MULTIPLEX PCR FOR DETECTION OF SUPERANTIGENIC TOXIN GENES IN METHICILLIN-SENSITIVE AND METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* ISOLATED FROM PATIENTS AND CARRIERS OF A HOSPITAL IN NORTHEAST THAILAND

Warawan Wongboot[1], Chariya Chomvarin[1], Chulapan Engchanil[1], and Prajuab Chaimanee[2]

[1]Department of Microbiology, Faculty of Medicine; [2]Clinical Laboratory Section, Srinagarind Hospital, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand

Abstract. The aims of this study were to develop multiplex PCR for simultaneous detection of five superantigenic toxin genes (*sea, seb, sec, sed* and *tst*-1) in *Staphylococcus aureus* isolated from 149 clinical samples and nasal swabs from 201 healthy subjects in Thailand, and to compare prevalence and expression of those genes between methicillin-resistant *S. aureus* (MRSA) and methicillin-sensitive *S. aureus* (MSSA). The sensitivity of multiplex PCR was 10^3 CFU/ml (60 CFU/PCR reaction) for DNA templates extracted by both boiling and extraction methods. *S. aureus* strains from patients (65%) harbored more superantigenic toxin genes than healthy subjects (54%). MRSA (80%) isolated from patients harbored more superantigenic toxin genes than MSSA (52%). *Sea* was the most frequently found gene in *S. aureus* strains from patients and carriers. MRSA isolates harbored *sea* and produced SEA more frequently than MSSA isolates ($p < 0.05$) and MRSA isolates (59%) from blood samples consisted of a higher number of superantigenic toxin producers than MSSA (9%) ($p < 0.05$). More *S. aureus* strains isolated from patients with severe septicemia contained superantigenic toxin genes (94%) and produced toxins (82%) than those from non-severe patients (64% and 57%, respectively). The multiplex PCR method described here offers a reliable tool for simultaneous detection of various staphylococcal toxin genes.

Keywords: multiplex PCR, superantigenic toxin, methicillin-resistant *S. aureus*, methicillin-sensitive *S. aureus*, patients, nasal carriers, Thailand

INTRODUCTION

Staphylococcus aureus is one of potentially virulent pathogens, which cause toxin-mediated diseases, such as food poisoning, toxic shock syndrome and staphylococcal purpura fulminans (Kravitz *et al*, 2005; Ferry and Etienne, 2009). Among the extracellular protein toxins produced by *S. aureus*, staphylococcal heat stable enterotoxin (SE) and toxic shock syndrome toxin-1 (TSST-1) are the most important virulent factors belonging to the superantigen family (Ortega *et al*, 2010;

Correspondence: Chariya Chomvarin, Department of Microbiology, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand.
Tel: +66 (0) 43 363808; Fax: +66 (0) 43 348385
E-mail: chariya@kku.ac.th

Pinchuk *et al*, 2010). Those superantigens can stimulate a high percentage of non-specific T-cells, and consequently they can stimulate the release of pro-inflammatory cytokines, such as interleukin (IL)-1, IL-2, interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) leading to fever, hypotension and shock (Kissner *et al*, 2010).

To date, more than 20 distinct superantigenic toxins are known to be produced by *S. aureus* (Ferry and Etienne, 2009; Vasconcelos and Cunha, 2010). Among these enterotoxins, staphylococcal enterotoxins SEA, SEB and SED are the most common causes of outbreaks of food poisoning (Lawrynowicz-Paciorek *et al*, 2007; Pinchuk *et al*, 2010), but SEB can also cause respiratory symptoms and in severe cases can lead to pulmonary edema and respiratory failure (Center for Food Security and Public Health, 2004).

Methicillin-resistant *S. aureus* (MRSA) has become a major problem in nosocomial infection worldwide (Lee *et al*, 2007). Most MRSA strains carry *mec*A encoding low affinity penicillin-binding protein PBP2a (or PBP2') (Hiramatsu *et al*, 2001). Both MRSA and methicillin-sensitive *S. aureus* (MSSA) can harbor one or more superantigenic toxin genes (Hu *et al*, 2008). MRSA is significantly more associated with mortality than MSSA (Melzer *et al*, 2003). The pathogenic mechanism and virulence factors are assumed to be different between MRSA and MSSA, but these issues are still controversial (Schmitz *et al*, 1997; Rozgonyi *et al*, 2007).

Uniplex PCR has been used for detection of superantigenic toxin genes but is time-consuming (Blaiotta *et al*, 2004). On the other hand, multiplex PCR has been developed for the simultaneous detection of several superantigenic toxin genes, but some primers show improper specificity when examined using BLAST (Schmitz *et al*, 1998; Mehrotra *et al*, 2000). Thus, this study describes the development of a multiplex PCR assay for simultaneous detection of *S. aureus* superantigenic toxin genes *sea, seb, sec, sed* and *tst*-1 using newly designed primers, comparison of the superantigenic toxin genes in MRSA and MSSA isolated from patients and carriers in Thailand, and evaluation of genotyping and phenotyping methods for detection of *S. aureus* superantigenic toxins.

MATERIALS AND METHODS

Bacterial strains

A total of 186 *S. aureus* isolates were collected from 149 patients who admitted to Srinagarind Hospital, Khon Kaen University, Thailand, and 37 isolates were from anterior nares of 201 healthy medical personnels who had not taken any antimicrobial agents for at least 7 days prior to obtaining nasal swabs. Of the *S. aureus* isolates, 71 were MRSA and 115 were MSSA (Table 1). For blood samples, 31 were collected from septicemia patients and 3 samples had incomplete data.

Six superantigenic toxin-producing reference strains of *S. aureus*, ATCC 13565 (SEA), ATCC 14458 (SEB), ATCC 19095 (SEC), ATCC 23235 (SED) and ATCC 33586 (TSST-1), were used as positive controls, and ATCC 25923 (non toxin-producing strain) as a negative control. Among the toxin producing reference strains, *S. aureus* ATCC 13565, 19095 and 23235 were *mec*A-negative strains and were considered as MSSA reference strains.

S. aureus were identified as MRSA strains by *mec*A positivity and resistance to cefoxitin (30 μg) (CLSI, 2007). Bacteria used for specificity testing of *S. aureus* (*nuc*-species-specific gene) included

Table 1
Sources of *S. aureus* isolates.

Source	No. of specimens	No. of *S. aureus* isolates (%)	
		MRSA	MSSA
Patients (*n*=149)			
Blood[a]	34	21 (62)	13 (38)
Sputum	52	24 (46)	28 (54)
Pus from wound swab	52	19 (36)	33 (63)
Urine	11	6 (54)	5 (45)
Nasal carriers[b]			
Nasal swab	37	1 (3)	36 (97)
Total	186	71 (38)	115 (62)

[a] From septicemia patients except for 3 samples without clinical data.
[b] From 201 healthy medical personnels without recent history of taking antibiotics.

Table 2
Primers used for multiplex PCR.

Gene	Primer	Primer sequence	Size (bp)	Reference
nuc	SAnuc-F	GCTTGCTATGATTGTGGTAGCC	423	This study
	SAnuc-R	TCTCTAGCAAGTCCCTTTTCCA		
*mec*A	M1-F	GATGGCTATCGTGTCACAATCG	312	Modified from (Vannuffel *et al*, 1995)
	M2-R	ATCTGGAACTTGTTGAGCAGAG		
sea	SAsea-F	ACCGTTTCCAAAGGTACTGTA	135	This study
	SAsea-R	TGGTACACCAAACAAAACAGC		
seb	SAseb-F	CCTAAACCAGATGAGTTGCAC	592	This study
	SAseb-R	CAGGCATCATGTCATACCAAA		
sec	GSECR-1	AGATGAAGTAGTTGATGTGTATGG	454	Modified from (Mehrotra *et al*, 2000)
	GSECR-2	CTTCACACTTTTAGAATCAACCG		
sed	SAsed-F	GCTTGTACATATGGAGGTGTCA	263	This study
	SAsed-R	GACCCATCAGAAGAATCAAACT		
tst-1	SAtst1-R	GGCAGCATCAGCCTTATAATTT	371	This study
	SAtst1-F	GTGGATCCGTCATTCATTGTT		

Escherichia coli ATCC 25922, *Vibrio mimicus* ATCC 33653, *Vibrio fluvialis* DMST 19347, *Enterobacter* spp, *Klebsiella* spp, *Proteus vulgaris*, *Plesiomonas shigelloides*, *Aeromonas hydrophila* and *Pseudomonas aeruginosa*.

This study has been approved by the Research Ethics Committee of Khon Kaen University, Thailand, according to the Declaration of Helsinki and ICH GCP (HE 521115).

Multiplex PCR assay

Total *S. aureus* genomic DNA was prepared either by boiling method or

Table 3
Bacterial strains used for specificity test of PCR primers by uniplex PCR.

Bacterial strain	No. of strains	Target gene					
		nuc	*sea*	*seb*	*sec*	*sed*	*tst*-1
Staphylococcus aureus ATCC 13565 (SEA)	1	+	+	-	-	-	-
Staphylococcus aureus ATCC 14458 (SEB)	1	+	-	+	-	-	-
Staphylococcus aureus ATCC 19095 (SEC)	1	+	-	-	+	-	-
Staphylococcus aureus ATCC 23235 (SED)	1	+	-	-	-	+	-
Staphylococcus aureus ATCC 33586 (TSST-1)	1	+	-	-	-	-	+
Staphylococcus aureus ATCC 25923	1	+	-	-	-	-	-
Staphylococcus aureus (clinical isolates)	10	+	+	+	-	-	-
Staphylococcus aureus (environmental isolates)	2	+	-	-	-	-	-
Staphylococcus epidermidis	5	-	-	-	-	-	-
Streptococcus pyogenes	1	-	-	-	-	-	-
Micrococcus spp	1	-	-	-	-	-	-
Bacillus subtilis	1	-	-	-	-	-	-
Streptococcus pneumoniae	1	-	-	-	-	-	-
Vibrio parahaemolyticus ATCC 17802	2	-	-	-	-	-	-
Vibrio alginolyticus DMST 14800	1	-	-	-	-	-	-
Vibrio vulnificus ATCC 27562	1	-	-	-	-	-	-
Shigella serogroup A (DMST 15111), B (DMST 4423), C (DMST 28180) and D (ATCC 11060)	4	-	-	-	-	-	-
Escherichia coli (EIEC) DMST 30545	1	-	-	-	-	-	-
Vibrio cholerae	5	-	-	-	-	-	-
Salmonella spp	5	-	-	-	-	-	-
Other gram-negative bacteria	9	-	-	-	-	-	-

+, PCR positive; -, PCR negative

Pure Gene DNA purification kit (Qiagen, Valencia, CA) using 0.5 ml of *S. aureus* overnight culture. For the former procedure, 100 µl aliquot of overnight *S. aureus* culture was sedimented at 13,000*g* for 30 seconds, and the pellet was suspended in 200 µl of sterile distilled water and heated at 100ºC for 15 minutes. Then, the bacterial lysate was centrifuged at 5,000*g* for 1 minute. A 5 µl aliquot of bacterial lysate was directly used as PCR template. DNA samples were stored at -20ºC until used.

The oligonucleotide primers for amplification of *S. aureus*-species specific *nuc* (encoding thermonuclease) (Kateete *et al*, 2010), and four superantigenic toxin genes, *sea, seb, sed* and *tst*-1, were newly designed using Primer 3 program (http://fokker.wi.mit.edu/primer3/input.htm), whereas primers for *sec* and *mec*A were modified from the previously published *S. aureus* DNA sequence (Bohach and Schlievert, 1987; Vannuffel *et al*, 1995; Mehrotra *et al*, 2000). The primer sequences and expected amplicon sizes used in this study are listed in Table 2.

Multiplex PCR consisted of two sets. In one set duplex PCR was designed to amplify *nuc* and *mec*A, which is specific for *S. aureus* and MRSA, respectively; and the other multiplex PCR was designed to detect the 5 superantigenic toxin genes in

Table 4
Distribution of superantigenic toxins of *S. aureus* strains from patients and carriers.

Source	No. of *S. aureus* isolates	No. of toxin positive isolates (%) and types of toxin															
		Gene[a]								Toxin[b]							
		a	*b*	*b+c*	*b+d*	*a+b+c*	*b+c+t*	*b+c+d+t*	Total	A	B	C	B+D	A+ C	C+T	C+D+T	Total
Patient	149	67(45)	17(11)	3(2)	0(0)	7(5)	2(1)	1(1)	97(65)	52(35)	17(11)	3(2)	0(0)	7(5)	2(1)	1(1)	82(55)
Carrier	37	14 (38)	3 (8)	0 (0)	1 (3)	2 (5)	0 (0)	0 (0)	20(54)	11 (30)	2 (5)	0 (0)	1 (3)	2 (5)	0 (0)	0 (0)	16(43)
Total	186	81(43)	20(11)	3(2)	1(0)	9(5)	2(1)	1(0)	117(63)	63(34)	19(10)	3(2)	1(0)	9(5)	2(1)	1(0)	98(53)

[a]*a, b, c, d* and *t* is *sea, seb, sec, sed* and *tst*-1, respectively, detected by multiplex PCR.
[b]A, B, C, D and T is SEA, SEB, SEC, SED and TSST-1, respectively, detected by RPLA assay.

S. aureus isolates. The optimum PCR condition in 25 µl volume consisted of 5 µl of bacterial lysate (50 ng of genomic DNA), 1X PCR buffer [10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.1 mg/ml BSA and 10 mM $(NH_4)_2SO_4$], 2.5 mM $MgCl_2$, 300 µM each dNTP (Amresco, Solon, OH), 0.5 µM each primer (*sea, seb, sec, tst*-1) or 0.1 µM *sed* primer, 2 U of *Taq* DNA polymerase (RBC, Taipei, Taiwan). For *nuc* and *mecA*, duplex PCR were conducted as described above except that 1.5 mM $MgCl_2$, 200 µM each dNTP, 0.2 µM each primer and 1 U *Taq* DNA polymerase were used. Both multiplex PCR and duplex PCR thermocycling were conducted using a thermocycler (Veriti 96 well Thermo Cycler, Applied Biosystem, Foster City, CA) as follows: 94ºC for 7 minutes, 35 cycles of 94ºC for 30 seconds, 58ºC for 30 seconds and 72ºC for 45 seconds; and final step at 72ºC for 7 minutes. Amplicons were electrophoresed in 2% agarose gel, stained with ethidium bromide and visualized under a transilluminator.

Specificity and sensitivity of multiplex PCR

Specificity of the five primer pairs for amplification of *nuc*, *sea*, *seb*, *sed* and *tst*-1 and the two pairs of primers for *mec*A and *sec* were evaluated using BLAST in NCBI database. For sensitivity evaluation, 1.0 ml aliquot of mid-log phase *S. aureus* reference strain cultures was pooled and 1.0 ml aliquot of pooled bacterial suspension was centrifuged at 13,000*g* for 1 minute. Then, the pellet was resuspended in 1.0 ml of sterile distilled water and 10-fold serial dilutions were made in sterile distilled water. Bacterial concentrations ranging from 10^5 CFU/ml to 10^0 CFU/ml were used to determine sensitivity. A 3-5 µl aliquot of bacterial lysate or DNA from each dilution was used as DNA template for multiplex PCR amplification.

Detection of MRSA by disc diffusion method

Antimicrobial susceptibility of *S. aureus* isolates were tested using cefoxitin disc (30 µg) for determining resistance against methicillin according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2007). *S. aureus* ATCC 25923 was used as control strain.

Superantigenic toxin detection by reverse passive latex agglutination assay (RPLA)

SEA, SEB, SEC, SED and TSST-1 superantigenic toxins were detected using commercial standardized RPLA test kit (Oxoid, Wesel, Germany) according to the manufacturer's instructions. Briefly, *S. aureus* was grown in brain heart infusion broth and incubated at 37ºC for 18-24 hours with shaking. The overnight culture was centrifuged at 900*g* for 20 minutes at 4ºC. Then, a 25 µl aliquot of supernatant was mixed with 25 µl of the latex suspension of each *S. aureus* superantigenic toxin control in a V-well microtiter plate. If any toxin is present (positive result), agglutination occurs resulting in the formation of a lattice structure.

Statistical analysis

χ^2-test was performed using SPSS program version 11.5 for Windows for examining correlation among the types of *S. aureus* superantigenic toxin genes isolated from clinical specimens and healthy carriers, types of *S. aureus* (MSSA and MRSA), and type of clinical specimens (blood, pus, sputum, urine, nasal swab). A p-value < 0.05 was considered statistically significant.

RESULTS

Specificity and sensitivity of multiplex PCR

BLAST search showed that the sequences of the amplicons generated by each primer set did not show any homology to other bacterial genes and had the expected size of the target gene, indicating all the primer pairs used in this study were highly specific (Table 3). Sensitivity of multiplex PCR for simultaneous amplification of 5 superantigenic toxin genes in DNA prepared using an extraction kit or boiling method had the same detection limit of 10^3 CFU/ml (60 CFU/PCR reaction) (Fig 1), and for uniplex PCR, the detection limit was 1-10 CFU/ml (1 CFU/PCR reaction).

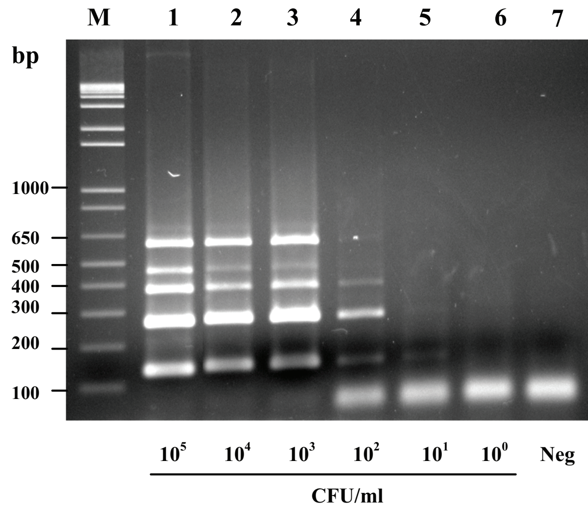

Fig 1-Sensitivity of multiplex PCR for simultaneous detection of five superantigenic toxin genes. Expected amplicon sizes are *sea* 135 bp, *seb* 592 bp, *sec* 454 bp, *sed* 263 bp, *tst*-1 371 bp. Lane M, 1 kb plus DNA ladder; lanes 1-6, PCR amplicons from pooled five reference strains of *S. aureus* ranging from 10^5 to 10^0 CFU/ml; lane 7, negative control. Band at bottom of lanes 4-7 is of primer dimer.

Superantigenic toxins and genes in *S. aureus* isolated from patients and carriers

Of the 149 *S. aureus* isolates from patients and of 37 *S. aureus* isolates from

Table 5
Distribution of superantigenic toxin in MRSA and MSSA isolates from various clinical sources.

Source	No. of isolates	No. of toxin positive isolates (%)					
		Gene[a]		Total	Toxin[b]		Total
		MRSA	MSSA		MRSA	MSSA	
Blood	34	20 (59)	6 (18)	26 (76)	20 (59)[c]	3 (9)	23 (68)
Sputum	52	20 (38)[d]	13 (25)	33 (63)	17 (33)[e]	9 (17)	26 (50)
Pus/wound swab	52	13 (25)	21 (40)	34 (65)	13 (25)	17 (33)	30 (58)
Urine	11	3 (27)	1 (9)	4 (36)	2 (18)	1 (9)	3 (27)

[a]*sea*, *seb*, *sec*, *sed* and *tst*-1, detected by multiplex PCR.
[b]SEA, SEB, SEC, SED and TSST-1, detected by RPLA assay.
[c]More frequent in MRSA than MSSA (χ^2= 9.174; p = 0.005; OR 11.667 ; 95% CI 2.075-65.595).
[d]More frequent in MRSA than MSSA (χ^2= 7.590; p = 0.006; OR 5.769 ; 95% CI 1.564-21.283).
[e]More frequent in MRSA than MSSA (χ^2= 7.738; p = 0.012; OR 5.127 ; 95% CI 1.568-16.765).

carriers, 97 (65%) and 20 (54%), respectively, harbored the superantigenic toxin genes (Table 4). There is no significant difference in the numbers and types of superantigenic toxins in both groups. SEA and *sea* was the most frequent toxin and gene, respectively, detected in *S. aureus* isolates from both patients and carriers.

Similarly, *sea* and SEA were most frequently found in all clinical specimens except urine samples, with the latter having equal prevalence of *sea* and *seb* but higher SEB prevalence than that of SEA (Table 4). Detection of superantigenic toxin gene-positive or toxin producing *S. aureus* strains was highest in blood specimens. However, no significant differences were observed among the sources of specimens. In sputum and blood, the frequency of superantigenic toxin-producing isolates is significantly higher in MRSA than the MSSA strains (Table 5).

Detection of superantigenic toxins and MRSA by genotyping (superantigenic toxins genes and *mec*A) and by phenotyping (RPLA and disc diffusion) was 77% and 100%, respectively. Interestingly, 16 isolates containing a combination of *seb* with other superantigenic toxin genes did not produce SEB, whereas 20 isolates harboring *seb* alone produced SEB (Table 4).

Superantigenic toxin genes present in MRSA and MSSA isolates were 79% (56/71) and 53% (61/115), respectively, with *sea* being more frequent in MRSA than MSSA isolates (p = 0.000; OR = 4.206), which corresponded with production of the toxin (p = 0.000; OR = 6.483) (Table 6). Combination of superantigenic toxin genes was found in both MRSA (3%) and MSSA (13%) isolates.

Relationship between toxin gene/protein expression and disease severity

In order to determine the relationship between severity of septicemia in patients and *S. aureus* haboring superantigenic toxin genes and toxin production, 31 *S. aureus* isolated from blood samples of septicemia patients were examined. The presence of *S. aureus* harboring any superantigenic toxin gene and toxin is significantly higher in patients with severe

Table 6
Presence of superantigenic toxins in MRSA and MSSA isolates.

S. aureus	No. of isolates	No. of toxin positive isolates (%) and types of toxin															
		Gene[a]								Toxin[b]							
		a	*b*	*b+c*	*b+d*	*a+b+c*	*b+c+t*	*b+c+d+t*	Total	A	B	C	B+D	A+ C	C+T	C+D+T	Total
MRSA	71	46 (65)[c]	8(11)	0(0)	0(0)	2(3)	0(0)	0(0)	56(79)	42(59)[d]	8(11)	0(0)	0(0)	2(3)	0(0)	0(0)	52(73)
MSSA	115	35(30)	12(10)	3(3)	1(1)	7(6)	2(2)	1(1)	61(53)	21(18)	11(10)	3(3)	1(1)	7(6)	2(2)	1(1)	46(40)

[a]*a, b, c, d* and *t* is *sea, seb, sec, sed* and *tst*-1, respectively, detected by multiplex PCR.
[b]A, B, C, D and T is SEA, SEB, SEC, SED and TSST-1, respectively, detected by RPLA assay.
[c]Harboring significantly more *sea* in MRSA than MSSA (χ^2= 21.074; p = 0.000; OR 4.206 ; 95% CI 2.243-7.884).
[d]significantly higher in MRSA than MSSA (χ^2= 32.775; p = 0.000; OR 6.483 ; 95% CI 3.320-12.685).

septicemia than in those with non-severe clinical features (Table 7). SEA or *sea* was most frequently found in septicemia patients (data not shown).

DISCUSSION

Regardless of the DNA extraction methods (boiling or extraction kit), multiplex PCR for simultaneous amplification of *S. aureus* 5 superantigenic toxin genes developed in this study is a highly sensitive method with a detection limit of 60 CFU/PCR reaction (6 pg of DNA) and as low as 1-10 CFU/ml (1 fg of DNA) by uniplex PCR. This sensitivity is superior than that of TaqMan assay reported by Klotz *et al* (2003) with a detection limit for enterotoxin A of 10^3 CFU. Multiplex PCR was more time-saving than RPLA assay. Additionally, as template DNA obtained from boiling and extraction method showed the same sensitivity, the boiling method is recommended for use in multiplex PCR because it is rapid and cost-effective.

Although detection rate of superantigenic toxin genes appeared to be higher in MRSA (79%) than in MSSA isolates (53%), it is not statistically significant, in agreement with studies of Hu *et al* (2008) and Schmitz *et al* (1997). Further investigations into the relationship between methicillin resistance and acquisition of superantigenic toxin genes in *S. aureus* may not be warranted.

In this study, *sea* and SEA were most frequently found in *S. aureus* isolates from both patients and carriers, in agreement with previous report that SEA alone or combined with SEB is detected with high frequency in *S. aureus* isolated from food samples in Khon Kaen, Thailand (Chomvarin *et al*, 2006). However, it has been reported that *sec,* combination of *sec* and

Table 7
Severity of septicemia and *S. aureus* isolated from blood samples.

Severity of disease[a]	No. of isolates	No. of *S. aureus* toxin positive isolates (%)	
		Gene[b]	Toxin[c]
Severe	17	16 (94)	14 (82)
Not-severe	14	9 (64)	8 (57)
Total	31	25 (81)	22 (71)

[a]Criteria for the severity of septicemia were as follows: 1) Patient had long-term treatment (more than 1 month), 2) patients had recurrent or persistent infection and/or 3) mortality with or without disseminated intravascular coagulation. The conditions were normalized to base line of all patients who were immuno-compromised hosts or had underlying diseases, such as diabetes, heart disease, renal failure, and cancer. All cases were treated with fusidic acid and vancomycin.
[b]*sea*, *seb, sec, sed* and *tst*-1, detected by multiplex PCR.
[c]SEA, SEB, SEC, SED and TSST-1, detected by RPLA assay.

sea, and *sea* alone are frequently found in *S. aureus* isolated from food samples in Bangkok (Pumtang-on *et al*, 2008). In Germany, *sec* has the highest prevalence in *S. aureus* isolated from patients (Klotz *et al*, 2003), and in Taiwan *tst*-1 followed by *sea*, *seb*, *sec*, and *sed* are found in *S. aureus* strains isolated from patients with staphylococcal food-poisoning (Chiang *et al*, 2008). The variable distribution of *S. aureus* superantigenic toxin genes in different areas may be explained by the fact that the superantigenic toxin genes are mostly carried by mobile genetic elements (Balaban and Rasooly, 2000), which can be exchanged among bacteria of the same or different species (Varshney *et al*, 2009; Malachowa and DeLeo, 2010), accouting for differences in the geographical distribution of *S. aureus* superantigenic toxin genes.

In this study, MRSA isolated from blood samples with superantigenic toxin are significantly higher than the MSSA ($p < 0.05$). But when the severity of septicemia patients were compared between those with and without superantigenic toxin gene containing *S. aureus*, there is no statistically significant difference between the two groups. However, the role of *S. aureus* superantigenic toxins in the severity of septicemia patients should not be discounted as *sea* is significantly associated with severity of sepsis caused by *S. aureus* (Ferry *et al*, 2005). This may be due to the persistent binding of SEA to antigen presenting cells and T cells via MHC II to cause a more prolonged exposure than other toxins, thereby triggering over-expression of inflammatory mediators leading to shock (Pless *et al*, 2005). Thus SEA might be a novel superantigen target for the treatment of *S. aureus*-related sepsis patients.

Genotypic detection rate of *S. aureus* harboring superantigenic toxin genes is higher than that of phenotypic detection of *S. aureus* producing toxins. Absence of toxin production may be due to growth conditions and environmental factors (Klotz *et al*, 2003; Schelin *et al*, 2011), production below assay detection level (Sharma *et al*, 2000), or mutation in regulatory or gene coding region (Sharma *et al*, 2000).

SEA or *sea* was found highest in all types of clinical specimens except urine, in disagreement with a previous study in which TSST-1 toxin is associated with septic shock and toxic shock syndrome (Uchiyama *et al*, 1989) and *sec*-harboring isolates are mostly found in wound infections (Uchiyama *et al*, 1994).

In summary, our study indicates that the multiplex PCR assay is a reliable, rapid, highly specific and sensitive tool for simultaneous detection of the superantigenic toxin genes in *S. aureus* strains isolated from clinical sources and can be used for surveillance of *S. aureus* virulence strains in hospitals. Multiplex PCR assay can be applied directly to blood or biological fluids from patients suspected of having *S. aureus* infection. SEA and *sea* were most frequently found in *S. aureus* isolates in northeastern Thailand. As severity of septicemia seemed, at least in part, to be related to *sea*-positive *S. aureus* strains, superantigenic toxin gene-positive *S. aureus* should also be monitored in hospitals.

ACKNOWLEDGEMENTS

This project was supported by an invitation research grant (2010/I52222) from the Faculty of Medicine, Khon Kaen University, Thailand. The authors thank Prof Dr Yukifumi Nawa for suggestions of the manuscript. We also thank staff of the Clinical Microbiology Laboratory Unit, Srinagarind Hospital, Faculty of Medicine, Khon Kaen University, for collecting the clinical bacterial isolates.

REFERENCES

Balaban N, Rasooly A. Staphylococcal enterotoxins. *Int J Food Microbiol* 2000; 61: 1-10.

Blaiotta G, Ercolini D, Pennacchia C, *et al.* PCR detection of staphylococcal enterotoxin genes in *Staphylococcus* spp. strains isolated from meat and dairy products. Evidence for new variants of *seG* and *seI* in *S. aureus* AB-8802. *J Appl Microbiol* 2004; 97: 719-30.

Bohach GA, Schlievert PM. Nucleotide sequence of the staphylococcal enterotoxin C1 gene and relatedness to other pyrogenic toxins. *Mol Gen Genet* 1987; 209: 15-20.

Center for Food Security and Public Health (CFSPH). Staphylococcal enterotoxin B. Ames, IA 2004: 1-2. [Accessed 2009 July 7]. Available from: URL: http://www.cfsph.iastate. edu/Factsheets/pdfs/staphylococcal_enterotoxin_b.pdf

Chiang YC, Liao WW, Fan CM, *et al.* PCR detection of Staphylococcal enterotoxins (SEs) N, O, P, Q, R, U, and survey of SE types in *Staphylococcus aureus* isolates from food-poisoning cases in Taiwan. *Int J Food Microbiol* 2008; 121: 66-73.

Chomvarin C, Chantarasuk Y, Srigulbutr S, Chareonsudjai S, Chaicumpar K. Enteropathogenic bacteria and enterotoxin-producing *Staphylococcus aureus* isolated from ready-to-eat foods in Khon Kaen, Thailand. *Southeast Asian J Trop Med Public Health* 2006; 37: 983-90.

Clinical and Laboratory Standards Institute (CLSI). Performance standard for antimicrobial susceptibility testing; Seventeenth Informational Supplement M100-S17. Wayne, Pennsylvania: CLSI, 2007.

Ferry T, Etienne J. Toxin-mediated Syndromes. In: Crossley KB, Jefferson KK, Archer GL, Fowler VG, eds. Staphylococci in human disease. 2nd ed. Singapore: Fabulous Printers, 2009: 484-96.

Ferry T, Thomas D, Genestier AL, *et al.* Comparative prevalence of superantigen genes in *Staphylococcus aureus* isolates causing sepsis with and without septic shock. *Clin Infect Dis* 2005; 41: 771-7.

Hiramatsu K, Cui L, Kuroda M, Ito T. The emergence and evolution of methicillin-resistant *Staphylococcus aureus*. *Trends Microbiol* 2001; 9: 486-93.

Hu DL, Omoe K, Inoue F, *et al.* Comparative prevalence of superantigenic toxin genes in methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* isolates. *J Med Microbiol* 2008; 57: 1106-12.

Kateete DP, Kimani CN, Katabazi FA, *et al.* Identification of *Staphylococcus aureus*: DNase and mannitol salt agar improve the efficiency of the tube coagulase test. *Ann Clin Microbiol Antimicrob* 2010; 9: 23.

Kissner T, Cisney E, Ulrich R, Fernandez S, Saikh K. Staphylococcal enterotoxin A induction of pro-inflammatory cytokines and lethality in mice is primarily dependent on MyD88. *Immunology* 2010; 130: 516-26.

Klotz M, Opper S, Heeg K, Zimmermann S. Detection of *Staphylococcus aureus* enterotoxins A to D by real-time fluorescence PCR assay. *J Clin Microbiol* 2003; 41: 4683-7.

Kravitz G, Dries D, Peterson M, Schlievert P. Purpura fulminans due to *Staphylococcus aureus*. *Clin Infect Dis* 2005; 40: 941-7.

Lawrynowicz-Paciorek M, Kochman M, Piekarska K, Grochowska A, Windyga B. The distribution of enterotoxin and enterotoxin-like genes in *Staphylococcus aureus* strains isolated from nasal carriers and food samples. *Int J Food Microbiol* 2007; 117: 319-23.

Lee SS, Kim HS, Kang HJ, Kim JK, Chung DR. Rapid spread of methicillin-resistant *Staphylococcus aureus* in a new hospital in the broad-spectrum antibiotic era. *J Infect* 2007; 55: 358-62.

Malachowa N, DeLeo FR. Mobile genetic elements of *Staphylococcus aureus*. *Cell Mol Life Sci* 2010; 67: 3057-71.

Mehrotra M, Wang G, Johnson WM. Multiplex PCR for detection of genes for *Staphylococcus aureus* enterotoxins, exfoliative toxins, toxic shock syndrome toxin 1, and methicillin resistance. *J Clin Microbiol* 2000; 38: 1032-5.

Melzer M, Eykyn SJ, Gransden WR, Chinn S. Is methicillin-resistant *Staphylococcus aureus* more virulent than methicillin-susceptible *S. aureus*? A comparative cohort study of British patients with nosocomial infection and bacteremia. *Clin Infect Dis* 2003; 37: 1453-60.

Ortega E, Abriouel H, Lucas R, Galvez A. Multiple roles of *Staphylococcus aureus* enterotoxins: pathogenicity, superantigenic activity, and correlation to antibiotic resistance. *Toxins* 2010; 2: 2117-31.

Pinchuk IV, Beswick EJ, Reyes VE. Staphylococcal enterotoxins. *Toxins* 2010; 2: 2177-97.

Pless DD, Ruthel G, Reinke EK, Ulrich RG, Bavari S. Persistence of zinc-binding bacterial superantigens at the surface of antigen-presenting cells contributes to the extreme potency of these superantigens as T-cell activators. *Infect Immun* 2005; 73: 5358-66.

Pumtang-on P, Nuanualsuwan S, Chuanchuen R, *et al.* Identification of enterotoxin encoding genes of *Staphylococcus aureus* isolated from Thai-style fermented pork sausage (Nham-mhoo). Bangkok: The 15th Congress of FAVA, FAVA - OIE Joint Symposium on Emerging Diseases, 2008: 37-40.

Rozgonyi F, Kocsis E, Kristof K, Nagy K. Is MRSA more virulent than MSSA? *Clin Microbiol Infect* 2007; 13: 843-5.

Schelin J, Wallin-Carlquist N, Cohn MT, *et al.* The formation of *Staphylococcus aureus* enterotoxin in food environments and advances in risk assessment. *Virulence* 2011; 2: 580-92.

Schmitz F, MacKenzie CR, Geisel R, *et al.* Enterotoxin and toxic shock syndrome toxin-1 production of methicillin resistant and methicillin sensitive *Staphylococcus aureus* strains. *Eur J Epidemiol* 1997; 13: 699-708.

Schmitz F, Steiert M, Hofmann B, *et al.* Development of a multiplex-PCR for direct detection of the genes for enterotoxin B and C, and toxic shock syndrome toxin-1 in *Staphylococcus aureus* isolates. *J Med Microbiol* 1998; 47: 335-40.

Sharma N, Rees C, Dodd C. Development of a single-reaction multiplex PCR toxin typing

assay for *Staphylococcus aureus* strains. *Appl Environ Microbiol* 2000; 66: 1347-53.

Uchiyama T, Tadakuma T, Imanishi K, *et al.* Activation of murine T cells by toxic shock syndrome toxin-1. The toxin-binding structures expressed on murine accessory cells are MHC class II molecules. *J Immunol* 1989; 143: 3175-82.

Uchiyama T, Yan XJ, Imanishi K, Yagi J. Bacterial superantigens mechanism of T cell activation by the superantigens and their role in the pathogenesis of infectious diseases. *Microbiol Immunol* 1994; 38: 245-56.

Vannuffel P, Gigi J, Ezzedine H, *et al.* Specific detection of methicillin-resistant *Staphylococcus* species by multiplex PCR. *J Clin Microbiol* 1995; 33: 2864-7.

Varshney A, Mediavilla J, Robiou N, *et al.* Diverse enterotoxin gene profiles among clonal complexes of *Staphylococcus aureus* isolates from the Bronx, New York. *Appl Environ Microbiol* 2009; 75: 6839-49.

Vasconcelos NG, Cunha MLRS. Staphylococcal enterotoxins: Molecular aspects and detection methods. *J Public Health Epidemiol* 2010; 2: 29-42.