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Subtyping of Enterotoxin C Strains Isolated from Food Poisoning Outbreaks in Taiwan

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ABSTRACT

Staphylococcus aureus is one of the important foodborne pathogens that caused food poisoning worldwide. There are several serologically distinct staphylococcal enterotoxins (A, B, C, D, E). SEC is subdivided into SEC1, SEC2, SEC3 based on their minor epitopes. Besides the enterotoxins, there are over thirty categories of extracellular proteins and toxins secreted by *S. aureus*, including coagulase, hemolysins, nuclease, and protein A. Among which, coagulase and protein A are critical biochemical characteristics of *S. aureus*. Total of 229 *S. aureus* strains were collected from 36 food poisoning outbreaks occurred in the Taiwan area and only ten SEC isolates were identified. Subtyping of these strains was analyzed by the polymorphism of the SEC, coagulase and protein A genes performed by polymerase chain reaction (PCR). Three strains belonged to SEC2 and seven belonged to SEC3 and PCR of the *sec* gene also showed to have subtypes. Two types of coagulase gene were detected by PCR and PCR-RFLP methods. Typing for the protein A gene by PCR revealed that the amplified, products digested by *Hin*d III or not, were identical. These rapid subtyping methods developed in this study would be valuable in analyzing the sources of contaminated food in the poisoning outbreaks.

Key words: *Staphylococcus aureus*, polymerase chain reaction (PCR), staphylococcal enterotoxin C (SEC), restriction fragment length polymorphism (RFLP), coagulase gene, protein A gene

INTRODUCTION

Staphylococcus aureus is a pathogen that ranked in top threes for causing food poisoning outbreaks in Taiwan. In Japan, on June 2000, a food poisoning outbreak occurred due to a low-fat dairy product made by Snow Brand Milk Products Co., LTD contaminated with *Staphylococcus aureus* resulting in 14,780 people infected[1]. The food poisoning outbreaks caused by staphylococcal enterotoxins have received great attention since these outbreaks occurred worldwide, even in Europe and America where are advance in food technology and safety. *Staphylococcus aureus* is a specie of gram-positive and a family of Micrococcaceae, a grape-like cluster with 1.0 μm diameter that can be observed under microscope. It is an asporogenous, nonmotile bacterium, and has no capsule for most of strains. Some strains are able to produce heat-stable enterotoxins, which often lead to food poisoning outbreaks. Enterotoxin, an exotoxin, which is resistant to heat and protease e.g. trypsin, chymotrypsin, papain, and rennin, with molecule weight less than 30 kDa. It also carries superantigen characteristic. Vomiting is the most common clinical symptom after ingestion enterotoxins for 1-8 hr. Other clinical symptoms, such as nausea, abdominal pain, diarrhea, and weakness, are usually observed. However, not all the cases of enterotoxins toxication can lead to the above clinical symptoms. Some of them may cause headache, muscle ache, and temporary blood pressure alteration. Ingestion of 1.0 μg of enterotoxin can be onset of digestion system disease[2]. Enterotoxins may stimulate the emetic receptor in intestinal track to develope symptoms including vomiting and diarrhea[3].

Staphylococcus aureus is heat-labile, but its enterotoxins are quite heat-stable. Among the staphylococcal enterotoxins, SEC shows the highest heat stability followed by SEB and SEA[4]. Therefore, even by heat treatment at 60°C for 30 min could not inactivate SEC[5]. The methodology in detection of enterotoxins can be divided into three categories. They are biological method, immunoassay, and polymerase chain reaction method. Animal study is necessary for performing biological method, which is therefore restricted on operation. Immunoassay is the commonly-used method for detection of staphylococcal enterotoxins. Its detection relies on a binding between antigen and antibody accompanied with a biochemical reaction. There are several serologically distinct enterotoxins. Among them, enterotoxins, A, D, E, G, H, and I share over 50% sequence homology and are immunologically cross-reactive proteins. Enterotoxins B and C1-C3 also share extensive sequence homology and perform cross-reaction in immunology too[6]. They demonstrate similarity both in physical and chemical properties[2, 7, 8]. According to Couch and Betley[9], SEC3 has 98% nucleotide sequence homology with SEC1, while SEC1and SEB exhibit 68% amino acids homology. The chemical structures with high similarity between SEB and SEC1 are also observed, especially the functional groups close to N and C terminals of polypeptides. Furthermore, they are similar in antigen

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decision position and molecular weight. Both carry one disulfide bond and no free sulfhydryl groups[10-13]. A cross-reaction occurrence among N terminals of polypeptides in SEB and SEC1 has been observed as detected by antibody[14]. A more than 97% similarity among three subtypes (C1, C2, and C3) of enterotoxin C has been reported[15]. Immunoassay detection on above enterotoxins is likely to cause a cross-reaction. Among *Staphylococcus* spp., *S. intermedius* and *S. hyicus* can also produce enterotoxins in addition to *S. aureus*[16].

Coagulase, an extracellular protein excreted by *S. aureus*, is a pathogenic factor. Its C terminals of gene sequence are composed of several 81-bp sequence sets. Subtyping of *S. aureus* may easily be achieved by taking advantage of above characteristics. A genetic subtyping of coagulase from *S. aureus* has been reported by Schwarzkopf *et al.*[17] Using PCR method, twelve *S. aureus* strains were isolated clinically and typed into two subtypes, which were then subdivided into four subtypes by digesting with a restriction enzyme, *Alu* I[17]. Another pathogenic factor, protein A, induced by *S. aureus* can block the Fc site of immunoglobulin that effectively keeps immunoglobulin from an antigen-antibody reaction. A short sequence repeat including several 24-bp sets has been found on the C terminals of protein A gene. Subtyping of *S. aureus* can be achieved by differentiation of these short sequence repeats among strains[18]. Therefore, both coagulase and protein A genes are critical targets for the detection of *S. aureus*.

The epidemiological markers are set up for disease prevention purpose. In epidemiological study, subtyping of pathogens is conducted using various techniques for tracking the sources of pathogens and investigating the characteristics of strain spreading areas. Using subtyping technique, the subtypes of pathogens isolated from the samples of animals or patients in food poisoning outbreak can be compared and identified. In case of new outbreak, the pathogen sources can be tracked immediately and the spreading areas can be thus controlled. In the mean time, the differences among pathogenic strains can be differentiated. There are several traditional subtyping methods, such as bacteriophage typing, serotyping, toxin typing, and antibiotic sensitivity test. Because of the development in molecular biology, subtyping technique has been upgraded to a molecular level. The following techniques have been applied to the study on the molecular epidemiology[19-22]. They are ribotyping, pulsed-field gel electrophoresis (PFGE), random amplified polymorphic DNA assay (RAPD), multilocus enzyme electrophoresis (MLEE), restriction fragment length polymorphism (RFLP), microrestriction analysis (MRA), differential PCR/16S rRNA, and internally transcribed spacer (ITS).

S. aureus is capable of producing the following toxins and extracellular proteins: enteroxins, coagulase, protein A, hemolysins (including α-, β-, γ-, and δ-), toxic shock syndrome toxin, lysostaphin, staphylococcal lysozyme, fibrinolysin, penicillinase, nuclease, pyrogenic exotoxin, and stphylokinase[23]. Production of coagulase is one of the important biochemical characteristics of *S. aureus*. *S. aureus* coagulase typing kits are commercially available. However, they are only able to type those strains into 8 subtypes. Some of the *S. aureus* strains are unable to be typed with these kits. Several genetic typing methods have been documented. Among them, Restriction Fragment Length Polymorphism (RFLP) method has been reported to be easy in operation and time saving. To take these advantages, RFLP technique was selected to be the genetic typing method in this study and expected to be applied to the detection and subtyping of *S. aureus* in food poisoning outbreak. The contamination sources and strains deviation as well as *S. aureus* epidemiological distribution in food can be further investigated by using the established method. In this study, the characteristics of high specificity of primers in polymerase chain reaction (PCR) to gene sequence were applied on the subtyping of staphylococcal enterotoxins, and detection of its coagulase and protein A genes in food poisoning outbreak. The PCR-RFLP method was used to identify coagulase and protein A genes and find their relationship with enterotoxin C producing strains, in order to establish the database of *S. aureus* strains in food poisoning outbreaks occurred in Taiwan.

MATERIALS AND METHODS

I. *Strains*

Total of 229 *S. aureus* strains were collected from the food poisoning outbreaks in Taiwan and 10 of them were identified to be enterotoxin C producing strains. This study used 10 strains including: NLFDSa144, NLFDSa385, NLFDSa403, NLFDSa511, NLFDSa512, NLFDSa513, NLFDSa514, NLFDSa515, NLFDSa516, and NLFDSa517, as well as another 4 enterotoxin C producing strains (CCRC12654, CCRC13826, CCRC13827, and CCRC13828), obtained from the Culture Collection and Research Center (CCRC).

II. *DNA Preparation*

The strains were inoculated in 3 mL of Trypticase Soy Broth (TSB), cultivated at 37°C overnight, and centrifuged at 13,200 x g for 3 min. The suspensions were then decanted and the precipitate, which contained pellet cells, was ready for DNA extraction. Procedures for DNA extraction were followed according to the instructions in the manual of Puregene DNA Isolation Kits (Model D-6000A, Gentra System, Inc., Minneapolis, USA).

III. *Polymerase Chain Reaction (PCR)*

The extracted DNA (1 μL) as template was added with a reaction mixture containing 1X reaction buffer (10 mM Tris-HCl, pH 8.8 at 25°C, 1.5 mM $MgCl_2$, 50 mM KCl, and

0.1% Triton X -100), 200 μM deoxynucleotide triphosphates, 500 nM each primer, and 1U/100 μL Recombinant Taq polymerase (Takara Shuzo Co., LTD, Shiga, Japan) to a final volume of 100 μL. Table 1 lists the Primer sequences[24-26]. A PCR reactor (GeneAmp, PCR system 9700, Applied Biosystems, Foster City, CA, USA) was used. Reaction temperatures were programmed as follows: (1) at 95˚C for 10 min; (2) at 95˚C for 1 min; (3) at 58˚C for 1.5 min; (4) at 72˚C for 1.5 min. The steps from (2) to (4) were repeated for 35 cycles and then finished with the step at 72˚C for 7 min. The resulting products were analyzed using a 2% agarose gel electrophoresis.

IV. *Restriction Fragment Length Polymorphism (RFLP)*

For the differentiation of the PCR products of coagulase and protein A genes, the PCR products (10 μL of each) were treated with restriction enzymes *Alu* I and *Hin*d III in a thermal oven for 4 hr and analyzed with a 2% agarose gel electrophoresis. A 100 bp DNA Ladder (Promega Co., Madison, WI, USA) was used as a molecular weight marker.

V. *DNA Sequencing*

(I) *PCR products recovering*

The PCR products from enterotoxin C and coagulase genes of CCRC13828 and CCRC13827 strains were separated by using a 2% low melting point agarose (Promega) gel electrophoresis. The agarose gel was cut and the PCR products in cut gels were then recovered using a DNA purification system kit (Promega).

(II) *Cloning*

A ligation reaction between above recovered DNA fragments and pGEM-T Easy vector (Promega) was proceeded overnight to generate a ligation plasmid named pGEM-T-828, which was then introduced into a host, *E. coli* (JM109). The host containing pGEM-T-828 was cultivated in a Luria-Bertani (LB) medium, on which it was spread with iso-propyl-β-D-thioglactopyranoside (IPTG), 5-bromo-4-chloro-3-indolyl-β-D-glactoside (X-Gal), and ampicillin for screening purpose. After overnight cultivation, the white colonies were selected to be the targets of plasmid extraction.

(III) *Plasmid purification*

Above selected colonies were inoculated in 3 mL of LB and cultivated at 37˚C overnight. One mL of cultivated fluid was then transferred and centrifuged at 12,000 rpm for 3 min. The liquid suspension was decanted and the precipitate was kept for Plasmid DNA purification following the instructions of the manual of Wizard plus SV Minipreps DNA Purification System (Promega). The extracted Plasmid DNA was analyzed using 0.7% agarose gel electrophoresis to confirm if a successful ligation between PCR products and vector was achieved.

(IV) *DNA sequencing and DNA sequence alignment*

The primers in SP6 and T7 RNA polymerase promoters from the pGEM-T Easy vector were the targets for DNA sequencing. A Beckman CEQ Dye Terminator Cycle Sequencing (DTCS) Quick start Kit (P/N 608120) was used for DTCS reaction. After reaction, the reaction products were then precipitated and analyzed using Beckman CEQ 2000XL capillary electrophoresis. The nucleic acid sequences were then aligned according to program BLAST from the website supplied by National Center for Biotechnology Information (NCBI).

RESULTS AND DISCUSSION

I. *Specificity Test of Enterotoxin C Subtyping Primers*

In this study, total of 229 *S. aureus* strains were isolated from 36 food poisoning outbreaks and 10 of them were identified to contain enterotoxin C genes by Multiplex PCR enterotoxins gene screening. They were further confirmed by using ELISA, to be able to produce enterotoxin C protein[27]. Therefore, these strains were used for subtyping. Four *S. aureus* strains, CCRC12654 (SEC1), CCRC13826 (SEC1), CCRC13827 (SEC2), and CCRC13828 (SEC3), which were obtained from the Culture Collection and Research Center (CCRC), were used as references. Subtyping of the enterotoxins produced by

Table 1. Specific primers used for the detection of specific factors in *S. aureus* by PCR method

Gene	Primer	Sequence	Primer source reference
Coagulase	COAG2	5'-CGAGACCAAGATTCAACAAG-3'	Goh, *et al.*[24]
	COAG3	5'-AAAGAAAACCACTCACATCA-3'	
Protein A	SPA-1	5'-CAAAGATCAACAAGCGCC-3'	Schwarzkopf, *et al.*[25]
	SPA-2	5'-CGAAGGATCGTCTTTAAGGC-3'	
Enterotoxin C	C1	5'-AAATTCACTGGTTTGATGGAA-3'	Haiao[26]
	C21	5'-AATAAGAGTCGATTTATTTCATGC-3'	
	C22	5'-GTACCAGTAAACTCACTTGA-3'	
	C3	5'-TATAAGAGATTATTTATTTCACGT-3'	This study
	C4	5'-TATTCCTCCATACATACAAG - 3'	

the above four strains were achieved by ELISA testing.

The specific primers were selected to carry on PCR testing. The strains of CCRC13826 and CCRC13827 were used to detect enterotoxins C1 (C1/C4 primer) and C2 (C21/C22 primer) in PCR reaction, respectively. They were able to generate fragments 300 bp and 137 bp, respectively. A PCR reaction of CCRC13828 for the detection of enterotoxin C3 (C3/C4 primer) unexpectedly generated a fragment 137 bp, which was supposed to be the product in detecting C2 enterotoxin (C21/C22), while fragment 426 bp, which was expected to appear, was missing. However, the strain NLFDASa511 isolated from food poisoning outbreak was able to produce fragment 426 bp for the detection of C3 enterotoxin (C3/C4 primer) after PCR reaction. The testing of each primer in subtypes of enterotoxin C showed satisfactory reproducibility and specificity (Figure 1).

II. *Subtyping and Sequencing of Coagulase and Protein A Genes*

The above result has shown that CCRC13828 was able to produce enterotoxin C2 instead of C3. To reconfirm this result was not due to cross-contamination with CCRC13827 (SEC2), the subtypes of coagulase and protein A genes of the above two strains were identified and compared. PCR subtyping results indicated that both CCRC13828 and CCRC13827 carried the same types of coagulase as shown in Figure 2 (lanes 2 and 3). However, their RFLP patterns were different when their PCR products were treated with restriction enzyme *Alu* I (lanes 2 and 3 in Figure 3). After treatment of PCR products from the subtypes of protein A genes with restriction enzyme *Hin*d III, the above two strains also showed different RFLP patterns (Figure 4). Furthermore, the enterotoxin C3 genes of CCRC13828 were sequenced and the results were compared to *S. aureus* enterotoxin C3 genes (Accession No. X51661) listed in the NCBI database. Ninety-one percent similarity was shown between them. However, as compared to the enterotoxin C2 genes in database, it showed 100% similarity. Therefore, CCRC13828, identified to be enterotoxin C3 strain by ELISA method, needs to be corrected to be enterotoxin C2 strain. This study has demonstrated the advantage of using PCR method for subtyping of enterotoxin C. The identification results generated by ELISA method is inconsistent with gene detection method possibly due to a cross-reaction occurred in using ELISA method.

PCR detection of coagulase genes in 14 *S. aureus* enterotoxin C strains revealed that their coagulase genes could be subtyped into three types. They were designated to be X, Y, and Z, and the fragments of PCR products were about 970 bp, 840 bp, and 910 bp, respectively, in agarose gel electrophoresis as shown in Figure 2. The Y type could be further divided into two types after treating their PCR products from coagulase genes with restriction enzyme *Alu* I. Therefore, in total, four types of PCR-RFLP coagulase genes, X1, Y1, Y2, and Z1, from *S. aureus* enterotoxin C strains were thus obtained (Figure 3). After treating with restriction enzyme *Alu* I, X1 type gene was digested into three fragments, 90 bp, 390 bp, and 490 bp; Y1 type gene remained intact; Y2 type was digested to 220 bp, 240 bp, and 380 bp fragments; while Z1 was digested to four

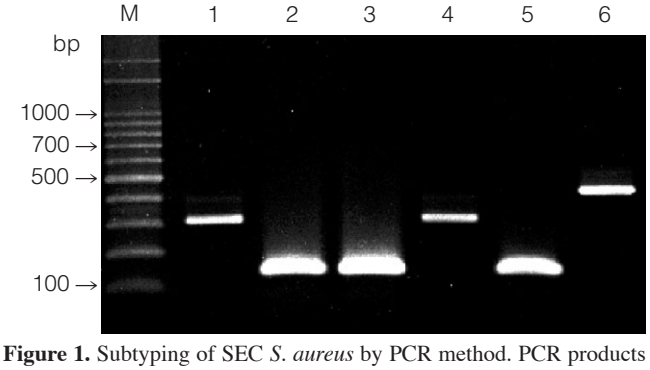

Figure 1. Subtyping of SEC *S. aureus* by PCR method. PCR products was loaded on 2.0% agarose gel and electrophoresed. Lane M: 100 bp ladder markers; lane 1: CCRC13826 (C1); lane 2: CCRC13827 (C2); lane 3: CCRC13828 (C2); lane 4: CCRC12654 (C1); lane 5: NLFDSa144 (C2); lane 6: NLFDSa511 (C3).

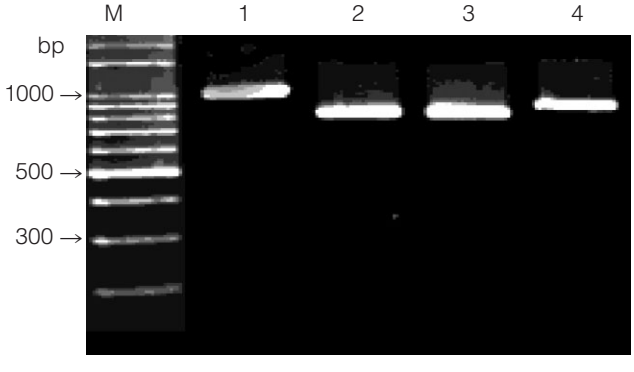

Figure 2. Detection of the coagulase gene in SEC *S. aureus* strains by PCR method. Lane M: 100 bp ladder markers; Lane 1: CCRC13826 (X); Lane 2: CCRC13827 (Y); Lane 3: CCRC13828 (Y); Lane 4: NLFDSa144 (Z).

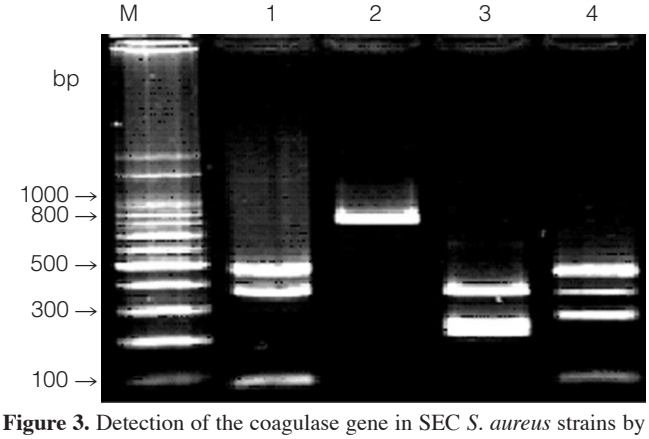

Figure 3. Detection of the coagulase gene in SEC *S. aureus* strains by PCR-RFLP. With RFLP, each strain had a different profile of *Alu* I restriction fragments. Lane M: 100 bp ladder markers; Lane 1: CCRC13826 (X1); Lane 2: CCRC13827 (Y1); Lane 3: CCRC13828 (Y2); Lane 4: NLFDSa144 (Z1).

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fragments, 80 bp, 90 bp, 270 bp, and 470 bp. In Figure 3, two fragments, 80 bp and 90 bp, on lane 4 were close to each other and an unexpected fragment appeared at 360 bp position suggesting an incomplete reaction with restriction enzyme.

Both Y1 and Y2 genes were sequenced to study why the restriction enzyme *Alu* I could only digest Y2 but not Y1 although they originated from the same Y type (840 bp) of the coagulase fragments of PCR products. Results showed the gene sequence in relative position of being digested by *Alu* I for Y1 type was CGCT, as compared to Y2 type which showed AGCT. Hence, restriction enzyme can only work on Y2. The Y type of RFLP coagulase gene was thus further divided into Y1 and Y2 subtypes. Gene sequencing is capable of differentiating the nucleic acid sequences among genes. However, it is time-consuming, labor intensive, and more expensive compared to PCR-RFLP method, which is recommended in this study. In addition, problems with the application of this technique on gene sequencing the direct recovered PCR products need to be solved. It needs highly skillful operation in cloning and plasmid recovering.

Subtyping of 14 *S. aureus* enterotoxin C strains were also conducted on the basis of protein A gene identification. Two types, type I and II, of genes at positions 620 bp and 450 bp, respectively, in agarose gel electrophoresis were determined. After treated with restriction enzyme *Hin*d III, the RFLP patterns showed type I gene was further digested into three fragments (type I-1), 170 bp, 190 bp, and 260 bp. Type II gene was digested into two fragments (type II-1), 190 bp and 260 bp as shown in Figure 4. Therefore, a PCR reaction of protein A gene could subtype those enterotoxin C strains into two types. Further subtyping with RFLP could also result in two types. Subtyping using PCR-RFLP method could generate a clear-cut result and therefore is suggested to be a reference method for basic classification.

III. *Subtyping of S. aureus Enterotoxin C Producing Strains Isolated from Food Poisoning Outbreaks in Taiwan*

Among 229 *S. aureus* strains isolated from 36 food poisoning outbreaks in Taiwan, 27% of them were deter-

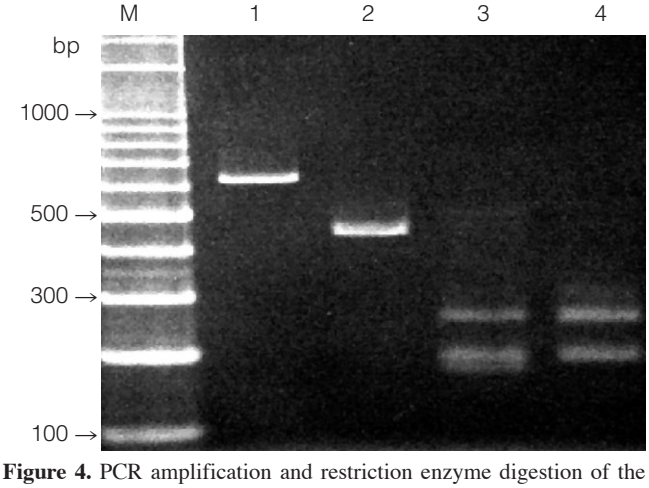

Figure 4. PCR amplification and restriction enzyme digestion of the protein A gene products from SEC *S. aureus* strains. Lane M: 100 bp ladder markers; Lane 1: PCR products from CCRC13827; Lane 2: PCR products from CCRC13828 ; *Hin*d III restriction is shown in lane 3 and lane 4. Lane 3: CCRC13827; Lane 4: CCRC13828.

Table 2. Subtyping of SEC *S. aureus* strains by PCR and PCR-RFLP for coagulase and protein A genes

Strain	Enterotoxin C subtype[a]	Polymorphism of coagulase gene[b]		Polymorphism of protein A gene[c]		Subtypes by combined method[d]
		PCR	PCR-RFLP	PCR	PCR-RFLP	
CCRC 13826	C1	X	X1	I	I-1	S1
CCRC 13827	C2	Y	Y1	I	I-1	S2
CCRC 13828	C2	Y	Y2	II	II-1	S3
CCRC 12654	C1	X	X1	I	I-1	S1
NLFDSa144[e]	C2	Z	Z1	I	I-1	S4
NLFDSa385[f]	C2	Z	Z1	I	I-1	S4
NLFDSa403[f]	C2	Z	Z1	I	I-1	S4
NLFDSa511[g]	C3	Y	Y1	I	I-1	S5
NLFDSa512	C3	Y	Y1	I	I-1	S5
NLFDSa513	C3	Y	Y1	I	I-1	S5
NLFDSa514	C3	Y	Y1	I	I-1	S5
NLFDSa515	C3	Y	Y1	I	I-1	S5
NLFDSa516	C3	Y	Y1	I	I-1	S5
NLFDSa517	C3	Y	Y1	I	I-1	S5

a: Subtyping of *sec* genes by PCR method.

b: PCR and PCR-RFLP patterns of coagulase gene: X: 970 bp; Y: 840 bp; Z: 910 bp. X1: 90 bp, 390 bp and 490 bp; Y1: 840 bp; Y2: 220 bp, 240 bp and 380 bp; Z1: 80 bp, 90 bp, 270 bp and 470 bp.

c: PCR and PCR-RFLP patterns of protein A gene: I: 620 bp; II: 450 bp. I-1: 170 bp, 190 bp and 260 bp; II-1: 190 bp and 260 bp.

d: Patterns of subtypes by *sec* genes, and PCR and PCR-RFLP patterns of coagulase and protein A genes: S1:C1X1I-1; S2: C2Y1I-1; S3: C2Y2II-1; S4: C2Z1I-1; S5: C3Y1I-1.

e: Isolated from the swab of sink, outbreak occurred in Taoyoan, Taiwan, December 1998.

f: Both strains were isolated from the swab of the food handler (hands), outbreak occurred in Keelung, Taiwan, August 1994.

g: All seven strains below were isolated from steamed eggs, outbreak occurred in Taipei, Taiwan, January 2002.

mined to be enterotoxin A producing strains[27]. Ten strains isolated from three food poisoning outbreaks were found to be enterotoxin C producing strains. Table 2 lists the results for subtyping of SEC *S. aureus* by PCR and PCR- RFLP methods for coagulase and protein A genes. There were 3 enterotoxin C2 strains isolated from two outbreaks and 7 enterotoxin C3 strains were isolated from the same outbreak. According to Chen *et al.*[29], 39 *S. aureus* entertoxin C producing strains, isolated from the feces of patients in food poisoning outbreaks during 1995 to 2000 in Taiwan, were subtyped into enterotoxins C2 and C3 by PCR method. Their subtyping results were similar to the results in this study although the sources of test samples were varied.

Subtyping of coagulase gene by PCR and PCR-RFLP methods showed that 3 enterotoxin C2 strains belonged to types Z and Z1; while 7 enterotoxin C3 strains belonged to types Y and Y1. Subtyping of protein A gene by PCR and PCR-RFLP methods revealed that both enterotoxins C2 and C3 were classified into type I. These results indicated that the pathogen sources of *S. aureus* enterotoxin C for the above three poisoning outbreaks were simple and were not contaminated with other types of enterotoxin C pathogens. In summary, the 4 CCRC strains tested in this study could be subtyped into three types, while the 10 native strains could only be subtyped into two according to the results of subtyping enterotoxin C, coagulase gene, and protein A gene. These subtypes were all different from those of reference strains.

CONCLUSION

The subtypes of enterotoxin C are a group of highly conserve protein. Among 239 amino acids, which consist enterotoxin C protein, only four amino acids differences have been shown between C2 and C3 subtypes[30]. Therefore, a false positive testing result is easy to occur due to the cross-reaction in immunoassay for reference strains testing as mentioned in this paper. A highly specificity primers used in this study was capable of differentiating enterotoxins C1, C2, and C3 without any cross-reaction that may occur as with immunoassay, even though these subtypes have 98% similarity in gene sequence. Except for enterotoxins C1, C2, and C3[31], other types of enterotoxins can still be differentiated by immunoassay without cross-reaction although they are similar in enterotoxin structures and immuno-biological characteristics[32]. Subtyping of enterotxoin C producing strains through their coagulase gene can obtain three subtypes with PCR method and get four subtypes with PCR-RFLP method. However, both the PCR and PCR-RFLP subtyping protein A gene can only obtain two subtypes. Therefore, the subtyping of enterotoxin C producing *S. aureus* strains can be assisted by subtyping of coagulase and protein A genes in order to investigate the simplicity of contamination source in food poisoning outbreaks.

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REFERENCES

1. Shimizu, A., Wakita, Y. and Igarashi, H. 2001. Molecular epidemiology of *Staphylococcus aureus* coagulase type VII isolation from staphylococcal food poisoning outbreaks 1980-1995 in Tokyo, Japan, by pulsed-field gel electrophoresis. Food Sanitation Res. 51: 45-52.
2. Reiser, R., Conaway, D. and Bergdoll, M. S. 1974. Detection of staphylococcal enterotoxins in foods. Appl. Microbiol. 27: 83-85.
3. Sugiyama, H. and Hayama, T. 1965. Abdominal viscera as site of emetic action for staphylococcal enterotoxin in the monkey. J. Infect. Dis. 115: 330-336.
4. Tibana, A., Rayman, K., Akhtar, M. and Szabo, R. 1987. Thermal stability of staphylococcal enterotoxins A, B, and C in a buffered system. J. Food Prot. 50: 239-242.
5. Borja, C. R. and Bergdoll, M. S. 1967. Purification and partial characterization of enterotoxin C produced by *Staphylococcus aureus* strain 137. Biochemistry. 6: 1463-1467.
6. Iandolo, J. J. 1989. Genetic analysis of extracellular toxins of *Staphylococcus aureus*. Anu. Rev. Microbiol. 43: 375-402.
7. Bergdoll, M. S. 1967. The staphylococcal enterotoxins. In Biochemistry of Some Foodborne Microbial Toxins. Mateles, R. I. and Wogan, G. N. eds. pp.1-25. MIT Press Cambridge, MA.
8. Schantz, E. J., Roessler, W. G., Wagman, J., Spero, L., Dunnery, D. A. and Bergdoll, M. S. 1965. Purification of staphylococcal enterotoxin B. Biochemistry 4: 1011-1016.
9. Couch, J. L. and Betley, M. J. 1989. Nucleotide sequence of the type C3 staphylococcal enterotoxin gene suggests that intergenic recombination causes antigenic variation. J. Bacteriol. 171: 4507-4510.
10. Bergdoll, M. S. and Schlievert, P. M. 1984. Toxic-shock syndrome toxin. Lancet ii: 691.
11. Spero, L., Morlock, B. A. and Metzger, J. F. 1978. On the cross-reactivity of staphylococcal enterotoxins A, B, and C. J. Immunol. 120: 86-89.
12. Spero, L., Griffin, B. Y., Middlebrook, J. L. and Metzger, J. F. 1976. Effect of single and double peptide bond scission by trypsin on the structure and activity of staphylococcal enterotoxin C. J. Biol. Chem. 251: 5580-5588.
13. Spero, L., Warren, J. R. and Metzger, J. F. 1973. Effect of single and double peptide bond scission by trypsin on the structure and activity of staphylococcal enterotoxin B. J. Biol. Chem. 248: 7289-7294.
14. Spero, L. and Morlock, B. A. 1979. Cross reactions

between tryptic polypeptides of staphylococcal enterotoxin B and C. J. Immunol. 122: 1285-1289.
15. Betley, M. J., Borst, D. W. and Regassa, L. B. 1992. Staphylococcal enterotoxins toxic shock syndrome toxin and streptococcal pyrogenic exotoxins: a comparative study of their molecular biology. Chem. Immunol. 55: 1-35.
16. Youmans, G. P., Palerson, P. Y. and Sommers, H. M. 1980. The Biologic and Clinical Basis of Infectious Diseases. 2nd ed. pp.639-648. W. B. Saunders Co., Philadelphia, U. S. A.
17. Schwarzkopf, A. and Karch, H. 1994. Genetic variation in *Staphylococcus aureus* coagulase genes: potential and limits for use as epidemiological marker. J. Clin. Microbiol. 32: 2407-2412.
18. Shopsin, B., Gomez, M., Montgomery, S. O., Smith, D. H., Waddington M., Dodge, D. E., Bost, D. A., Riehman, M., Naidich, S. and Kreiswirth, B. N. 1999. Evaluation of protein A gene polymorphic region DNA sequencing for typing of *Staphylococcus aureus* strains. J. Clin. Microbiol. 37: 3556-3563.
19. Deplano, A., Vaneechoutte, M., Verschraegen, G. and struelens, M. J. 1997. Typing of *Staphylococcus aureus* and *Staphylococcus* epidermidis strains by PCR analysis of inter-IS256 spacer length polymorphisms. J. Clin. Microbiol. 35: 2580-2587.
20. Skov, M. N., Pedersen, K. and Larsen, J. L. 1995. Comparison of pulsed-field gel electrophoresis, ribotyping, and plasmid profiling for typing of *Vibrio anguillarum* serovar O1. Appl. Environ. Microbiol. 61: 1540-1545.
21. Pedersen, K., Koblavi, S., Tiainen, T. and Griment, P. T. 1996. Restriction fragment length polymorphism of the pMJ101-like plasmid and ribotyping in the fish pathogen *Vibrio ordalii*. Epidemiol. Infect. 117: 385-391.
22. Griment, F. and Griment, P. A. D. 1986. Ribosomal ribonucleic acid gene restriction patterns as potential taxonomic tools. Ann. Inst. Pasteur/Microbiol. 137B: 165-175.
23. Hui, Y. H., Pierson, M. D. and Gorham, J. R. 2001. *Staphylococcus aureus* In: Food borne Disease Handbook. 2nd ed. pp.345-382. Marcel Dekber, Inc., U.S.A.
24. Goh, S. H., Byrne, S. K., Zhang j. L. and Chow A. W. 1992. Molecular typing of *Staphylococcus aureus* on the basis of coagulase gene polymorphisms. J. Clin. Microbiol. 30: 1642-1645.
25. Schwarzkopf, A., Karch, H., Schmidt, H., Lenz, W. and Heesemann, J. 1993. Phenotypical and genotypical characterization of epidemic clumping factor-negative, oxacillin-resistant *Staphylococcus aureus*. J. Clin. Microbiol. 31: 2281-2285.
26. Haiao, M. H. 1999. PCR Differentiation of enterotoxin C1, C2 and C3 strains and the development of specific primers for enterotoxin G, H, and I gene of *Staphylococcus aureus*. Master thesis. p. 40. Department of Food Science, National Chung-Shing University, Taiwan, R. O. C.
27. Huang, T. P., Lai, Y. J., Chang, Y. C., Wang, J. Y. and Shih, Daniel Y. C. 2000. Coagulase typing of *Staphylococcus aureus* by PCR-RFLP technique. Annual scientific report of National Laboratories of Food and Drugs, Department of Health, Executive Yuan, Taiwan, R. O. C.
28. Bohach, G. A. and Schlievert, P. M. 1989. Conservation of the biologically active portions of staphylococcal enterotoxins C1 and C2. Infect. Immun. 57: 2249-2252.
29. Chen, T. R., Hsiao, M. H., Chiou C. S. and Tsen, H. Y. 2001. Development and use of PCR primers for the investigation of C1, C2, and C3 enterotoxin types of *Staphylococcus aureus* strains isolated from food-borne outbreak. Int. J. Food Microbiol. 71: 63-70.
30. Hovde, C. J, Hackett, S. P. and Bohach, G. A. 1990. Nucleotide sequence of the staphylococcal enterotoxin C3 gene: sequence comparison of all three type C staphylococcal enterotoxins. Mol. Gen. Gent. 220: 329-333.
31. Avena, R. M. and Bergdoll, M. S. 1967. Purification and some physicochemical properties of enterotoxin C, *Staphylococcus aureus* strain 361. Biochemistry 6: 1474-1480.
32. Bergdoll, M. S., Robbins, R. N., Weiss, K., Borja, C. R., Huang, I. Y. and Chu, F. S. 1973. The staphylococcal enterotoxins: similarities. Contrib. Microbiol. Immunol. 1: 390-396.