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Contributed Paper

Detection of *Staphylococcus aureus* Enterotoxin Type I using qPCR

Chotika Jackranukul [a], Nathinee Panvisavas [b] and Soraya Chaturongakul*[c]

[a] Forensic Science Graduate Program, Faculty of Science, Mahidol University, Bangkok 10400, Thailand.

[b] Department of Plant Science, Faculty of Science, Mahidol University, Bangkok 10400, Thailand.

[c] Department of Microbiology, Faculty of Science, Mahidol University, Bangkok 10400, Thailand.

*Author for correspondence; e-mail: scsch@mahidol.ac.th

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ABSTRACT

Epidemiological surveillance reports revealed that *Staphylococcus aureus* ranked third among top bacterial pathogens causing food poisoning in Thailand during 2010-2012. Thailand Food and Drug Administration (FDA) abides by the international "zero tolerance" policy for staphylococcal enterotoxins (SEs). Specifically, SEs must be absent in 25 g of food, while the bacteria are tolerated at low level depending on the types of food. Increasing numbers of *S. aureus* isolates from foods carried the newly described staphylococcal enterotoxin type I (SEI) and type G (SEG) rather than the classic types (e.g., SEA and SEB) previously identified as the common cause of staphylococcal food poisoning. In this study, we developed a quantitative PCR (qPCR) for detection of SEI-encoding gene as SEI is the most prevalent enterotoxin in newly described SE subgroup 3 and is increasingly found in *S. aureus* isolates from food samples causing food poisoning. The designed *sei* primer and probe set is specific to *S. aureus sei* gene and can detect as low as 150 femtogram of the target (approximately 50 bacterial cells/ml of milk). We proposed that our *sei* primer and probe set can be used in multiplex qPCR assay along with the already developed primer and probe sets targeting classic *sea* and *seb* genes for enterotoxin detection in food safety surveillance.

Keywords: *Staphylococcus aureus*, enterotoxin, SEI

1. INTRODUCTION

Staphylococcus aureus is the one of the most common causes of bacterial foodborne illnesses worldwide . In Thailand, the largest number of staphylococcal food poisoning cases was reported in 1995, when 485 people were ill after consumption of SE-contaminated eclair [1]. Epidemiological Surveillance Reports of Thai Bureau of Epidemiology, Department of Disease Control in 2010-2012 revealed that *S. aureus* ranked third among top bacterial pathogens causing food poisoning, first being *Vibrio parahaemolyticus* and second being *Salmonella* spp., and that *S. aureus* was the causative agent of approximately 10 percent of food poisoning cases known to be caused by bacterial pathogens[2]. Thailand Food and Drug Administration reinforces the international "zero tolerance" policy for absence of SEs in foods. Based on Thailand Food Act, violations would result in withdrawal of manufacturer's license, and

imprisonment for 2 years or 20,000 Thai Baht fine, or both [3].

S. aureus can produce exotoxins including exfoliative toxins, leukocidins, toxic shock syndrome toxin-1 (TSST-1) and staphylococcal enterotoxins (SEs) [4]. The staphylococcal enterotoxins (SEs) are emetic toxins and are the causes of staphylococcal food poisoning. SEs are further divided into the classic SEA to SEE and newly identified SEG to SE*l*U [5]. Based on amino acid sequence alignment of the SEs, mature forms of SEs can be divided into 4 subgroups: (i) SEA, SED, SEE, SEH, SEJ, SE*l*N, SE*l*O, and SE*l*P; (ii) SEB, SEC, SEG, SE*l*R, and SE*l*U; (iii) SEI, SE*l*K, SE*l*L, SE*l*M, and SE*l*Q; and (iv) TSST-1 or SEF [6]. Previous studies have shown that classic SEs, such as SEA and SEB, were the major causes of foodborne illnesses [7]. However, newly identified enterotoxins (SEG-SE*l*U) were found in increasing numbers together with classic SEs (SEA-SEE) from *S. aureus* isolated from foods [8]. In recent studies, more of *S. aureus* isolates from foods carried the newly described enterotoxins SEI and SEG than classic SEs [9, 11]. For example, among 70 *S. aureus* isolates from raw milk samples, SE genes were found in 39 (55.7%) isolates and, among the SE genes found, *sei* was the most prevalent at 26 (38.6%) isolates followed by *seg*, *sea*, and *seb* [9]. In 2011, Kim et al. [11] showed that, among 197 *S. aureus* isolates from ready-to-eat (RTE) foods (such as Sushi, Kimbab and California roll), the most frequently found enterotoxin genes were *sei*, *seg*, *sea*, and *tst-1*.

In staphylococcal food poisoning, ingested SEs induce massive release of 5-hydroxytryptamine (5-HT) or serotonin, a neurotransmitter in the small intestine that causes movements of intestinal muscles and secretion of fluids into the lumen, leading to abdominal cramp and diarrhea. Overwhelming stimulation of 5-HT_3 receptor by the 5-HT ligands results in emetic responses such as nausea and vomiting [12]. Such symptoms of food poisoning can occur within 2 to 6 hours after ingestion of SE contaminated foods depending on the dose. The emetic doses of SEA and SEI are approximately 20 and 200 μg per kilogram weight in house musk shrew animal model, respectively [12, 13]. Such doses could be reached if *S. aureus* producing enterotoxins is present more than 10^5 colony forming unit (CFU) per gram of food [14].

SEs are resistant to many protein denaturing factors such as heat, pH variation, and various proteases in gastrointestinal tract; therefore, elimination of SEs is difficult to manage. Early detection of SEs in foods is necessary for risk management and surveillance in food safety. In recent years, Enzyme-Linked Immunosorbent Assay (ELISA), latex agglutination, PCR and Multiplex PCR [15, 16] have been developed for detection of classic SEs such as SEA to SEE but the results are relatively more time-consuming, less specific and less sensitive than qPCR [17]. In this study, we focused on SEI because SEI is the most prevalent enterotoxin in SE subgroup 3 and is increasingly found in *S. aureus* isolates causing food poisoning. SEI is encoded by *sei* gene with 729 nucleotides in length [18]. Genes encoding SEI are frequently found along with genes encoding SEA, SEC, SEG, SE*l*L, SE*l*M, SE*l*N, SE*l*O, SE*l*P and TSST-1 [19]. *S. aureus* isolates containing *sei* gene are commonly associated with foods such as raw milk [20], meat [21], dairy products [21], and some are also clinical isolates [22]. In this study, we developed qPCR assay for detection of *sei* using TaqMan technology. We proposed that our *sei* primer and probe set can be used in multiplex qPCR assay along with primer and probe sets for detection of the *sea* and *seb* genes [17] for simultaneous screening of SEs contamination in large

amount of food samples with an increased sensitivity of SE detection.

2. MATERIALS AND METHODS

2.1 Sequence Alignment and Target Selection

DNA sequences of *S. aureus sei* genes were obtained from GenBank (i.e., *S. aureus* JH1 accession CP000736.1, *S. aureus* JH9 accession CP000703.1, *S. aureus* Mu3 accession AP009324.1, *S. aureus* Mu50 accession BA000017.4, and *S. aureus* N315 accession BA000018.3). BioEdit Sequence Alignment Editor V.7.0.4 program [23] was used to identify the conserved regions of *sei* genes before specific primer and probe set was designed based on the following criteria e.g., primers with melting temperature [Tm] of 58-60°C, probe with Tm of 68-70°C, and GC content of 20-80%. Sequences of primers and probe are showed in Table 1. Specificity of the *sei* primer and probe set was also confirmed by BLAST search.

2.2 Bacterial Strains and Culture Media

Bacterial strains used in this study were *S. aureus* ATCC25923 (SEI variant positive), *S. aureus* RF122 (SEI positive), *S. aureus* ATCC29213, *S. aureus* ATCC25178, *S. aureus* ATCC29740, *S. aureus* FPR3757, *S. aureus* COL, and other bacterial pathogens as negative controls (i.e., *Escherichia coli* ATCC25922, *Streptococcus pyogenes*, *Streptococcus agalactiae*, *Pseudomonas fluorescens*, *Listeria monocytogenes*, *Bacillus subtilis*, and *Salmonella enterica* serotype Typhimurium). Bacterial cultures were streaked from -80°C frozen stocks onto tryptic soy agar (TSA; Becton, Dickinson, and Company, Sparks, MD, USA) and incubated at 37°C for 24 hours. Overnight culture was prepared by inoculation of a single colony into 5 ml of tryptic soy broth (TSB) and incubated in a shaking incubator (200 rpm) at 37°C for 16-18 hours.

2.3 Isolation of DNA

Genomic DNA was isolated from overnight cultures according to Wilson et al. [24], with minor modifications. Briefly, cells were collected from 2 ml overnight culture by centrifugation at 10,000 rpm for 10 min. Supernatant was discarded and pellet was resuspended in 432 μl of Tris-EDTA (TE) buffer. An aliquot of 30 μl of 50 mg/ml lysozyme (Sigma-Aldrich, St. Louis, MO, USA) at a final amount of 1.5 mg was added to the resuspended cells instead of lysostaphine, and incubated at 37°C for 1 h. Then, 7.5 μl of 20

Table 1. Primer and probe sequences used in qPCR assay.

Target		Sequence (5'->3')	Product size (base pairs)
SEI	Forward	GTTTGCCATTAACCCAAAGATTAA	134
	Reverse	GAAACTGGATATTTTTGGCATTGA	
	Probe	FAM-TATTGTCCTGATAAAGTGGCCCCTCCA-BHQ1	
IPC[a]	Forward	AAGCGTGATATTGCTCTTTCGTATAG	77
	Reverse	ACATAGCGACAGATTACAACATTAGTATTG	
	Probe	NED-TACCATGGCAATGCT-MGB-NFQ	

[a] Internal positive control (IPC) for qPCR is from Hudlow et al. [25]. FAM and NED are fluorescent dyes with excitation/emission wavelengths at 495/520 nm and 546/575 nm, respectively. BHQ or black hole quencher is a dark quencher and NFQ is a non-uorescent quencher linked to MGB (minor groove binder) sugar moiety (Biosearch Technologies, Novato, CA, USA).

mg/ml Proteinase K (USB, Cleveland, OH, USA) at a final amount of 150 μg was added and further incubated at 37°C for 1 h. A 30-μl aliquot of 10% (w/v) SDS was added to the lysate, and incubated at 65°C for 30 min prior to phenol-chloroform extraction. The top aqueous was transferred into a new sterile microfuge tube. DNA was then recovered by ethanol precipitation in the presence of 3M sodium acetate. The pellet was resuspended in 250 μl of TE buffer. DNA concentration was measured by spectrophotometry using NanoDrop™ spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

2.4 PCR and qPCR

Amplifications of *sei* target region by PCR and qPCR were carried out in a final volume of 25 μl containing the following components: 900 nM of *sei* forward/reverse primer, 250 nM *sei* probe, TaqMan RT-qPCR VeriQuest™ Probe One-Step RT-qPCR Master Mix (2X) with ROX dye (USB), RNase free water, and 2.5 μl of DNA sample at varying concentrations (e.g., containing 10^7, 10^5, and 10^3 copies of chromosomal DNA for standard curve). In order to ensure amplification in *sei*-negative samples, Internal Positive Control (IPC) primers and probe (Table 1) were also added (i.e., 100 nM of IPC forward/reverse primer and 100 nM of IPC probe) into the reaction. Thermocycling condition for qPCR was 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 sec, 60°C for 1 min. After qPCR was completed, threshold value was automatically determined by ABI 7500 Software (Life Technologies, Grand Island, NY, USA). Ct value of each sample was reported. Samples with Ct values above 35 were considered *sei*-negative. Experiments were performed at least in duplicates.

2.5 Detection of *S. aureus* in Milk

Ten-fold serial dilutions were performed by adding 100 μl of the overnight culture into 900 μl of PBS (Phosphate Buffered Saline). Density of overnight cultures (CFU/ml values) was determined using plate spreading and bacterial count in the following day. Aliquots of 100 μl of diluted *S. aureus* cultures were spiked into 900 μl of non-fat milk to give final cell density of approximately 10^7, 10^5, 10^3 and 10 CFUs/ml. DNA was extracted from spiked milk samples using PrepSEQ™ Rapid Spin Sample Preparation Kit (Life Technologies) according to manufacturer's instruction with modification of lysis buffer. Lysis buffer for *Staphylococcus* spp. containing 100 μg/ml lysostaphine was used instead lysis buffer for *Salmonella* spp. provided in the kit. Experiments were performed in triplicates.

3. RESULTS AND DISCUSSION

To design TaqMan probe and primers specific to *S. aureus* enterotoxin type I gene, we aligned and selected a 134-bp conserved region (position 345 to 478 bp) of *sei* genes from *S. aureus* strains JH1, JH9, Mu50, N315 and Mu3, to perform BLAST search. Results showed that the designed probe and primer set can detect *sei* genes from at least 30 different *S. aureus* strains with E-values ranging from 6^{e-63} to 4^{e-55}. After testing probe and primer set on selected *S. aureus* strains in qPCR, the designed oligos can detect *sei* target sequences including those in *S. aureus* that show polymorphisms (Figure 1). Although sequences of forward and reverse primers are not identical to those in *S. aureus* RF122 and ATCC25923 *sei* genes, the calculated PCR efficiencies (Equation 1) are 1.90 for ATCC25923, 1.96 for RF122, and 1.97 for ATCC29213, corresponding to 95%, 98% and 98% amplification. These values suggest that even if polymorphisms are present in

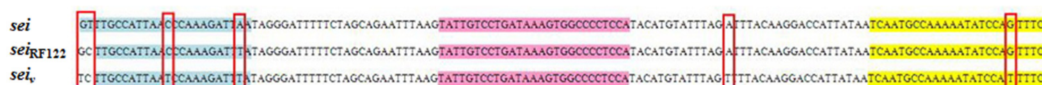

Figure 1. Nucleic acid alignment (position 345 to 478) of *S. aureus sei* genes from strain Mu50 (*sei*), RF122, and ATCC25923 (sei_v). Polymorphisms are shown in red boxes. Sequences of forward primer, TaqMan probe, and reverse primer are highlighted in blue, pink, and yellow, respectively.

unknown food samples, the designed *sei* probe and primer set can be used to detect these alleles at high efficiencies.

$$E = 10^{(-1/slope)}; \qquad \text{Equation 1}$$

where E is PCR efficiency and E equals 2 when 100% amplification is achieved [26]. Slope is based on DNA standard curve (Figure 2).

To verify that the designed primer and probe set is only specific for *sei* genes in SEI producing *S. aureus*, the oligos were also tested on *S. aureus* strains not producing SEI such as *S. aureus* ATCC29740 and *S. aureus* FPR3757. As expected, the primer and probe set could not detect *sei* targets as fluorescence was not detected in samples containing DNA from these strains. In addition, non-*S. aureus* bacteria such as *Streptococcus pyogenes*, *Listeria monocytogenes*, and *Salmonella enterica* serotype Typhimurium were also tested. The designed oligos could not detect these other bacterial strains (i.e., Ct values were more than 35). To

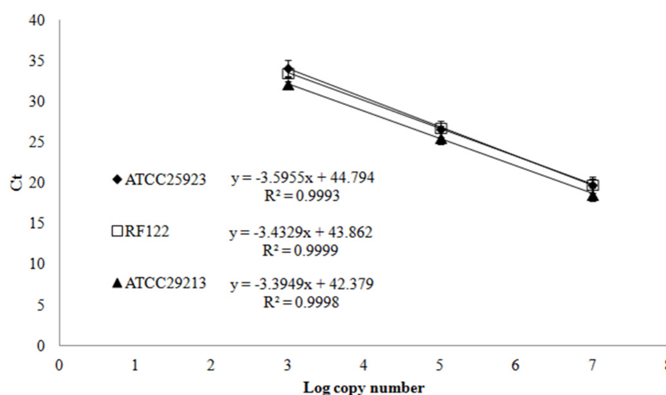

Figure 2. Standard curves of threshold cycle (Ct) values vs. log copy numbers of bacterial chromosome based on qPCR results. Error bars indicate standard deviations.

confirm the validity of samples showing negative qPCR analytical results, an internal positive control (IPC) was developed in duplex qPCR with *sei* primer and probe set. Approximately 10 picogram of IPC oligonucleotide template was spiked into the qPCR reaction, and IPC signal was obtained at Ct of 25-28. Sensitivity of this duplex qPCR detection for *sei*-positive *S. aureus* was as low as 3 pg of DNA or 10^3 CFU. Furthermore, a triplex qPCR was also accomplished by adding species-specific *nuc* primer and probe set to the developed duplex described above (data not shown), indicating that this *sei* primer and probe set could be incorporated to the routine multiplex qPCR detection of the *S. aureus* species and presence of multiple SE genes.

To demonstrate that the *sei* qPCR primer and probe set can be used in food matrices, the designed oligos were applied to analyze non-fat milk spiked with SEI

producing *S. aureus* ATCC25923 in varying concentrations (5×10^7 to 50 CFU/ml). For samples containing approximately 5×10^7 CFU/ml of bacteria, qPCR signals for *sei* gene detection was obtained at Ct ~ 20. As expected, Ct values increased as spiked bacterial densities decreased. Specifically, at 5×10^5 and 5×10^3 CFUs/ml, average Ct values were 25 and 31, respectively. The detection limit of *sei* qPCR primer and probe set was at Ct 34.5, equivalent to samples containing as low as 50 CFU/ml (or 150 fg of DNA) of SEI producing *S. aureus*. In the presence of IPC probe and primer set (duplex qPCR), the *sei* qPCR set also showed positive detection signal at Ct 27-30. We have demonstrated here that the designed *sei* probe and primers could effectively in detect SEI producing *S. aureus* in liquid foods.

4. Conclusions

The *sei* primer and probe set is specific to *S. aureus* producing enterotoxin I and I variant strains. This *sei* detection set can detect presence of the gene at as low as femtogram range. Screening samples such as milk and other liquid or semi-solid foods could be more rapid in order to surveillance food processing for food safety. We proposed that our *sei* detection set can be used in multiplex qPCR assay along with *sea* and *seb* primer and probe sets for screening large food sample sizes for contamination of SE-producing *S. aureus* throughout production stages in food processing.

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