P-BS002

MULTIPLEX QUANTITATIVE PCR FOR DETECTION OF *Staphylococcus aureus* IN FOODS

Orawan Atthasopa[1,*], Nathinee Panvisavas[2], Soraya Chaturongakul [3,#]
[1]Forensic Science Graduate Program, Faculty of Science, Mahidol University, Thailand
[2]Department of Plant Science, Faculty of Science, Mahidol University, Bangkok, Thailand
[3]Department of Microbiology, Faculty of Science, Mahidol University, Bangkok, Thailand
*e-mail: orawan6900@gmail.com, #e-mail: soraya.cha@mahidol.ac.th

Abstract

Based on Thai Department of Disease Control (DDC), *Staphylococcus aureus* has ranked third as a causative agent of food poisoning in Thailand. Staphylococcal food poisoning occurs when food contaminated with staphylococcal enterotoxin is ingested. The toxin accumulates as the bacterial contamination exceed approximately 10^5 colony forming unit per gram of food. The objective of this study is to detect *S. aureus* in food using rapid, sensitive, and specific multiplex quantitative PCR for detection of *S. aureus*. Hydrolysis probe and primer sets were designed to target conserved regions of specific genes which are *nuc* and *mecA* and the designed oligos were used in detection of different *S. aureus* strains. The probe of *nuc* and *mecA* genes were tested by qPCR with labeled Quasar 670 and TAMRA as reporter dye, respectively. Spiked milk was used as a model for detection of *S. aureus* in simple food matrix. The detection limit of *nuc* qPCR primer and probe set was at approximately 5,000 CFU/ml (equivalent to 15 pg of DNA).

Keywords: *Staphylococcus aureus*, multiplex quantitative RT-PCR, *nuc* gene, *mecA* gene

Introduction

Staphylococcus aureus is a Gram-positive bacterium that generally is ubiquitously found as normal flora on skin and in nasal cavity. However, *S. aureus* could cause a wide range of illnesses such as impetigo, scalded skin syndrome, pneumonia, toxic shock syndrome, and food poisoning (Peter *et al.*,1986). *S. aureus* can resist in the high quantity of salt and sugar (Jeffrey, 2007). *S. aureus* can be found in several kinds of foods such as meat, meat products, poultry, egg products, salad, tuna, milk, dairy products, chocolate, and sandwich (Yang *et al.*, 2007). It can grow in the low water activity (a_w of 0.83 to 0.99) (Bergey *et al.*, 2009). Some strains of *S. aureus* can produce the thermal resistant enterotoxins which can cause food poisoning (Elena *et al.*, 2003). The staphylococcal gastroenteritis is self-limiting, with the person recovering in 8–24 hours.Todar, 2011 Symptoms include nausea, vomiting, diarrhea, and major abdominal pain (Ertas *et al.*, 2010). In addition, *S. aureus* could also be multi-drug resistant such as those called methicillin-resistant *S. aureus* (MRSA) which contains altered penicillin binding proteins (Jone *et al.*, 2002). Prevalence of food contaminated with MRSA has shown to be on the increase (Pinto, 2005) indicating that treatment of infected consumers would become more difficult.

According to Thai Department of Disease Control (DDC), *S. aureus* is the third cause of food poisoning in Thailand. The universal food code CodexAlimentarius has suggested food safety criteria for the numbers of *S. aureus* that can be found in foods. In Thailand, Food and Drug Administration (FDA) abides by Codex recommendation and, for example, regulates that *S. aureus* must not be found in 0.1 gram of milk or dairy product while *S. aureus* enterotoxin cannot be found in any types of foods (DDC, 2010). In addition, it has been shown that the toxin accumulates as the bacterial contamination exceed approximately 10^5 colony forming unit (CFU) per gram (or ml) of food (Tereza *et al.*, 2009). Therefore, detection for *S. aureus* presence and quantification of its numbers in foods is a crucial step towards controlling *S. aureus*-borne food poisoning.

In this study, hydrolysis probe and primer sets were designed for use in rapid and quantitative *S. aureus* detection. The targets were species-specific thermonuclease gene (*nuc*) and penicillin binding protein gene (*mecA*). The designed probe and primer sets were tested on different *S. aureus* strains and other species were also used as negative controls. In order to mimic detection of *S. aureus* in foods, milk samples were spiked and the designed probe and primer sets were used in multiplex quantitative detection.

Methodology

Sequence alignment and design of probe and primer sets. Locus identification numbers of DNA sequences of *nuc* and *mecA* genes used in alignment are listed in Table 1. DNA sequences were downloaded from www.cmr.jcvi.org. Conserved regions within each gene was identified using BioEdit Sequence Alignment Editor Version 7.0.4. The conserved sequences were selected to design hydrolysis TaqMan probe and primer sets using Primer Express Program Version 3.0. The sequences are shown in following Table 2.

Table 1. Locus ID numbers of *nuc* and *mecA* genes used in probe and primer design

***S. aureus* strain**	**Locus ID**	
	nuc	***mecA***
MRSA252	SAR1334	SAR0039
Mu3	SAHV_1313	SAHV_0040
Mu50	SAV1324	SAV0041
MW2	MW1211	MW0031
N315	SA1160	SA0038
Newman	NWMN_1236	
RF122	SAB1182	
COL		SACOL0033
USA300-FPR3757	SAUSA300_1222	SAUSA300_0032

Bacterial strains and culture media. Bacterial strains used in this study were *S. aureus* strain ATCC25923, *S. aureus* strain RF122, *S. aureus* strain ATCC29213, *S. aureus* strain ATCC25178, *S. aureus* strain ATCC29740 and *S. aureus* strain COL (as *nuc* positive) and *S. aureus* strain FPR3757 (as *mecA* positive). Other bacteria were used 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) and incubated at 37°C for 24 h. Each overnight culture was prepared from a single colony that was inoculated into 5 ml of tryptic soy broth (TSB) and incubated at 37°*C* with shaking (200 rpm) for 16-18 h.

Isolation of DNA. Genomic DNA was isolated from an overnight culture, according to Wilson *et al.* (1991) with minor modifications. Briefly, 2 ml of an overnight culture was added to a new microfuge tube and centrifuged at 10,000 rpm for 10 min. Supernatant was discarded and the pellet was resuspended in 500 µl of PBS, then centrifuged at 10,000 rpm for 10 min. Supernatant was discarded and pellet was resuspended in 432 µl of TE buffer. An aliquot of 30 µl of 50 mg/ml lysozyme (final amount of 1.5 mg) was added to the resuspended cells, and incubated at 37°C for 1 h. Then, 7.5 µl of 20 mg/ml Proteinase K (final amount of 150 µg) was added and further incubated at 37°C for 1 h. An 30-µl aliquot of 10% (w/v) SDS was added to the lysate, and incubated at 65°C for 30 min prior to 1:1 phenol-chloroform extraction. The top aqueous layer was transferred into a sterile fresh microfuge tube. An aliquot of 50 µl 3M sodium acetate and 500 µl of ice-cold ethanol were added

and incubated at –20°C overnight. Prior to use, each sample was centrifuged at 12,000 rpm for 30 min at 4°C. Supernatant was discarded and pellet was washed in 500 µl of 70% ethanol. The pellet was resuspended in 250 µl of TE buffer. DNA concentration was measured by spectrophotometry using NanoDrop™ spectrophotometer (Thermo Scientific).

PCR assay. In order to test the *nuc* and *mecA* primer set, each oligo was dissolved in TE buffer to a stock concentration of 100 µM. The primer set was tested in a final volume of 25 µl containing the following components: 10 µM of forward/reverse primer, 12.5 µl of Master Mix (i.e., 5X reaction buffer pH 8.5, 10 mM dNTP, and 25 mM $MgCl_2$), RNase-free water, and 20 ng of DNA sample. GeneAmp® PCR System 9700 was set at an initial denaturing step of 5 min at 95°C followed by 35 cycles of 95°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec and then a final extension at 72°C for 7 min. PCR products were separated by 3% agarose gel electrophoresis at 70 V and 120 mA for 60 min with 1500 bp (*Promega)* and 100 bp (*SibEnzyme*) DNA ladder.

TaqMan qPCR assay. In this study, amplifications of *nuc* and *mecA* primer and probe sets by PCR and qPCR were carried out in a final volume of 25 µl containing the following components: 15 µM of *nuc* forward/reverse primer, 15 µM of *mecA* forward/reverse primer, 25 µM *nuc* probe, 25 µM *mecA* probe, FastStart Universal Probe master™ RT-qPCR Master Mix (2X) with ROX dye, RNase-free water, and 2.5 µl DNA sample at varying concentrations (e.g., containing 10^7, 10^5, and 10^3 copies of chromosomal DNA for standard curve). Internal Positive Control (IPC) primers and probe (Hudlow *et al.*, 2008) were also added (i.e., 100 nM of IPC forward/reverse primer and 100 nM of IPC probe) into the reaction in order to ensure amplification in *nuc* and *mecA*-negative sample. Applied Biosystems (ABI) 7500 was set at 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 1 min, 60°C for 1 min. After qPCR was complete, threshold value was automatically set based on ABI 7500 Software at the exponential portion of amplification curves. Ct value of each sample was reported. Samples with Ct values above 35 were considered *nuc* and *mecA*-negative or undetected. Experiments were performed in triplicates.

Evaluation of qPCR specificity and sensitivity. To evaluate sensitivity of *nuc* and *mecA* detection primer and probe set in *S. aureus* strains, ten-fold dilution series of DNA template was used. DNA samples were prepared at 125 ng/µl. In order to convert amount of DNA (in ng) into copy number of chromosome, the formula in Figure 1 was used. For this calculation, the genome of *S. aureus* is estimated to be 3 Mbp, molecular weight of dsDNA is 660 pg/pmol, and Avogadro's number of 6.022×10^{23} molecule/mole was used. Using 10-fold dilution series, approximately 10^7, 10^5, and 10^3 copy numbers or 31.25 ng, 312.5 pg, and 3.125 pg, respectively, were achieved.

$$\frac{(2.5\ \mu l \times 125\ ng/\mu l) \times 6.022 \times 10^{23}\ molecule/mole}{(3 \times 10^6\ bp \times 660\ pg/pmol)} = \sim 10^8\ copies\ of\ gDNA$$

Figure 1 Formula used for copy number calculation (e.g., 10^8 copies of gDNA)

Detection of S. aureus in milk. Ten-fold serial dilutions of overnight cultures were prepared. CFU/ml was determined using plate spreading and bacterial count. Aliquots of 100 µl of diluted *S. aureus* cultures were spiked into 900 µl of non-fat milk to give cell density of approximately 10^7, 10^5, and 10^3 CFUs/ml. DNA was extracted from spiked milk samples using PrepSEQ™ Rapid Spin Sample Preparation Kit (Applied Biosystems, USA). DNA samples were kept at -20°C until use. Experiments were performed in triplicates.

Results

To design TaqMan probe and primers specific for *S. aureus* gene, we aligned and selected the conserved sequences of *nuc* and *mecA* genes from several *S. aureus* strains. The amplicon size are 120 and 113 bp of *nuc* and *mecA* gene, respectively. Using the sequence of this amplicon to perform BLAST search, we found that our designed probe and primer set can detect *nuc* and *mecA* genes from at least 30 different *S. aureus* strains.

Using the designed *nuc* and *mecA* primer sets, the PCR product were at 120 and 113 bp as expected. The *nuc* primer set showed positive results in *S. aureus* strain ATCC25923, *S. aureus* strain ATCC29213, *S. aureus* strain ATCC25178, *S. aureus* strain ATCC29740, and *S. aureus* strain RF122, while the *mecA* primer set can detect *S. aureus* FRP3757. For other bacterial pathogens, primer sets cannot detect *Escherichia coli* ATCC25922, *Streptococcus pyogenes*, *Streptococcus agalactiae*, *Pseudomonas fluorescens*, *Listeria monocytogenes*, *Bacillus subtilis*, and *Salmonella enterica* serotype Typhimurium In a triplex qPCR, *nuc*, *mecA* and IPC primer and probe sets were used. Ct values for *nucA* and *mecA* targerts are as reported in Table 3. IPC probe and primer set detected 10 pg of positive control oligonulceotides at Ct range of 10 to 18.

Log copy number of gDNA	**Average threshold cycle (Ct)**					
	***nuc* gene**					***mecA* gene**
	***S. aureus* ATCC25923**	***S. aureus* ATCC29213**	***S. aureus* ATCC25178**	***S. aureus* ATCC29740**	***S. aureus* RF122**	***S. aureus* FPR3757**
7	18.06	21.15	17.06	23.85	18.56	16.48
5	23.74	27.39	23.16	30.71	25.04	24.14
3	29.66	35	29.26	36	30.84	30.56

Primer and probe sets were applied to analyze non-fat milk samples as they were spiked with *S. aureus* ATCC25178 in varying concentrations ($5x10^7$ to 50 CFU/ml). For samples containing approximately $5x10^7$ CFU/ml of bacteria, qPCR signals for *nuc* gene detection was obtained at Ct ~ 20 (Table 4). As expected, Ct values increased as spiked bacterial densities decreased. Specifically, at $5x10^5$ and $5x10^3$ CFUs/ml, average Ct values were 25.96 and 33.54, respectively. The detection limit of qPCR primer and probe sets was at Ct ~33.54 as it was able to detect samples containing approximately 5,000 CFU/ml (equivalent to 15 pg of DNA).

Table 4. Ct values from spiked milk samples using *nuc* probe and primer detection set

DNA copy number	**10^7**	**10^5**	**10^3**	**Negative**
Ct_1	20.6	26.07	33.93	Undetected*
Ct_2	20.47	25.85	33.14	
Average Ct	**20.54**	**25.96**	**33.54**	

* "Undetected" means Ct value is greater than 35.

Discussion and Conclusion

Thai Department of Disease Control (DDC) has ranked *Staphylococcus aureus* as the third cause of food borne illness in Thailand. Food and Drug Administration (FDA) controls the numbers of them in foods based on the Act of Legislation and Ministerial Regulations. So the *nuc* and *mecA* primer and probe sets were designed for rapid, sensitive, and specific multiplex quantitative RT-PCR for detection in foods. We found that primer and probe sets can detect presence of thermonuclease and methiciliin resistant *staphylococcal* strains. Although the detection limit with our method is still high (i.e., 5,000 CFU/ml), the designed probe and primer set can still be used to detect the bacteria well below the tolerable level of 10^5 per gram or ml of food. Screening samples such as milk and the other foods could be more rapid and sensitivity in order to food processing for food safety. Additional studies should be done in order to (i) lower the detection limit and (ii) assess the specificity of probe and primer set with more *S. aureus* strains.

References

1. Bergey, D. H., Garrity, G. M., Boone, D. R., Castenholz, R. W., Brenner, D. J., Krieg, N. R. (2009). Family VIII *Staphylococcaceae*. In K. H. Schilefer & J. A. Bell (Eds), *Bergey's manual of systematic bacteriology*. 7th ed. New York: Williams & Wilkins. p. 396-400.
2. Department of Disease Control MoPH. Food Administration, Ministrial Regulations and Announcement of Ministry of Public Health. In: Department of Disease Control MoPH, editor. Bangkok, Thailand: Department of Disease Control, Ministry of Public Health; 2010.
3. Elena R, *et al.* Mutiplex PCR for simultaneous detection of *enterococcal* genes *vanA* and *vanB* and staphylococcal genes *mecA*, *ileS-2* and *femB*. Int Microbiol. 2003;6:113-5.
4. Elizaquivel P, Aznar R. A multiplex RTi-PCR reaction for simultaneous detection of *Escherichia coli* O157:H7, *Salmonella* spp. and *Staphylococcus aureus* on fresh, minimally processed vegetables. Food Microbiol. 2008;25:705-13.
5. Ertas N, *et al.* Detection of *Staphylococcus aureus* enterotoxins in sheep cheese and dairy desserts by multiplex PCR technique. Int J Food Microbiol. [Evaluation Studies]. 2010;15;142:74-7.
6. Hudlow W.R., Chong M.D., Swango K.L., Timken M.D. and Buoncristiani M.R., A quadruplex real-time qPCR assay for the simultaneous assessment of total human DNA, human male DNA, DNA degradation and the presence of PCR inhibitors in forensic samples: A diagnostic tool for STR typing, *Forensic Sci. Int. Gen.*, 2008; 2(2): 108-125.
7. Jeffrey CP. Alcamo's Fundamentals of microbiology. 8th ed. USA: Jones and Barlett Publisher; 2007.
8. Jones TF, *et al.* An outbreak of community-acquired foodborne illness caused by methicillin-resistant *staphylococcus aureus*. Emerging Infect Dis. 2002;8(1):82-4.
9. Klotz M, *et al.* Detection of *Staphylococcus aureus* enterotoxins A to D by real-time Fluorescence PCR assay. Clinical Microbiology. 2003;41(10):4683-7.
10. Omoe K, *et al.* Comprehensive analysis of classical and newly described *Staphylococcal* superantigenic toxin genes in *Staphylococcus aureus isolates*. FEMS Microbiol Lett.2005;246(2):191-8.
11. Peter S, *et al.* Bergey's manual of systematic bacteriology. Peter HAS, editor. Baltimore, Maryland: Williams and Wilkins; 1986.
12. Pinto B. Identification and typing of food-borne by PCR-based techniques. Syst Appl Microbiol. 2005;28(4):340-52.
13. Riyaz-Ul-Hassan S, *et al.* Evaluation of three different molecular markers for the detection of *staphylococcus aureus* by polymerase chain reaction. Food Microbiol. 2008;25:452-9.
14. Sabet NS, *et al.* Detection of mecA and ermA genes and simultaneous identification of *Staphylococcus aureus* using triplex real-time PCR from Malaysian *S. aureus* collections. Int J Antimicrob Agents. 2007;29:582-5.

15. Tereza T, *et al.* Rapid and sensitive detection of *Staphylococcus aureus* in food using selective enrichment and real-time PCR targeting a new gene marker. 2009;2:241-50.
16. Todar K. *Staphylococcus aureus* and *Staphylococcal* disease 2011. Available from: http://textbookofbacteriology.net/staph.html.
17. Yang Y, *et al.* Detection of *Staphylococcus aureus* in Dairy Products by Polymerase Chain Reaction Assay. Agricultural Sciences in China. 2007;6(7):857-62.