC1	GACATAAAAGCTAGGAATTT	257 bp
	SEC2	AAATCGGATTAACATTATCC	
sed	SED1	CTAGTTTGGTAATATCTCCT	317 bp
	SED2	TAATGCTATATCTTATAGGG	
see	SEE1	TAGATAAAGTTAAAACAAGC	170 bp
	SEE2	TAACTTACCGTGGACCCTTC	
seg	SEG1	TGCTATCGACACACTACAACC	704 bp
	SEG2	CCAGATTCAAATGCAGAACC	
seh	SEH1	CGAAAGCAGAAGATTTACACG	495 bp
	SEH2	GACCTTTACTTATTTCGCTGTC	
sei	SEI1	GACAACAAAACTGTCGAAACTG	630 bp
	SEI2	CCATATTCTTTGCCTTTACCAG	
sej	ESJ1	CAGCGATAGCAAAAATGAAACA	426 bp
	ESJ2	TCTAGCGGAACAACAGTTCTGA	
sen	mpSEN-1	ATGAGATTGTTCTACATAGCTGCAAT	680 bp
	mpSEN-2	AACTCTGCTCCCACTGAAC	
seo	mpSEO-1	AGTTTGTGTAAGAAGTCAAGTGTAGA	180 bp
	mpSEO-2	ATCTTTAAATTCAGCAGATATTCCATCTAA C	
sem	mpSEM-1	CTATTAATCTTTGGGTTAATGGAGAAC	300 bp
	mpSEM-2	TTCAGTTTCGACAGTTTTGTTGTCAT	
16s rRNA	16sF	CCGCCTGGGGAGTACG	240 bp
	16sR3	AAGGGTTGCGCTCGTTGC	
Nuclease	Sa-1	GAAAGGGCAATACGCAAAGA	482 bp
	Sa-2	TAGCCAAGCCTTGACGAACT	

inactivated for 5 min at 94°C, as described in the commercial protocol.

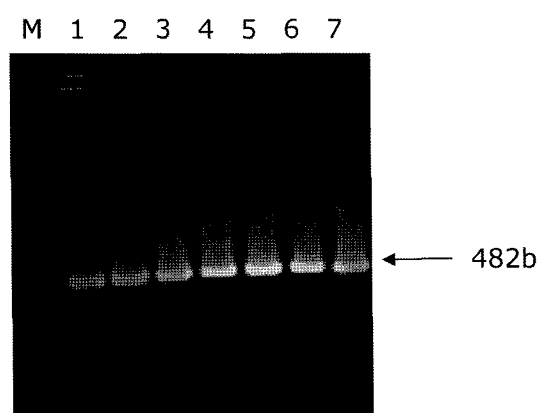

Fig. 1. Agarose gel electrophoresis of the *nuc* gene PCR product from *S. aureus* isolates.
M, molecular weight marker; 1, *S. aureus* KCCM12103; 2, *S. aureus* FI[a]6A; 3, *S. aureus* FI7A; 4, *S. aureus* FI14A; 5, *S. aureus* FI21A; 6, *S. aureus* CI[b]19; 7, *S. aureus* CI23; FI[a], Food *S. aureus* isolate; CI[b], Clinical *S. aureus* isolate.

Real-Time PCR

The real-time PCR analysis was performed using a LightCycler 2.01 Instrument system (Roche, Mannheim, Germany). The primers shown in Table 1 targeted the 16s rRNA and enterotoxin genes of *S. aureus*. Two µl of the relevant cDNA, 20 pmol of each primer, 100 mM KCl, 40 mM Tris-HCl (pH 8.4), 0.4 mM of each dNTP, 50 U/ml of iTaq DNA polymerase, 6 mM $MgCl_2$, and 20 nM SYBR Green I were all included in a 20 µl capillary PCR tube. The cycling program used was as follows: one denaturation cycle for 5 min at 95°C and 45 amplification cycles for 1 min at 95°C, annealing for 1 min at 55°C, extension for 1 min at 72°C, and termination for 5 min at 72°C. Fluorescence readings were taken after each extension step, which was followed by a melting curve analysis at 65–99°C (temperature transition rate of 0.1°C/sec) based on continuous fluorescence readings. The expression of the staphylococcal enterotoxin genes was calculated relative to the calibration sample and an endogenous control (16S rRNA) to normalize the sample input amount [40]. All the experiments were performed twice.

Table 2. Detection of staphylococcal enterotoxins using TECRA SET ID VIA.

	EnterotoxinA	Enterotoxin B	Enterotoxin C	Enterotoxin D	Enterotoxin E
S. aureus FI [a]6A	+	–	–	–	–
S. aureus FI 7A	–	–	–	–	–
S. aureus FI 14A	+	–	–	–	–
S. aureus FI 21A	+	–	–	–	–
S. aureus CI [b]19	–	–	+	–	–
S. aureus CI 23	–	–	+	–	–

+, Detected; –, not detected.
FI[a], Food *S. aureus* isolate; CI[b], Clinical *S. aureus* isolate.

Results and Discussion

Isolation and Identification of *S. aureus* from Ready-to-Eat *Kimbap*

To isolate *S. aureus* from 50 *kimbap* samples, the suspicious colonies were analyzed based on the biochemical characteristics of Gram stain, catalase, and coagulase tests (data not shown), and reconfirmed by a PCR. The isolates showed a 482-bp product in the PCR analysis, which was the same as *S. aureus* KCCM12103 (Fig. 1). Finally, four strains of *S. aureus* were isolated and identified from the 50 *kimbap* samples

Detection of Staphylococcal Enterotoxins Using Commercial Kit

The production of enterotoxins by the food and clinical *S. aureus* isolates was confirmed using a TECRA kit, which can detect staphylococcal enterotoxins A (SEA) to E (SEE) based on an immunoassay method. As a result of the toxin analysis using the TECRA kit, the toxin production pattern confirmed SEA in the food isolates *S. aureus* (FI)6A, *S. aurues* FI14A, and *S. aureus* FI21A, and SEC in the clinical isolates *S. aureus* (CI)19 and *S. aureus* CI23 (Table 2).

Detection of Staphylococcal Enterotoxin Genes Using PCR

To detect the enterotoxin genes for *sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, *sei*, *sej*, *sen*, *seo*, and *sem* in each isolate, a PCR was performed and the results compared with the enterotoxin genes (Table 3 and Figs. 2 and 3). Consequently, the *sei*, *sen*, *seo*, and *sem* genes were detected in *S. aureus* FI7A, *S. aureus* FI21A, *S. aureus* CI19, and *S. aureus* CI23, where the expressed toxins were also confirmed by the TECRA kit. Other studies involving enterotoxin genotype analyses [13, 17, 32, 39] have previously reported the possibility of *S. aureus* strains including two enterotoxins. In the present study, the food *S. aureus* isolates were found to have more than one enterotoxin gene, whereas the clinical isolates only had one enterotoxin gene. Thus, the food isolates were apparently more hazardous than the clinical isolates because of the various kinds of enterotoxin production.

Table 3. Staphylococcal enterotoxins detected in *S. aureus* isolates.

Detected strains	Enterotoxin gene
S. aureus FI6A	*sea, she*
S. aureus FI14A	*sea*
S. aureus FI17A	*seg, sei, sen, seo, sem*
S. aureus FI21A	*sea, seg, seh, sei, sen, seo, sem*
S. aureus CI19	*sed, sei, sen, seo, sem*
S. aureus CI23	*sed, sei, sen, seo, sem*

Expression of *S. aureus* Enterotoxin Genes Based on Reverse Transcription Real-Time PCR

After identifying the enterotoxins in the food isolates, the gene expressions were confirmed by a reverse transcription real-time PCR (Figs. 4, 5, 6), which also allowed examination of the relative gene expression between the target and reference genes, where the 16s rRNA was used as the

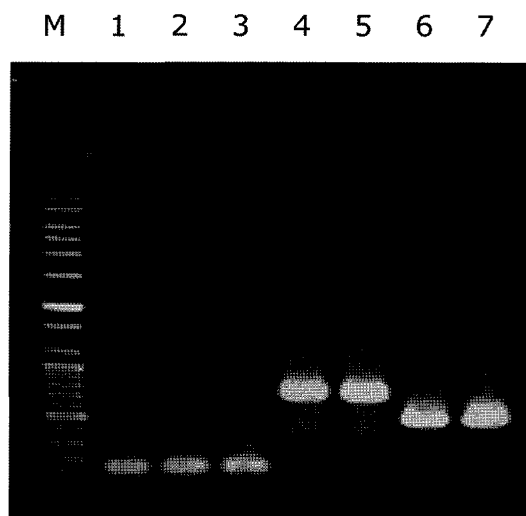

Fig. 2. Agarose gel electrophoresis of enterotoxin A, G, and H PCR products from *S. aureus* isolates.
M, molecular weight marker; 1, *S. aureus* FI6A; 2, *S. aureus* FI14A; 3, *S. aureus* FI21A; 4, *S. aureus* FI7A; 5, *S. aureus* FI21A; 6, *S. aureus* FI6A; 7, *S. aureus* FI21A. Lanes 1–3, *sea* gene (120 bp)*; lanes 4 and 5, *seg* gene (704 bp); lanes 6 and 7, *seh* gene (495 bp).

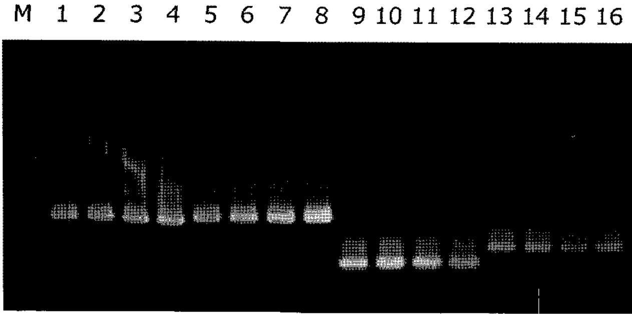

Fig. 3. Agarose gel electrophoresis of enterotoxin I, N, O, and M PCR products from *S. aureus* isolates.
M, molecular weight marker; 1, *S. aureus* FI6A; 2, *S. aureus* FI14A; 3, *S. aureus* FI21A; 4, *S. aureus* FI7A; 5, *S. aureus* FI21A; 6, *S. aureus* FI6A; 7, *S. aureus* FI21A. Lanes 1–4, *sei* gene (630 bp)*; lanes 5–8, *sen* gene (680 bp); lanes 9–12, *seo* gene (180 bp); lanes 13–16, *sem* gene (300 bp).

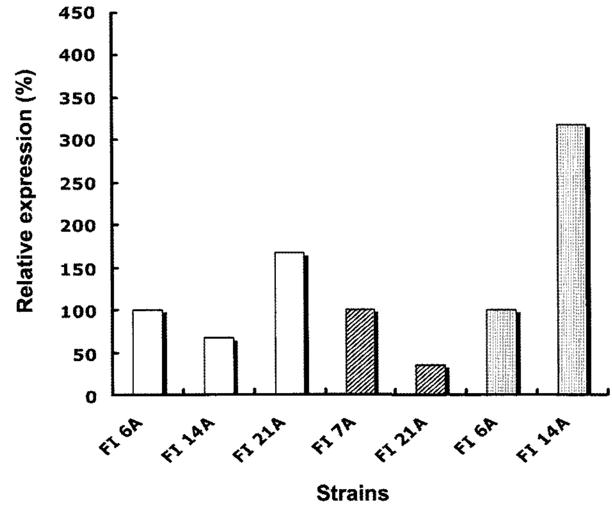

Fig. 5. Quantification of relative expression of staphylococcal enterotoxins based on reverse transcription real-time PCR.
□, *sea* gene; ▨, *seg* gene; ▥, *seh* gene.

reference gene and the enterotoxin genes as the target genes. The expressed internal reference gene is usually quantified at the same time, and the number of copies of the target gene normalized against the number of copies of the reference gene [14, 15, 34].

The relative expression of the enterotoxin genes among the food isolates was confirmed as follows. In the case of the *sea* gene, it was expressed 1.5 times more in *S. aureus* FI6A and 2.5 times more in *S. aureus* FI21A compared with *S. aureus* FI14A. For the *seg* gene, it was also expressed 2.8 times more in *S. aureus* FI7A than in *S. aureus* FI21A. Thus, a different risk was indicated according to the enterotoxins produced by each strain. There were also differences between the clinical and food isolates in the expression of the *sei*, *sen*, *seo*, and *sem* genes. In the case of the *sei* gene, it was expressed approximately 1.2 times more in *S. aureus* CI19, 1.5 times more in *S. aureus* CI23, and 4.2 times more in *S. aureus* FI21A compared with *S. aureus* FI7A, whereas the *sen* gene was expressed about 1.6 times more in *S. aureus* CI19, 1.8 times more in *S. aureus* CI23, and 3.74 times more in *S. aureus* FI7A compared with *S. aureus* FI21A. For the *seo* gene, it was also expressed 2.12 times more in *S. aureus* CI23, 4.4 times more in *S. aureus* FI21A, and 6.48 times more in *S. aureus* CI19 than in *S. aureus* FI7A, whereas the *sem* gene was expressed about 2.17 times more in *S. aureus* CI23, 4.34 times more in *S. aureus* FI21A, and 8.93 times more in *S. aureus* CI19 compared with *S. aureus* FI17A. Therefore, the expression results for the food *S. aureus* isolates artificially cultured in TSB exposed a dramatic difference between the strains. Thus, further studies are needed on the degree of enterotoxin expression in contaminated food, such as *kimbap*, along

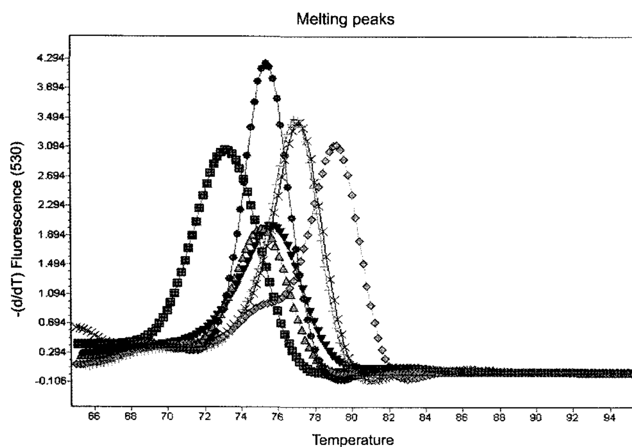

Fig. 4. Specificity of reverse transcription real-time PCR for amplification of *S. aureus* genes *sea*, *seg*, *seh*, *sei*, *sen*, *seo*, and *sem*, as determined by melting peak analysis.
×, *sea*; ▼, *seg*; ◆, *seh*; ■, *sei*; ▲, *sen*; ●, *seo*; †, *sem*.

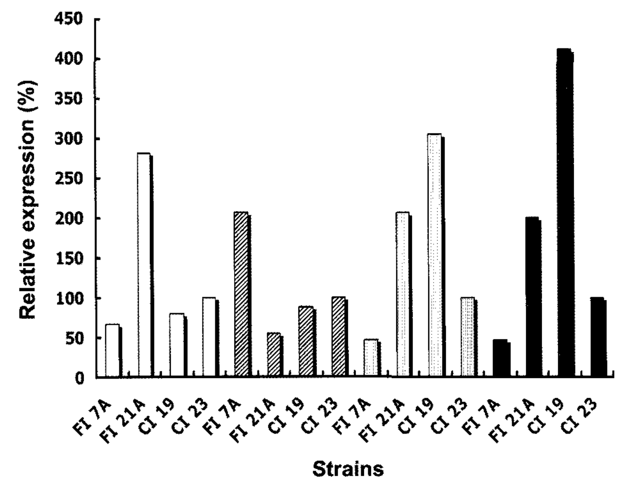

Fig. 6. Quantification of relative expression of staphylococcal enterotoxins based on reverse transcription real-time PCR.
□, *sei* gene; ▨, *sen* gene; ▥, *seo* gene; ■, *sem* gene.

with a comparative study of enterotoxin expression *in vivo*. Whereas expression is normally only investigated using a complicated and costly Western blot or Northern blot, the present study successfully examined the expression of *Staphylococcus aureus* producing enterotoxins at the RNA level using a reverse transcription real-time PCR. Although molecular biological techniques, such as a PCR, RFLP, AFLP, and PFGE, are normally used for the gene detection of enterotoxins A to E, rather than an immunoassay kit [7, 21, 22, 30, 35, 38], these techniques are limited with regards confirming the expression of enterotoxin genes. Nonetheless, verifying the actions of toxin genes in humans is very important. Therefore, the expression of enterotoxin genes in *S. aureus* was confirmed by a reverse transcription real-time PCR that facilitates detection on an RNA level. Consequently, comparing the expression of various staphylococcal enterotoxin genes can provide a more effective evaluation of the toxic hazards of *S. aureus*.

Acknowledgment

This work was supported by research funding of Dongguk University and Korea Food Research Institute.

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