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Novel PCR Primers for Specific Detection of C1, C2 and C3 Enterotoxin Genes in *Staphylococcus aureus*

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ABSTRACT

Staphylococcus aureus is the major pathogen that causes clinical infection as well as food poisoning. Enterotoxin produced by *Staphylococcus aureus* strains are, among others, staphylococcal enterotoxin (SE) A, SEB, SEC, SED, SEE, SEG, SEH, SEI, SEJ, SEK, SEL and SEM. For SEC, there are three major antigentically distinct SEC subtypes, i.e. SEC1, SEC2 and SEC3, in addition to other molecular variants. The nucleotide sequence homology between SEC1, SEC2, and SEC3 genes is higher than 97%; however, we were able to develop a second set of PCR primers that allowed us to differentiate the three SECs, i.e., SEC1, SEC2 and SEC3, *S. aureus* strains. These PCR primers and their combinations were C12F/C1BR, C12F/C23R and C3BF/C23R, for specific detection of the SEC1, SEC2 and SEC3 genes of *S. aureus* strains, respectively. Using these primers, we examined 39 SEC *S. aureus* isolates, which were obtained from foods that were likely the source of food-borne outbreaks between 1995 and 1997 in central Taiwan, and from the patients of the outbreaks. The results were consistent with those obtained from the first set of primers reported earlier, indicating that the present primers could be used for specific detection of these three toxin types.

Key words: *Staphylococcus aureus*, SEC1, SEC2, SEC3, PCR primers

INTRODUCTION

Staphylococcus aureus is a major pathogen that causes general infection and food poisoning. This organism produces one or more members of an enterotoxins family, staphylococcal enterotoxins (SEs) SEA, SEB, SEC, SED and SEE, the classical enterotoxins, as well as other newly found enterotoxins, such as SEG, SEH, SEI, SEJ SEK, SEL and SEM etc.[1-5]. Of these enterotoxins, SEC consists of a group of highly conserved proteins with significant immunological cross-reactivity[6]. Amino acid sequences of the mature form of SEC1, SEC2 and SEC3 and the nucleotide sequence of these SEC genes are known[7-9]. In addition to SEC1, SEC2 and SEC3, additional SEC variants exist[6].

Commercially available immunoassay kits, such as the staphylococcal enterotoxins reverse phase latex agglutination (SET-RPLA) kit (Denka Seiken, Tokyo, Japan), and the staphylococcal enterotoxin ELISA (SET-EIA) kit (Toxin Technology, Sarasota, Florida, USA or TECRA Bioenterprises Pty. Ltd, Roseville, Australia), only allow detection of classical SEs, namely SEA, SEB, SEC, SED and SEE, but not the specific individual subtypes of SEC. There are two DNA based methods available. One is DNA hybridization with probe and the other is polymerase chain reaction (PCR) with primers. Probes and primers specific for most of the staphylococcal enterotoxin genes have been reported[10-12]. To detect specific SEC subtypes by PCR, however, there is only one method available which was reported by Chen *et al*[13].

Perhaps due to the high homology of the amino acid sequences in SEC1, SEC2 and SEC3, the method to differentiate SEC subtypes[7,9,14] is not yet commercially available. Based on the nucleotide sequences of these enterotoxins, we have designed SEC subtype specific primers for the detection of these SEC genes individually[13]. We checked the specificity of these SEC subtype specific primers and used them to investigate the distribution of SEC1, SEC2 and SEC3 *S. aureus* strains in staphylococcal food poisoning outbreaks that occurred in central Taiwan between 1995 and 1997. With an attempt to show that more than one PCR primer set could be designed and used for the differentiation of these SEC genes highly homologous to each other, in this study, we designed a second set of PCR primers for SEC1, SEC2 and SEC3 detection. Here we report a successful designing of this set of PCR primers. In addition, the result for survey of the distribution of specific SEC subtypes of *S. aureus* in food poisoning outbreaks between 1995 and 1997 in central Taiwan is presented. The result is consistent with that obtained using the previous reported primers.

MATERIALS AND METHODS

I. *Bacterial Strains*

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S. aureus strains used in this study include standard isolates of *S. aureus* characterized as staphylococcal enterotoxin (SE) A, B, C, D, E, G, H, I and toxic shock syndrome toxin 1 (TSST-1) producing strains. These strains are CCRC 12657 (SEA), CCRC 12653 (SEB), CCRC 12654 (SEC), FRI 137 (SEC1), FRI 361 (SEC2), FRI 913 (SEC3), CCRC 12660 (SED), CCRC 12656 (SEE) and CCRC 13831 (TSST-1), FRI 572 (SEG), FRI 569 (SEH) and FRI 445 (SEI). In addition, non-enterotoxigenic *S. aureus* and 20 bacteria species other than *S. aureus* were also used. They were *S. aureus* CCRC 011, 029, 033, *S. epidermidis* CCRC 11030, *S. xylosus* CCRC 12930, *Streptococcus mutans* CCRC 10793, *Micrococcus varians* CCRC 11272; *Alcaligenes faecalis* CCRC 10828; *Brevibacterium linens* CCRC 10041, *Enterobacter aerogenes* CCRC 10370, *Erwinia carotovora* CCRC 11298, *Hafnia alyei* CCRC 10906, *Klebsiella pneumoniae* CCRC 10629, *Kluyvera ascorbata* CCRC 11645, *Micrococcus roseus* CCRC11577, *Morganella morganii* CCRC 10706, *Proteus vulgaris* CCRC 10728, *Salmonella enterica* serovar Enteritidis ATCC 13076, *S. enterica* serovar Typhimurium ATCC 14028, *Serratia marcescens* CCRC 13880, *Escherichia coli* ATCC 35401 and *Yersinia enterocolitica* CCRC 10807. These strains were obtained from the American Type Culture Collection (ATCC), Rockville, MD, USA, the Bioresources Collection and Research Center (BCRC, former CCRC), Shin-Chu, Taiwan, and the Food Research Institute (FRI), University of Wisconsin, Madison, WI, USA. In addition, 39 SEC producing *S. aureus* strains isolated from the stool samples of diarrhea patients involved in food-borne outbreaks in central Taiwan between 1995 and 1997 were obtained from the Third Branch Office, Center for Disease Control, Department of Health, Taichung, Taiwan.

S. aureus strains were isolated from food samples or stool samples (obtained with a cotton swab) of persons with diarrhea and identified by the methods described previously[13] which were essentially, the methods described in Bacteriological Analytical Manual (BAM)[15]. Test samples were cultured in tryptic soy broth (TSB), Baird-Parker agar (BPA), brain heart infusion (BHI) or tryptic soy agar (TSA) media (Difco, Detroit, MI, USA) and the bacteria grown were Gram stained and microscopic examined. They were further tested for coagulase, catalase, lysostaphin sensitivity, thermally stable nuclease and utilization of glucose as well as mannitol. Bacterial numbers were enumerated on plate count agar (PCA) plates by conventional methods.

II. *Media*

Media used were TSA, BP base, egg yolk tellurite (EY), BHI, PCA and peptone. All purchased from Difco Lab (Detroit, MI., USA). *Staphylococcus* species were grown in 5 mL of TSB or BHI at 37°C for 18-24 hr. Other bacteria were grown in TSB or BHI at 37°C for 12 hr.

III. *Detection of Enterotoxigenicity*

Enterotoxigenicity of *S. aureus* was detected with Staphylococcal enterotoxin A, B, C, D detection kit which utilized reversed passive latex agglutination (SET-RPLA) (Denka Seiken, Tokyo, Japan). The procedure described by the manufacturer was followed[13].

IV. *Recovery of DNA for PCR*

The bacteria were cultured by conditions described [13]. Total genomic DNA was extracted from *Staphylococcus* cells by the phenol chloroform extraction method as described by Ausubel *et al*.[16] with modification, and details of DNA recovery were described previously[13]. For non-staphylococcal bacteria, the same method without modification[16] was used.

V. *PCR*

PCR primers used for the detection of the genes of staphylococcal enterotoxins A, B, C, D, E and TSST-1 were those previously reported[10-12]. Primers EntCF (5'-AACAT TAGTG ATAAA AAACT GAAA-3', 235-258 bp) and EntCR (5'-TTGTA AGTTC CCATT ATCAA AGTG-3', 445-468 bp) used here were SEC gene specific primers previously reported[13]. They were originally termed as primer C1 and C2[13]. Primers for the specific detection of SEC1, SEC2 and SEC3 genes were designed from the published gene sequences, respectively, coded for staphylococcal enterotoxins C1, C2 and C3[7,9]. They were termed as primer C12F, C1BR, C23R and C3BF.

The sequence homology between the SEC1, SEC2 and SEC3 genes exceeded higher than 97%. That meant there would be minor heterogeneity amounting to about 3%. These regions were selected for the designing of PCR primers by methods similar to those previously described [13]. The combinations of these primers used were C12F/C1BR for the detection of SEC1; C12F/C23R for SEC2; and C3BF/C23R for SEC3. The sequences and locations of these genes that would be amplified are shown in Figure 1. Molecular weights of each PCR product were identical, all of the size 138 bp.

PCR conditions for the detection of SEA, SEB, SEC, SED and SEE genes of *S. aureus* were those described previously[11,12]. For PCR detection of SEC1, SEC2 and SEC3 genes of *S. aureus*, each reaction mixture contained the target DNA (approximately 1000 ng), 1 μL each of 10 mM dNTP, 5 μL of 10X PCR buffer (100 mM Tris -HCl, pH 8.3, 500 mM KCl, 60 mM $MgCl_2$, 0.1% gelatin and 1% Triton X-100), 1 μL each of the primers (50 μM) and 1 unit of ProZyme (PROtech. Technol. Taipei, Taiwan). For amplification of the SEC1, SEC2 and SEC3 genes with primers C12F/C1BR, C12F/C23R and C3BF/C23R, each PCR cycle was performed at 94°C/30 sec, 60°C/30 sec and 72°C/35 sec; 94°C/30 sec, 58°C/30 sec, 72°C/35 sec; and 94°C/30 sec, 55°C/30 sec, 72°C/35 sec, respectively. A

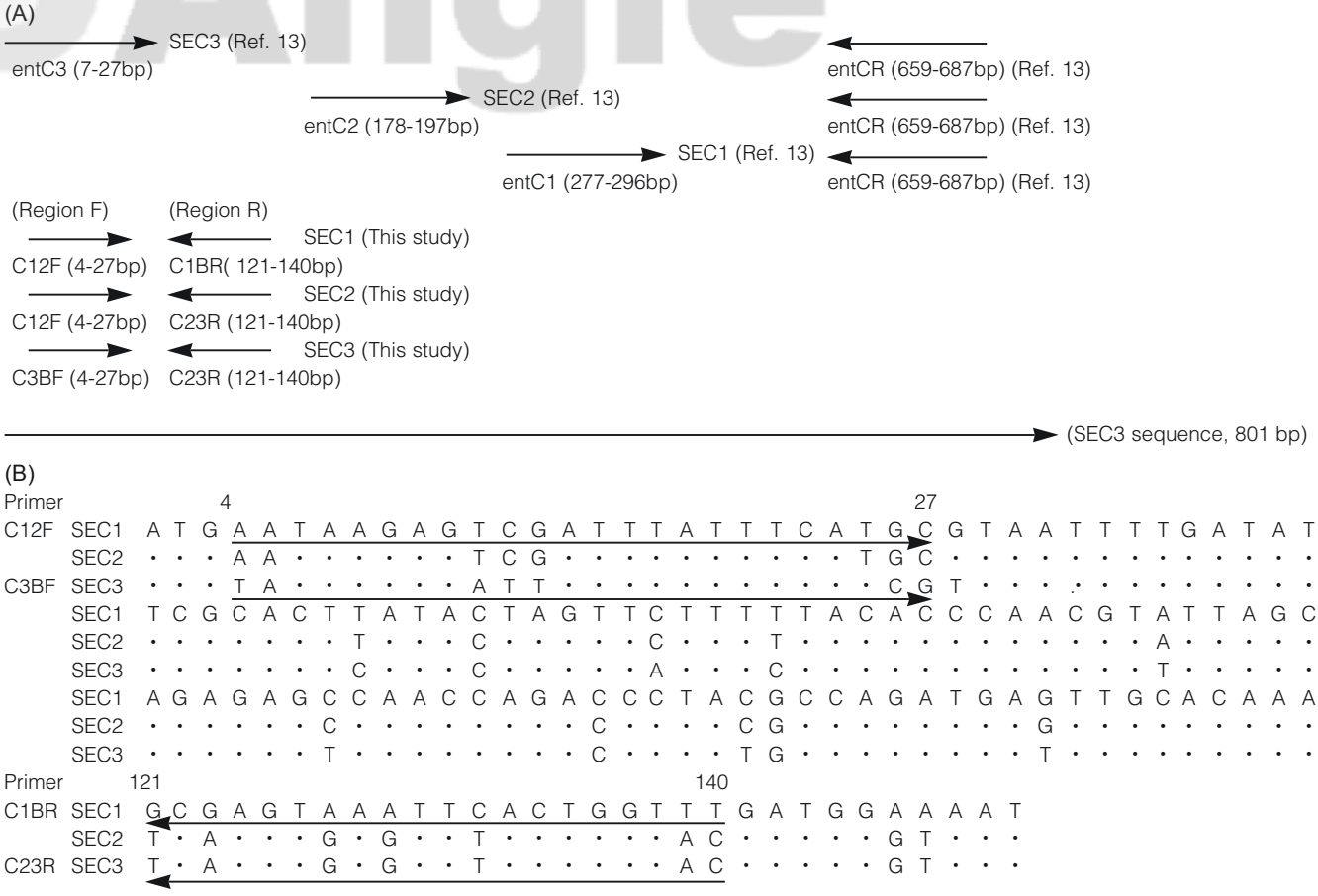

Figure 1. Location and sequence of the primers C12F, C3BF, C1BR and C23R. (A) Positions relative to the previously reported primer entC1, entC2, entC3 and entCR, (B) Alignment of the primers C12F, C3BF, C1BR and C23R with the corresponding SEC1, SEC2, SEC3 gene sequence. The sequences underlined are the sequences of primer C12F, C3BF, C1BR and C23R, respectively.

total of 35 cycles were performed with the first denaturation carried out at 94°C for 5 min and the final extension at 72°C for 7 min. The amplified products were electrophoresed in a 2% agarose gel for detection.

RESULTS AND DISCUSSION

I. *Specificity and Sensitivity of the Primers for Amplification of the SEC1, SEC2 and SEC3 Genes*

Specificity of the PCR primers C12F/C1BR, C12F/C23R and C3BF/C23R, were confirmed by amplification of the template DNAs isolated from enterotoxigenic *S. aureus* and other bacteria strains. Under the conditions described in Methods, only the target *S. aureus* strains, i.e. strains with SEC1 (strain FRI 137), SEC2 (strain FRI 361) and SEC3 (strain FRI 913), generated the expected PCR product. For example, when primers C12F/C1BR were used for PCR assay, only the SEC1 strain, i.e. strain FRI 137, generated the 138 bp product. None of the other *S. aureus* strains including SEC2 strain, i.e. strain FRI 361, and SEC3 strain, i.e. strain FRI 913, generated the 138 bp product (Figure 2A). Similarly, when primers C12F/C23R or primers C3BF/C23R were used for assay, only SEC2 strain FRI 361 or SEC3 strain FRI 913, generated the 138 bp product (Figure 2B and 2C). Toxin types for strains shown in Figure 2 and Table 1 have been confirmed with the kits of SET-RPLA, TSST-RPLA (Denka Seiken) and the PCR primers specific for these toxin genes. When strains other than *S. aureus*, including strains of other Staphylococcus spp., were assayed with these SEC1, SEC2 and SEC3 specific PCR primers, none of these strains generated positive results. PCR amplification with total DNA extracted from decimal dilution series of each target strain, i.e. SEC1, SEC2 or SEC3 *S. aureus* using the specific primer pairs, i.e. primers C12F/C1BR, C12F/C23R and C3BF/C23R, respectively, showed that under the experimental conditions, DNA from 1×10^0~9×10^0 CFU of enterotoxigenic *S. aureus* could be detected. An example with C3BF and C23R as PCR primers is shown (Figure 3). Similar detection limit could be obtained for the previously reported PCR primers. To assure the positive detection of target cells, on study of the specificity of these primers, DNA from 10^8 CFU cells per assay was used.

Molecular weights of the PCR products generated from primers C12F/C1BR, C12F/C23R and C3BF/C23R were all equal, i.e. 138 bp. Such results make the develop-

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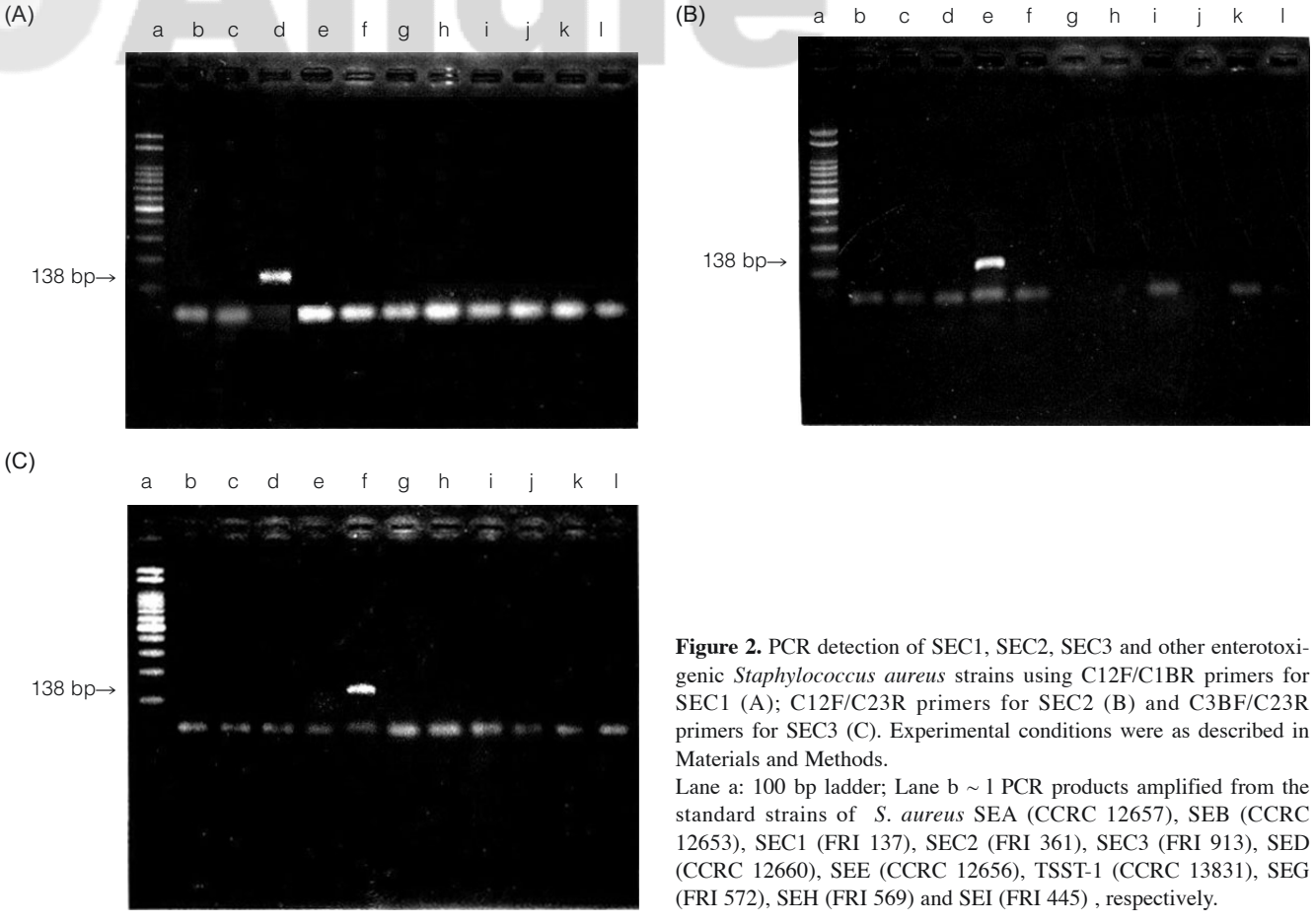

Figure 2. PCR detection of SEC1, SEC2, SEC3 and other enterotoxigenic *Staphylococcus aureus* strains using C12F/C1BR primers for SEC1 (A); C12F/C23R primers for SEC2 (B) and C3BF/C23R primers for SEC3 (C). Experimental conditions were as described in Materials and Methods.
Lane a: 100 bp ladder; Lane b ~ l PCR products amplified from the standard strains of *S. aureus* SEA (CCRC 12657), SEB (CCRC 12653), SEC1 (FRI 137), SEC2 (FRI 361), SEC3 (FRI 913), SED (CCRC 12660), SEE (CCRC 12656), TSST-1 (CCRC 13831), SEG (FRI 572), SEH (FRI 569) and SEI (FRI 445) , respectively.

Table 1. Specificity of the three PCR primers to detect the SEC1, C2 and C3 genes of *Staphylococcus aureus* strains

Source and strain No.	Enterotoxin type[a]	RPLA	EntCF/EntCR[b]	PCR for SEC subtype[b]		
				C12F/C1BR	C12F/C23R	C3BF/C23R
CCRC 12657	A	A	—	—	—	—
CCRC 12653	B	B	—	—	—	—
CCRC 12654	C	C	+	+	—	—
FRI 137	C1	C	+	+	—	—
FRI 361	C2	C	+	—	+	—
FRI 913	C3	C	+	—	—	+
CCRC 12660	D	D	—	—	—	—
CCRC 12656	E	ND[d]	—	—	—	—
CCRC 13831	TSST-1	TSST	—	—	—	—
FRI 572	G	ND	—	—	—	—
FRI 569	H	ND	—	—	—	—
FRI 445	I	ND	—	—	—	—
CCRC 011	Non-enterogenic	ND	—	—	—	—
CCRC 029	Non-enterogenic	ND	—	—	—	—
CCRC 033	Non-enterogenic	ND	—	—	—	—
Other bacteria[c]			—	—	—	—

[a]Enterotoxin types are determined according to the information from strain sources.
[b]PCR primers used were those described in Materials and Methods.
[c]Other bacteria strains are as shown in Table 1.
[d]ND: not determined.

ment of a multiplex PCR system using these primers for the simultaneous detection of SEC1, SEC2 or SEC3 strains to be impossible. However, these primers could be used in single pair for the detection of individual SEC1, SEC2 or SEC3 genes of *S. aureus* strains. In addition, if primers EntCF, EntCR, C12F, C1BR, C23R and C3BF were arrayed on the nitrocellulose membrane as probes followed by hybridization of the fluorescence or biotin labeled PCR products amplified with the multiplex PCR system (C12F, C1BR, C23R, C3BF as primers), or amplified with a pair of

universal primers flanking the regions of these C12F, C3BF, C1BR and C23R primers, i.e. the region from base number 4 to 140 bp (Figure 1A), to the oligonucleotides arrayed on the membrane, simultaneous detection of SEC1, SEC2 and SEC3 *S. aureus* may be possible. As compared to the locations of the previously reported primers[13], i.e. entC1, C2 and C3, primer C12F and C3BF were near the location of primer entC3[13], but 174 and 273 bases upstream from those of the primers entC2 and entC1, respectively. Also, primer C1BR and C23R were 538 base downstream (reverse and complementary strand) from the entCR primer. (Figure 1A)

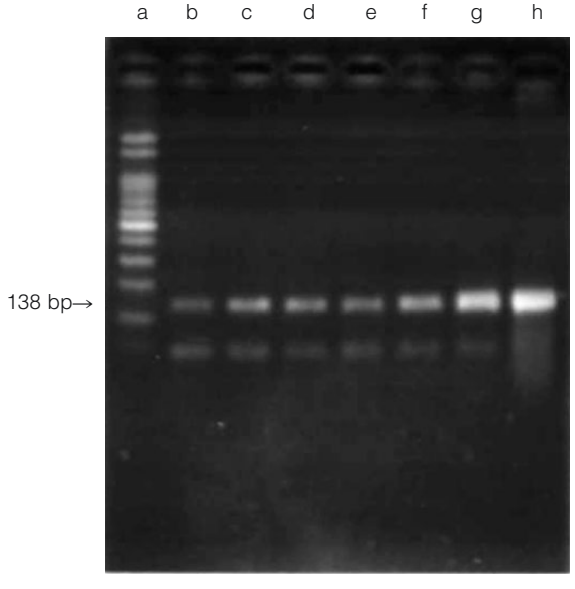

Figure 3. Sensitivity for PCR detection of SEC3 *Staphylococcus aureus* FRI 913 using C3BF/C23R primers. Experimental conditions were as described in Materials and Methods.
Lane a, 100 bp ladder; Lanes b through h represent PCR products amplified from (1-9) × 10^0 to (1-9) × 10^6 CFU/mL per assay of *S. aureus* cells.

II. *Distribution of the SEC1, C2 and C3 Subtypes for Type C Enterotoxigenic S. aureus Strains Isolated from Patients of Food Poisoning Outbreaks*

A total of 31 SEC *S. aureus* strains isolated from 31 randomly selected patients engaged in 14 food-borne outbreaks between January, 1995 and December, 1997 (3 years) in central Taiwan were investigated for their SEC subtypes using the primers here. Table 2 shows the dates and locations for those food-borne outbreaks, the ratio of diseased persons, and the type of food samples contaminated. Results shown in Table 2 indicate that for the 31 randomly selected SEC strains identified with SET-RPLA (Denka Seiken), 12 are SEC2 *S. aureus* and 13 are SEC3 *S. aureus* strains. Only one strain is SEC1 and 5 strains belong to other SEC subtypes. Also, these *S. aureus* strains were isolated from 1, 6 and 10 of the 14 outbreaks. Thus, SEC 3 and SEC2 are the major SEC subtypes which may cause food-borne outbreaks. The present result is consistent with the result reported previously using other set of PCR primers[13].

The SEC *S. aureus* was found in 1965[17]; however, the DNA sequences of the three genes, i.e. SEC1, SEC2 and SEC3 genes, were reported only recently[6,7,9]. Perhaps due to the fact that kits specific for SEC1, SEC2 and SEC3 genes or enterotoxins detection are not commercially available, reports regarding the distribution of SEC1, SEC2 and SEC3 subtypes of SEC *S. aureus* strains isolated from patients of food-borne outbreaks are rare, although incidents of type C *S. aureus* intoxication in staphylococcal

Table 2. PCR identification of the SEC1, C2 and C3 subtypes for randomly selected S. aureus strains obtained from patients of food-borne outbreaks occurred in central Taiwan between 1995-1997

Outbreaks No.	Ratio of diseased person[a]	Date	Location	SEC strains identified by SET-RPLA[b]	Food Samples associated	No. of strains for each SEC subtype[c]			
						C1	C2	C3	Other C
1	121 / 402	3 / 95	Yunlin	1	School lunch			1	
2	12 / 51	3 / 95	Nantou	4	Meal boxes		4		
3	66 / 405	3 / 95	Taichung	2	Meal boxes		2		
4	21 / 38	10 / 95	Miaoli	1	Meal boxes			1	
5	22 / 201	1 / 96	Taichung	1	Meal boxes			1	
6	34 / 393	1 / 96	Taichung	2	Meal boxes			2	
7	15 / 1935	3 / 96	Nantou	1	School lunch			1	
8	20 / 120	4 / 96	Taichung	1	Banquet			1	
9	20 / 279	1 / 97	Taichung	1	Meal boxes			1	
10	44 / 862	3 / 97	Taichung	3	Meal boxes		1	1	1
11	1036 / 1938	3 / 97	Taichung	9	Meal boxes	1	3	3	2
12	6 / 25	10 /97	Taichung	3	Meal boxes		1		2
13	23 / 300	12 / 97	Miaoli	1	Banquet			1	
14	3 / 3	12 / 97	Taichung	1	Lunch		1		
Total number of strains identified				31		1	12	13	5
Total No. of outbreaks associated with each SEC subtype						1	6	10	3

[a]The number of diseased persons over the number of total attendants.
[b]Fecal samples from selected diseased persons were collected and subjected to SET-RPLA assay by the Third Branch of National Center for Disease Control, Taichung, Taiwan. Randomly selected SEC strains were used in this study.
[c]SEC subtypes were determined by PCR. SEC strains not grouped in SEC1, C2 and C3 subtypes were termed as other SEC subtype.

poisoning cases have been reported[18]. Since it has been suggested that toxin heterotoxigenicity may be due to selection of modified SEC sequences that facilitate the survival of *S. aureus* isolates in their respective hosts[6], thus, it is of interest to know what are the major SEC subtypes for type C *S. aureus* strains from patients of food-borne outbreaks. Using the primers here and those previously reported[13], we have investigated the distribution of SEC subtypes for SEC strains isolated from food-poisoning outbreaks occurred in central Taiwan and consistent results were obtained. In conclusion, the new primers could be applied for the detection of the *S. aureus* strains with specific SEC subtype genes.

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