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Multiplex PCR for the Simultaneous Detection of the SEA, SEB, SEC, SED and SEE Genes of Enterotoxigenic Staphylococcus aureus

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ABSTRACT

The enterotoxins of *Staphylococcus aureus* (A, B, C, D and E) are known agents of food poisoning. Classical identification for these enterotoxigenic *S. aurreus* strains is laborious, time consuming and sometimes gives erronneous results. In this work, seven synthetic oligonucleotide primers were used in a multiplex PCR protocol to detect the genes encoding the Staphylococcal enterotoxins A to E simultaneously. The primers include two universal primers, which encode consensus sequences, and five primers, which encode unique sequences for toxin genes. None of the specific primer pairs (U2/A2, U1/B2, U1/C2, U2/D2 and U2/E2) cross-reacted with each other. Each primer was specific for the detection of its corresponding toxin gene, even though U2 and U1 are universal primers. The sizes of the amplified PCR products were 582 bp, 732 bp, 403 bp, 251 bp and 474 bp for enterotoxin genes, A, B,C,D and E, respectively. Unequivocal discrimination of the toxin genes was obtained by PCR using DNA extracted from strains of *S. aureus* whose toxigenicity had been previously established biologically and immunologically. When these primers were used to detect *S. aureus* in spiked foods containing10^2 to 10^3 cells per ml of food homogenate, all five types of genes could be identified after six to eight hours of preincubation.

Key words : multiplex PCR, enterotoxigenic *Staphylococcus aureus*

INTRODUCTION

Staphylococcus aureus is one of the common agents of food poisoning. It may produce a number of toxins. The most important ones with respect to foodborne illness belong to the family of heat-stable staphylococcal enterotoxins (SEs). Nine serologically distinct enterotoxins, SEA, SEB, SEC1, SEC2, SEC3, SED, SEE, SEG and SEH have been identified[1-6]. Among them, SEA, SEB, the SECs, SED and SEE are the most common types. It may be important to identify the toxin types for Staphylococcal infection from the epidemiological viewpoint and to determine if there is *Staphylococcus aureus* present in a suspected vehicle of a foodborne disease outbreak[7].

Conventional methods for the detection of *S. aureus* in food samples require four to five days to obtain presumptive results after initiation of sample analysis[8]. The polymerase chain reaction (PCR), which is a technique for the *in vitro* amplification of specific segments of DNA, offers a rapid, sensitive and specific identification method for the genes responsible for toxins produced by *Staph. aureus*[8-12]. Becker *et al.*[12] used multiplex PCR to detect Staphylococcal enterotoxin genes, exofoliative toxin gene and toxic shock syndrome toxin I gene. Mehrotra *et al.*[13] used multiplex PCR for the detection of genes coding *Staphylococcus aureus* enterotoxins, exofoliative toxin, toxic shock syndrome toxin I gene and methicillin resistance. However, all their assays were performed for pure culture only, not for any food samples.

In this paper, five specific primers: A2, B2, C2, D2 and E2[8,11,15], which were published earlier by other researchers, were employed for multiplex PCR detection of the staphylococcal toxin genes. To meet the requirements for distinguishable amplified bands by multiplex PCR, it was necessary to select new primers within the target genes. Two novel universal primers, U1 and U2, which encode consensus sequences of enterotoxin genes, were selected by computer analysis of nucleotide sequences for the corresponding SEA to SEE genes[1-6]. Five primer pairs, U2/A2, U1/B2, U1/C2, U2/D2 and U2/E2, were then used in multiplex PCR for the assay of culture mix of *Staphylococcus aureus* and foodborne samples. Our results showed that multiplex PCR using the five primer sets were specific for the detection of SEA, SEB, SEC, SED and SEE genes.

MATERIALS AND METHODS

I. *Bacterial Strains*

Bacterial strains including reference isolates of *Staphylococcus aureus* characterized as Staphylococcal enterotoxin (SE) A, B, Cs, D and E producing strains were used. These *S. aureus* strains were CCRC 13824, CCRC 12657 (SEA); CCRC 13825, CCRC 12653 (SEB); CCRC 13826, CCRC 12654 (SEC); CCRC 13829, CCRC 12660 (SED) and CCRC 13830, CCRC 12656 (SEE). Other *S. aureus* strains used included toxic shock syndrome toxin (TSST)-producing strains (CCRC 13831) and those not pro-

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ducing enterotoxin. A set of isolates representing the family *Enterobacteriaceae*, such as *Escherichia coli*, *Salmonella* sp. *Proteus vulgaris*, as well as Gram positive bacteria, such as *Bacillus cereus*, *B. subtilis*, were also used. All these strains were obtained from American Type Culture Collection (ATCC) (Rockville, Maryland, U.S.A)., the Culture Collection and Research Center (CCRC) (Hsinchu, Taiwan), and Dr. H.Y. Tsen of Chung-Hsin University (Taichung, Taiwan). The non-enterotoxin producing bacterial strains used in this study are described in Table 1. The lack of enterotoxigenicity was confirmed with a staphylococcal enterotoxin detection kit by reversed passive latex agglutination kit (SET-RPLA, Denka Seiken, Tokyo, Japan)

II. *PCR Primers Design*

The PCR primer sets used for the simultaneous detection of enterotoxins genes of *S. aureus* were: U2/A2, U1/B2, U1/C2, U2/D2 and U2/E2, as listed in Table 2. Among them, U1 encodes a consensus sequence of the SEB and SECs genes, while U2 encodes a consensus sequence of the SEA, SED and SEE genes. Both of them were designed by computerized sequence analysis of SEA to SEE genes[1-6]. The five reverse primers, A2, B2, C2, D2 and E2, have been shown to be specific for the SEA, SEB, SEC, SED and SEE genes, respectively[8,11,15].

III. *DNA Preparation for PCR Amplification*

Total genomic DNA was prepared according to a modified phenol -chloroform-isoamyl alcohol (25:24:1) extraction method originally described by Ausubel *et al*.[16]. Five milliliters of overnight culture were centrifuged and washed with 0.5 mL of Tris-buffer saline (50 mM Tris-HCl, 150 mM NaCl, pH7.5). The pellet was then resuspended and incubated at 37˚C for at least 2 hrs in 0.25 mL of Tris-buffer saline with 10 mM lysostaphin buffer containing about 10 unit/mL of lysostaphin (Sigma) and 20 µg RNase (Sigma). Then, 25 µL proteinase K (1 mg/mL, Boeheringer, Germany) was added and incubation was continued at 65˚C for 2 hrs. Phenol-chloroform extraction and ethanol precipitation was then performed. Before use, the DNA was suspended in TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) buffer and stored at 4˚C.

Table 1. Non-enterotoxigenic Staphylococcus aureus and non-Staphylococcus aureus strains used for the multiplex polymerase chain reaction (m-PCR)

Microorganism strains	
Staphylococcus aureus (TSST)a	CCRC 13831
Staphylococcus. aureus (non-enterotoxigenic)[b]	029, 052, 094, 126, 224, 281, 293, 326,
Escherichia coli	ATCC 25922, ATCC 11775
Citrobacter freundii	ATCC 8090
Salmonella typhi	CCRC 12948
Salmonella typhimurium	CCRC 12947
Shigella flexneri	CCRC 10772
Enterobacter cloacae	ATCC 23355
Kluyvera ascorbata	CCRC 11645
Bacillus subtilis	ATCC 21778
Proteus vulgaris	ATCC 8427
Psedumonas cepacia	CCRC 10735
S. haemolyticus	ATCC 12923
S. xylosus	ATCC 12930

a: Toxic shock syndrome toxin (TSST)-producing strain.

b: Laboratory isolates from Dr. H.Y. Tsen of Chung-Hsin University, Taichung, Taiwan were confirmed by reversed passive latex agglutination kit (SET-RPLA, Denka Seiken, Tokyo, Japan).

IV. *Multiplex PCR*

PCR reactions were performed in a thermal cycler (Perkin-Elmer GeneAmp PCR System 2400). The amplification reactions were performed in a final volume of 50 µL, containing 0.5 µg of genomic DNA, 2.5 units of Taq polymerase (Promega, Madision, WI.), 2 µg each of 10 mM dATP, dTTP, dCTP and dGTP, 5 µL of 10 X reaction buffer (50 mM KCl, 10 mM Tris-HCl (pH 8.3 at 25˚C), 0.01% Triton X-100, 0.01% gelatin, 6.0 mM $MgCl_2$), 90 pmol of primer D2, 100 pmol of primer U1, 190 pmol of primer U2 and 50 pmol of primers A2, B2, C2, and E2. To prevent evaporation, 2 drops of mineral oil was layered on top of the reaction mixture. The reaction mixture was first denatured by heating at 94˚C for 2 minutes, followed by 35 cycles (45 seconds at 94˚C for denaturation, 55 seconds at 52˚C for annealing, and 55 seconds at 72˚C for extension). At the end of the cycles, the reaction mixture was maintained at 72˚C for 2 mins.

The amplification products were loaded onto a 1.8% agarose gel. After electrophoresis in 1X TBE (Tris-Borate-EDTA) buffer at 50 volts, the gel was stained with ethidium bromide before photographed using ultraviolet illumination.

V. *Sensitivity for the Detection of Enterotoxigenic S. aureus*

Overnight cultures of enterotoxigenic *S. aureus* in brain heart broth (BHI) were diluted with sterile water in decimal series. One milliliter of the diluent containing A-E enterotoxigenic types of *S. aureus* strains was used for the total DNA extraction and the PCR amplification with the procedures described earlier.

VI. *PCR Detection of Enterotoxigenic S. aureus in Food Samples*

25 g of minced food sample was mixed with 225 mL of 0.1% peptone water and homogenized. For evaluation of sensitivity for this method, various concentrations (0, 10^1-10^4 cells/mL) of each enterotoxigenic type of *S. aureus* were simultaneously added to the homogenate. To increase the sensitivity of detection, 1 mL of the sample mixture was mixed with 9 ml of tryptic soy broth (TSB) containing 10% NaCl, and then incubated at 37˚C for 6 to 8 hrs. All the samples were analyzed by preparing DNA as for the multiplex PCR described above.

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Table 2. Oligonucleotide primers used for the simultaneous amplification of the SEA, SEB, SEC, SED and SEE enterotoxin genes of enterotoxigenic *Staphylococcus aureus*

Enterotoxin	Primers[a]	Oligonucleotide Sequence (5'→3")	Location (nucleotide)	Predicted size of PCR (bp)
SEA	A2	ATTAACCGAAGGTTCTGTAGA	637-657	582
	U2	TTGCGTAAAAAGTCTGAATT	76-94	
SEB	B2	TTTTTCTTTGTCGTAAGATAA	1040-1124	732
	U1	CCAACGTTTTAGCAGAGAAG	311-329	
SEC	C2	TAAGTTCCCATTATCAAAGTG	568-589	403
	U1	CCAACGTTTTAGCAGAGAAG	186-204	
SED	D2	TAATGCTATATCTTATAGGG	602-622	251
	U2	TTGCGTAAAAAGTCTGAATT	372-391	
SEE	E2	TAAACCAAATTTTCCGTG	561-578	474
	U2	TTGCGTAAAAAGTCTGAATT	105-123	

a: U1 and U2 primers were derived from the published sequences of SEA, SEB, SEC, SED and SEE genes[1-6]. A2, B2, C2, D2, and E2 were derived from Tsen *et al.*[11,15] and Johnson *et al.* [8].

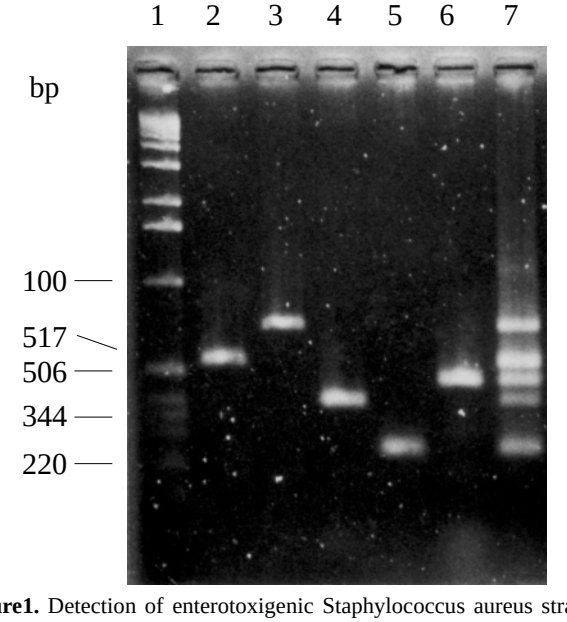

Figure1. Detection of enterotoxigenic Staphylococcus aureus strains using the multiplex PCR. Lane 1:1 Kb DNA ladder; lanes 2 to 7: PCR products amplified from strains containing the SEA gene (CCRC 13824) (lane 2), the SEB gene (CCRC 13825) (lane 3), the SEC gene (CCRC 13826) (lane 4), the SED gene (CCRC 13829) (lane 5), the SEE gene (CCRC13830) (lane 6) and a mixture of strains producing each corresponding enterotoxigenic gene (CCRC 13824/CCRC 13825/CCRC 13826/CCRC 13829/CCRC 13830) (lane 7).

Table 3. Sensitivity of the multiplex PCR for the detection of SEA, SEB, SEC, SED and SEE genes of *Staphylococcus aureus*

Genotype[a]	Mixtures of enterotoxigenic *Staphylococcus aureus* (CFU/strain)						
	0	10^1	10^2	10^3	10^4	10^5	10^6
SEA	–	–	+	+	+	+	+
SEB	–	–	+	+	+	+	+
SEC	–	–	–	+	+	+	+
SED	–	–	–	±[b]	±	+	+
SEE	–	–	–	+	+	+	+

a: SEA, SEB, SEC, SED, and SEE genes of *Staphylococcus aureus* were from CCRC 13824, CCRC 13825, CCRC 13826, CCRC 13829 and CCRC13830, respectively.

b: The band intensity of this gene on the agarose gel electrophoresis was much weaker than other enterotoxin gene products.

RESULTS AND DISCUSSIONS

I. *Specificity of the Multiplex PCR*

To investigate the specificity of this method, both non-enterotoxigenic *Staphylococcus aureus* and non- *S. aureus* strains listed in Table 1 were employed as negative control along with known enterotoxigenic *S. aureus* in the test. These negative reference strains included *S. aureus* that produces TSST (CCRC 13831), *S. aureus* strains that do not produce any enterotoxin, and some isolates that commonly contaminate food.

The five pairs of the synthetic toxin-specific oligonucleotide primers for multiplex PCR were termed U2/A2, U1/B2, U1/C2, U2/D2 and U2/E2 (Table 2). Among them, A2, B2, C2, D2, and E2, were derived from other researches [8,11,15] and had been demonstrated to be specific for SEA, SEB, SEC, SED, and SEE, respectively in PCR[12]. U1 encodes a consensus sequence of the SEB and SEC genes , while U2 encodes a consensus sequence of the SEA, SED and SEE genes. Our result showed that under the carefully selected conditions as described in Materials and Methods, their application for the detection of staphylococcal enterotoxin gene was specific. The relative molecular sizes of the PCR products amplified from SEA (CCRC 13824), SEB (CCRC 13825), SEC (CCRC 13826), SED (CCRC 13829) and SEE (CCRC 13830) genes of S. aureus with the multiple primers were 582 bp, 732bp, 403 bp, 251 bp, and 474 bp, respectively (Figure 1). This result was consistent with those predicted from the primer design (Table 2). However, this system is unable to distinguish the SEC subtypes, i.e. SEC1, SEC2 and SEC3 [2,3] genes. This may be due to the high homology in sequence (98%) among SEC1, SEC2 and SEC3 genes. In contrast, none of the primer pairs reacted with any of the non- enterotoxigenic *S. aureus* or non-staphylococcal strains listed in Table 1 (data not shown).

II. *Sensitivity of the Multiplex PCR System*

From various tests, we found that a template mixture containing as little as 10 ng/each of genomic DNA from

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SEA, SEB, SEC, and SEE as well as 100 ng of SED can be detected successfully by the multiplex PCR. When equal amount of genomic DNA was applied in multiplex PCR, 180 pmol of D2 primer in stead of 90 pmol as described in Materials and Methods was required to achieve the same band intensity as the other enterotoxin genes (data not shown). This sensitivity is compatible with that obtained from Mehrotra *et al.*[13].

To further test the sensitivity of the assay, the PCR templates were prepared by mixing equal amounts of bacteria cultures followed by DNA extraction as described in Materials and Methods. The result was shown in Table 3. It showed that DNA extracted from as little as 10^3 CFU/each of enterotoxigenic *S. aureus* could produce positive results. Among the PCR products, the amplification generated from SED gene was weaker than those from the other enterotoxin genes (Figure 2). The disparity of band intensity disappeared when higher numbers (> 10^5) of SED S. aureus were tested.

The reaction conditions for the multiplex PCR assay were optimized to ensure that all of the target gene sequences were satisfactorily amplified. The primers used in each set had almost equal annealing temperature[8,11], which reduced the possibility of occurrence of unwanted bands originating from nonspecific amplification. In our conditions, the low sensitivity of SED gene may result from competition of primers and templates. This may explain why we needed more SED template and D2 primer to achieve successful multiplex PCR.

III. *Simultaneous Detection of Enterotoxigenic S. aureus in Foods*

In an attempt to further evaluate the specificity of the PCR primers and to investigate the possibility of using these primers for the simultaneous detection of various enterotoxigenic *S. aureus* cells in foods, food samples, including beef, chicken and fish purchased from a local open markets, were used for *S. aureus* detection. In general, these raw food samples were highly contaminated with natural microflora. For example, the viable counts in the food samples ranged from 7 x 10^4 to 1 x 10^5 CFU per gram of sample. Table 4 shows that using inoculation ranging from 10^1 to 10^3 cells per

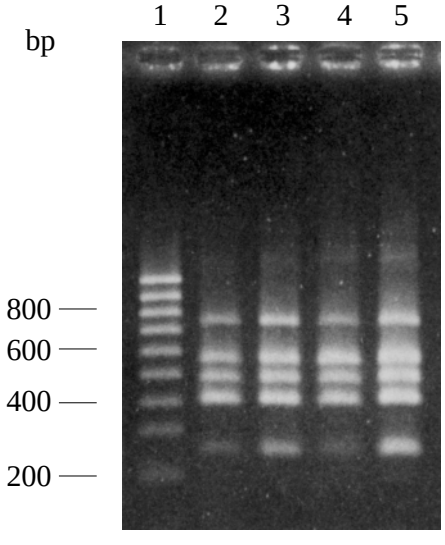

Figure 2. Sensitivity of the multiplex PCR for the detection of enterotoxigenic genes of Staphylococcus aureus. Lane 1: 100 bp ladder; lanes 2 and 3: target DNA isolated from culture mixtures containing 103 and 106 cells of each of the following strains, CCRC 13824, CCRC 13825, CCRC 13826, CCRC 13829 and CCRC 13830, respectively ; lanes 4 and 5: target DNA isolated from culture mixtures containing 104 and 106 cells of each of the following strains, CCRC 12657, CCRC 12653, CCRC 12654, CCRC 12660 and CCRC 12656, respectively.

Table 4. Multiplex PCR detection of enterotoxigenic Staphylococcus aureus with artificially inoculated food samples after enrichment

Food	No. of tested samples	Inoculated Cells (CFU)/ sample	No. of positive samples detected by multiplex PCR				
			SEA	SEB	SEC	SED	SEE
Chicken	5	0[a]	0	0	0	0	0
	5	10^1	4	4	3	2	3
	5	10^2	5	5	4	3	5
	5	10^3	5	5	5	4	5
	5	10^4	5	5	5	5	5
Fish	5	0	0	0	0	0	0
	5	10^1	5	5	3	3	4
	5	10^2	5	5	4	4	5
	5	10^3	5	5	5	5	5
	5	10^4	5	5	5	5	5
Beef	5	0	0	0	0	0	0
	5	10^1	5	4	4	2	3
	5	10^2	5	5	4	4	5
	5	10^3	5	5	5	5	5
	5	10^4	5	5	5	5	5

a: Under the experimental conditions, food samples were without inoculation and free of *S. aureus* confirmed by the conventional culture method.

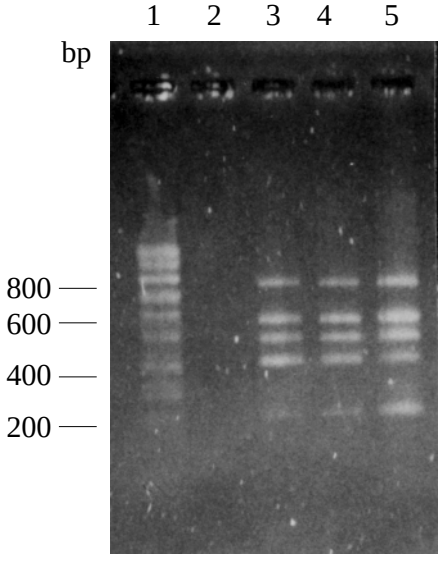

Figure 3. Sensitivity for the detection of enterotoxigenic S. aureus in beef sample. Lane 1: 100 bp ladder marker; Lane 2: PCR product for beef sample without inoculation of target cell; Lanes 3 to 5: PCR products amplified from 10^1-10^3 target cells.

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strain per ml of food homogenates, enterotoxigenic *S. aureus* could be detected after pre-enrichment for 8 hr (Figure 3). Herein we found the same phenomena as those obtained from pure culture detection: the highest sensitivity was found in SEA and SEB genes while the lowest was found in SED gene.

Vantarakis *et al.*[17] reported that the sensitivity of detection of *Salmonella* sp. with multiplex PCR without pre-enrichment was very high (< 10 cell per ml of homogenate), whereas that of *Shigella* sp. was rather low at 10^3 cell per ml of homogenate. In this study, the detection sensitivity for SED gene of *S. aureus* was similar to that of the latter, and pre-enrichment could increase the sensitivity of detection. Although Becker e*t al.*[12] and Mehrotra *et al.*[13] have used multiplex PCR to detect staphylococcal enterotoxin genes simultaneously, their assays were performed using pure cultures of *S. aureus* isolated from the clinical samples not from food samples. For food samples, their compositions are complicated and how to enrich the target bacteria to reach the detection limit is a major difficulty in the multiplex PCR. In addition, the sensitivity of detection for the multiplex PCR method usually varied with the food samples[11]. This might be caused by microbial competition for growth and the characteristics of the various primers[17].

In conclusion, multiplex PCR provides a rapid and convenient method for the simultaneous detection of a large variety of microorganisms. However, the limitation of the application of multiplex PCR is that certain target bacteria remain below the detection sensitivity after enrichment. This limitation may be caused by competition between microorganisms and other foodborne bacteria in food, as well as the distinctiveness of different food samples and primers. Nonetheless, although pre-PCR culture is required, detection sensitivity can be significantly increased if appropriate primers are used [18]. The results of the present investigation provide experimental evidence that in the future, multiplex PCR may be used for routine assay of microorganisms.

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以多套式聚合酶鏈反應同時檢測具腸毒素基因之*Staphylococcus aureus*

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摘　要

腸毒素型金黃色葡萄球菌（*Staphylococcus aureus*）（含A, B, C, D及E腸毒素），常引起食物中毒。傳統分析檢測費時、費力，且易有偽結果發生。本研究設計七段合成的寡核苷酸引子（primer），以多套式聚合酶鏈反應（multiplex PCR）探討同時檢測腸毒素A, B, C, D及E的可能性。這七段寡核苷酸引子中包括二段共同引子（U1、U2）及五段具特異性的毒素基因引子（A2、B2、C2、D2及E2）。結果顯示，組合成的五組引子（A2/U2、B2/U1、C2/U1、D2/U2及E2/U2）中，雖然含U1、U2兩個共同引子，但仍可產生五段具特異性的PCR產物，大小分別為582 bp、732 bp、403 bp、251 bp及474 bp。將已知的各類腸毒素型金黃色葡萄球菌混合培養後，亦可利用此多套式PCR法成功的將各類毒素型檢測出來。進一步應用於食品微生物的檢驗時，我們發現食品均質液中若接入含SEA-SEE *Staphylococcus aureus* 10^2-10^3cells/mL經6-8小時培養後，即可利用此法檢測出正確的腸毒素型。

關鍵詞：多套式聚合酶鏈反應，腸毒素型金黃色葡萄球菌