RESEARCH NOTE

PHENOTYPIC AND GENOTYPIC DETECTION OF ENTEROTOXINS, TOXIC SHOCK SYNDROME TOXIN-1 AND OF METHICILLIN RESISTANCE IN *STAPHYLOCOCCUS AUREUS* ISOLATED FROM RETAIL READY-TO-EAT FOODS IN NORTHEASTERN THAILAND

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Abstract. Toxigenic *Staphylococcus aureus* contamination of ready-to-eat (RTE) foods is a leading cause of foodborne illness in Thailand. From 151 RTE food samples randomly collected from food vendors and food shops in Khon Kaen municipality, Thailand and after culture-based identification of *S. aureus* isolates, pentaplex PCR was used for simultaneous detection of super-antigenic toxin (SE) genes (*sea*, *seb*, *sec*, *sed* and *tst*-1) and presence of their toxins by reversed passive latex agglutination assay. *S. aureus* was identified in 57 isolates, of which 60% and 25% was positive for presence of super-antigenic toxin genes and toxins, respectively; and among the former isolates *sea* was the most common (46%), as well as its product (SEA) (14%) among the latter group. Methicillin resistance *S. aureus mec*A was not found in any of the isolates using both PCR and disk diffusion methods. These results showed that pentaplex PCR is a useful tool for detection of SE-encoding genes in *S. aureus* isolates from RTE food.

Keywords: methicillin resistance *Staphylococcus aureus*, enterotoxins, ready-to-eat food, toxic shock syndrome toxin-1

INTRODUCTION

Staphylococcus aureus is a human and animal pathogen, which can cause a wide range of infections (Al-Talib *et al*, 2009; Indrawattana *et al*, 2013). It is one of the pathogens responsible for foodborne disease outbreaks worldwide (Le Loir *et al*, 2003). *S. aureus* produces such toxins as toxic shock syndrome toxin-1 (TSST-1), staphylococcal enterotoxins (SEs) and exfoliative toxin (Ortega *et al*, 2010; Pinchuk *et al*, 2010). In Thailand, between January and September, 2013, the Bureau of Epidemiology, Ministry of Public Health reported a morbidity rate of diarrhea of 1,322.04 per 100,000 population and food poisoning of 155.66 per 100,000 (Bureau of Epidemiology, 2013).

The major virulence factors causing

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staphylococcal food poisoning are SEs and, traditionally, they have been divided into 5 major types, namely SEA, SEB, SEC, SED and SEE, encoded by *sea*, *seb*, *sec*, *sed* and *see*, respectively (Pinchuk *et al*, 2010) Another enterotoxin, SEF, is biochemically identical to TSST-1 (Fueyo *et al*, 2005; Pinchuk *et al*, 2010). The SEs and TSST-1 are known as bacterial super-antigens, which can cause a massive release of pro-inflammatory cytokines, such as interleukin (IL)-1, IL-6, interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α), leading to fever, hypotension and shock (Kissner *et al*, 2010). Although new SEs, including SEG - SER and SEU, have been reported (Vasconcelos and Cunha, 2010), SEA - SEE are the major enterotoxins associated with foodborne outbreaks (Le Loir *et al*, 2003; Argudin *et al*, 2010), and TSST-1 (which can be considered among the SEs) has been isolated from nasal swabs from healthy carriers (Fueyo *et al*, 2005; Wongboot *et al*, 2013), and from food handlers (Sospedra *et al*, 2012). SEs are produced during active growth of *S. aureus* in food and are heat stable, persisting in cooked food (Argudin *et al*, 2010). The onset of symptoms (diarrhea and/or food poisoning) is approximately 1-6 hours after ingestion of *S. aureus*-contaminated food, depending on the amounts of toxin consumed and sensitivity of the individuals to the toxins (Argudin *et al*, 2010; Pinchuk *et al*, 2010; Patel and Myers, 2013).

Various ready-to-eat (RTE) foods have become increasingly popular worldwide including Thailand. RTE foods can be easily contaminated with various microorganisms (including *S. aureus*) resulting in food poisoning (Oh *et al*, 2007; Xing *et al*, 2014). The popular Thai traditional green papaya salad ("somtum"), and fermented pork mixed with rice ("nam krug") are local RTE foods. Seafood and fresh fruit juice are also popular around the world. Bacterial contamination of these RTE foods may come from the raw materials used or may occur during food handing, depending on the types of food (Jay, 2000).

Although the production of SEA - SED and TSST-1 has been studied in Thai RTE foods using conventional phenotypic methods [*viz*, reversed passive latex agglutination (RPLA)] the frequency of the genes encoding these SEs has not been investigated. Additionally, the presence of these genes and their products in *S. aureus* isolated from diarrheal patients in Thailand has not been demonstrated.

The use of antimicrobial agents to control diseases has contributed extensively to the emergence of drug-resistant bacterial strains, such as methicillin-resistant *S. aureus* (MRSA), in hospitals, the community and food (Weese *et al*, 2010). In a previous study, a multiplex PCR assay was employed for detection of these *S. aureus* five super-antigenic toxin genes in clinical specimens (Wongboot *et al*, 2013). In the current study, we investigated the prevalence of five superantigenic toxins (SEA-SED and TSST-1) and their corresponding genes (*sea*, *seb*, *sec*, *sed*, *tst*-1) in *S. aureus* isolates from RTE foods in Khon Kaen municipality, Thailand and to detect MRSA among these isolates.

MATERIALS AND METHODS

Bacterial strains

Four enterotoxin- and TSST-1-producing *S. aureus* reference strains [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 the negative control.

Food sample collection and processing

A total of 151 food samples, randomly

collected from food vendors and food shops in Khon Kaen municipality, Thailand were grouped into three categories: (cat. 1) 50 samples of local foods (27 of green papaya salad, "somtum") and 23 of fermented pork mixed with rice ("nam krug"); (cat. 2) 50 samples of seafood; and (cat. 3) 51 samples of fruit juices and beverages. The food samples were collected aseptically and placed in sterile containers at 4°C prior to transfer to the laboratory.

A 25 g portion from each sample (solid samples were first cut into small pieces) was added to 225 ml of sterile Trypticase soy broth (TSB; Oxoid, Hamshire, UK) and the mixture (food sample in TSB) incubated at 37°C for 18-24 hours, and then streaked on Baird-Parker plate containing egg yolk tellurite emulsion (Biomark, Pune, India) and incubated at 37°C for a further 48 hours (Bennett and Lancette, 2001). Colonies typical of *S. aureus* (gray to jet-black surrounding opaque zone) were identified using biochemical tests, including catalase activity, coagulase production, PR-glucose and PR-mannitol fermentation tests (Murray *et al*, 2007). *S. aureus* colonies were confirmed by PCR assay (see below).

Detection of five super-antigenic toxin genes and *mec*A by multiplex PCR

S. aureus genomic DNA was prepared using a boiling method (Perez-Roth *et al*, 2001). In brief, a 100 µl aliquot of overnight culture was centrifuged at 13,000*g* for 30 seconds and pellet suspended in 200 µl of sterile distilled water, heated at 100°C for 15 minutes and centrifuged at 5,000*g* for 1 minute. A 5 µl aliquot of the bacterial lysate was used directly as PCR template. Multiplex PCR consisted of two sets of reactions, a pentaplex PCR designed for detection of the five super-antigenic toxin genes (*sea* - *sed* and *tst*-1) and a duplex PCR for detection of *S. aureus nuc* and MRSA *mec*A. Primer sequences, PCR mixtures, thermocycling conditions and expected amplicon sizes are listed in Table 1.

Detection of *S. aureus* enterotoxins and TSST-1

Each *S. aureus* coagulase-positive colony was cultured in 1 ml of brain heart infusion broth (Difco, Detroit, MI) at 37°C for 24 hours, followed by centrifugation at 900*g* for 20 minutes at 4°C and the supernatant then was examined for the presence of enterotoxins SEA, SEB, SEC, SED and TSST-1 using an RPLA kit (SET-RPLA, Oxoid).

Detection of antibiotic resistance

All *S. aureus* were tested for antibiotic resistance against cefoxitin (30 µg) using a disk diffusion assay (CLSI, 2007).

RESULTS

S. aureus was identified in cultures from 57/151 (38%) of the RTE food samples, with 34 (60%) *S. aureus* isolates harboring super-antigenic toxin genes (*sea* - *sed* and *tst*-1) and 14 (25%) produced these toxins (Table 2). Toxin SEA was the most frequently detected, following by SEB, SEC and other combinations of toxins, such as SEB + SED. All isolates harboring *seb* and *sed* expressed their respective toxins, whereas 28% and 33% carrying *sea* and *sec*, respectively expressed SEA and SEC. One isolate contained *tst*-1 but did not express the toxin. No isolates were positive for *mec*A or resistant to cefoxitin (data not shown).

DISCUSSION

SEA, SEB and SED have been identified as the toxins most frequently associated with food poisoning (Lawrynowicz-

Table 1
Primers and PCR conditions used for detection of staphylococcal super-antigenic toxins and methicillin resistance genes.

Gene	Primer	Primer sequence	GenBank accession no.	Size (bp)	Concentration of PCR component					PCR condition	Reference
					PCR buffer	$MgCl_2$ (mM)	dNTPs (mM)	Primers (μM)	Taq (U)		
nuc	SAnuc-F SAnuc-R	GCTTGCTATGATTGTGGTAGCC TCTCTAGCAAGTCCCTTTTCCA	NC_009641	423	1X	1.5	0.2	0.2	1	Duplex PCR 94ºC ; 7 min 35 cycles of	(Wongboot *et al*, 2013)
mecA	M1-F M2-R	GATGGCTATCGTGTCACAATCG ATCTGGAACTTGTTGAGCAGAG	-	312						94ºC ; 30 sec 58ºC ; 30 sec 72ºC ; 45 sec 72ºC ; 7 min	Modified[a]
sea	SAsea-F SAsea-R	ACCGTTTCCAAAGGTACTGTA TGGTACACCAAACAAAACAGC	NC_009641	135	1X	2.5	0.3	0.5	2	Pentaplex PCR 94ºC ; 7 min 35 cycles of	(Wongboot *et al*, 2013)
seb	SAseb-F SAseb-R	CCTAAACCAGATGAGTTGCAC CAGGCATCATGTCATACCAAA	NC_002951	592				0.5		94ºC ; 30 sec 58ºC ; 30 sec 72ºC ; 45 sec	(Wongboot *et al*, 2013)
sec	GSECR-1 GSECR-2	AGATGAAGTAGTTGATGTGTATGG CTTCACACTTTTAGAATCAACCG	NC_009782	454				0.5		72ºC ; 7 min	Modified[b]
sed	SAsed-F SAsed-R	GCTTGTACATATGGAGGTGTCA GACCCATCAGAAGAATCAAACT	M28521	263				0.1			(Wongboot *et al*, 2013)
tst-1	SAtst1-R SAtst1-F	GGCAGCATCAGCCTTATAATTT GTGGATCCGTCATTCATTGTT	NC_009782	371				0.5			(Wongboot *et al*, 2013)

[a,b]Primers for *mecA* and *sec* was modified from Vannuffel *et al* (1995) and Mehrotra *et al* (2000), respectively.

Table 2
Occurrence of staphylococcal super-antigenic toxins and genes in *S. aureus* isolates from RTE food.

Source	Number of samples	Number of *S. aureus* isolates (%)	Number of toxin-positive isolates (%) and type of toxin											
			Gene						Protein					
			sea	*seb*	*sec*	*seb*+*sed*	*sec*+ *tst*-1	Total	SEA	SEB	SEC	SEB+SED	SEC+TSST-1	Total
Local food	50	25 (50)	10	1	1	2	1	15	4	1	0	2	0	7
Seafood	50	17 (34)	8	1	1	0	0	10	1	1	1	0	0	3
Fruit juice and beverage	51	15 (29)	8	1	0	0	0	9	3	1	0	0	0	4
Total	151	57 (38)	26 (46)	3 (5)	2 (3)	2 (3)	1 (2)	34 (60)	8 (14)	3 (5)	1 (2)	2 (3)	0	14 (25)

Paciorek *et al*, 2007; Pinchuk *et al*, 2010). In Spain, *S. aureus* isolates from nasal carriers and handled foods contained SEs (21.2%), TSST-1 (3.7%) and combinations of these toxins (3.7%) (Fueyo *et al*, 2005). In other studies, enterotoxigenic staphylococci isolated from different kinds of food samples (ice cream, raw chicken meat, cake and boiled rice) produced different types of enterotoxins (Gücükoğlu *et al*, 2013; Nagaraj *et al*, 2013). Oh *et al* (2007) reported that 8.6% of RTE food samples in Korea are contaminated with *S. aureus* and 48% of the isolates produce one or more toxins, of which SEA is the most frequently found (90%).

A previous report showed that enterotoxin type A, alone or in combination with other enterotoxins, is the most common (29%) of *S.aureus* in food samples from Khon Kaen, northeastern Thailand tested using only the phenotypic assay method (Chomvarin *et al*, 1993). In the current study, although being carried out in Khon Kaen more than twenty years later, *S. aureus* enterotoxins were still found using the phenotypic assay in nearly the same proportion of food samples as in the previous report, whereas more than half of the isolates harbored genes encoding these toxins. The local foods, "nam krug" and green papaya salad, gently heated and uncooked foods may promote *S. aureus* contamination from unhygienic hand contact and/or raw materials (Jay, 2000). A previous study conducted in Bangkok reported that *sec* or *sea* alone, or a combination of them, are frequently found in *S. aureus* isolated from food samples (Pumtang-on *et al*, 2008).

The low correlation between the presence of genes and protein expression of SEA and SEC may be due to a requirement for prophage induction for SEA (Borst and Betley, 1994; Cao *et al*, 2012) and the

lack of an accessory gene regulator (*agr*) for SEC expression (Tremaine *et al*, 1993). Our results are in agreement with those of Gücükoğlu *et al* (2013).

MRSA is a major nosocomial pathogen found in many hospitals the world over (Lee *et al*, 2007). In 2011, it was reported that community acquired MRSA infections were increasing in Asia, representing 25.5% of community-associated *S. aureus* infections and 67.4% of healthcare-associated infections (Song *et al*, 2011). In Thailand in 1998, MRSA accounted for 44.3% and 39%, of cases of *S. aureus* in a hospital in Bangkok and a hospital in Khon Kaen, respectively (Chomvarin *et al*, 1998), and MRSA accounted for 15.1% of *S. aureus* isolates from nasal swabs taken from medical staff and students (Chomvarin *et al*, 1998). In the current study, MRSA was not detected in RTE food samples indicating that the MRSA has not spread into the environment (food) in this area.

In summary, our findings indicate that RTE foods carried a risk for staphylococcal food poisoning and that SEA was the major SE in Khon Kaen, Thailand. Appropriate hygienic measures should be taken by local and national public health organizations to reduce the risk posed by *S. aureus* or other foodborne pathogens in RTE foods.

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