Characterisation of classical enterotoxins, virulence activity, and antibiotic susceptibility of *Staphylococcus aureus* isolated from Thai fermented pork sausages, clinical samples, and healthy carriers in northeastern Thailand

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Abstract

Introduction: Contamination by *Staphylococcus aureus* of food produced from animal sources may have diverse and multifactorial causes that depend on geographical distribution. The goal of this study was to isolate and characterise *S. aureus* strains from contaminated fermented pork sausage, which is a local food of northeastern Thailand. **Material and Methods:** *S. aureus* strains were isolated from local pork sausage, and the presence of classical enterotoxins was determined by PCR and reversed passive latex agglutination. These results were compared with strains derived from hospitalised patients and healthy carriers. Additionally, production of extracellular enzymes and haemolysin, biofilm formation, and antibiotic susceptibility were assessed. **Results:** *S. aureus* was identified in 36 sausage isolates (60%). The strains positive for staphylococcal enterotoxin A were more frequently found in isolates from sausage and healthy carriers than in those from patients. All tested *S. aureus* strains were positive for DNase, lipase, proteinase, haemolysin, and biofilm formation; notably, strains isolated from food and healthy carriers displayed similar values. Most isolates were resistant to penicillin and ampicillin, while none were to methicillin. **Conclusions:** Thai fermented pork sausages are associated with a high risk of staphylococcal food poisoning, which may be linked to contamination caused by carriers. Dissemination of knowledge regarding best practices in sanitation and hygiene is important in local communities.

Keywords: food, pork, *Staphylococcus aureus* enterotoxins, Thailand.

Introduction

Staphylococcus aureus is a facultative anaerobic Gram-positive bacterium and a well-known causative agent of community or hospital-acquired infections and food poisoning. This bacterium is found widely in humans and animals (mainly colonising the nares and skin), the environment, and food produced from raw materials of animal origin, such as pork, beef, or chicken (18). Its prevalence contributes to worldwide foodborne diseases resulting from food contamination *via* poor slaughtering sanitation or inappropriate food processing, including food handling, mincing, preparation, wrapping, and storage (11). Fermented pork sausage, naem moo, is a local food of northeastern Thailand. It has a sour flavour that the local population prefers for raw consumption. Raw minced pork is commonly used as the main ingredient and is mixed with several local spices. It has been suggested that this local food could be contaminated with several pathogens, including *S. aureus*, as a result of unclean ingredients or inappropriate processing. Characterisation

of *S. aureus* contaminants of this local food may lead to the development of strategies for surveillance and prevention of food poisoning.

Some enterotoxigenic *S. aureus* strains associated with food poisoning have been shown to produce staphylococcal enterotoxins (SEs), members of the large family of superantigenic toxins (2). SEs are highly heat stable, are not degraded by cooking, and also persist through harsh environmental conditions such as freezing and drying (16). More than 20 *S. aureus* enterotoxins have been investigated, of which the classical SEs are considered to be these five: SEA, SEB, SEC, SED, and SEE (14, 16). They are the most common causes of staphylococcal food poisoning, and among them, SEA is particularly frequent (24). SEs have been documented to induce several clinical manifestations following a short incubation period, including fever, nausea, vomiting, and diarrhoea. Some *S. aureus* strains produce an enterotoxin-like protein such as toxic shock syndrome toxin-1 (TSST-1, SEF), a manifestation featuring severe, acute symptoms and causing the dysfunction of multi-organ systems (7). In addition to enterotoxins, *S. aureus* strains produce haemolysin (pore-forming toxin) and several extracellular enzymes (including coagulase, protease, lipase, and DNase), and can form biofilms; these features are thought to facilitate survival and growth under a variety of stressful conditions and have associated consequences, such as immune evasion, bacterial dissemination, food spoilage, and tolerance to cold temperatures or high concentrations of salt and sugar during food production (8, 15).

To date, no studies have been performed to characterise *S. aureus* contaminants of Thai fermented pork sausage, which is popularly consumed raw in northeastern Thailand. This study aimed to isolate *S. aureus* from Thai fermented pork sausage and subsequently determine the presence of classical SE genes, comparing their prevalence against clinical and healthy carrier strains. Production of haemolysin and extracellular enzymes, biofilm formation, and antibiotic susceptibility of isolates obtained from three different sources were also investigated. Such determination of the genetic and phenotypic characteristics of *S. aureus* isolates is important for epidemiological surveillance and prevention of food contamination, especially in local foods produced from raw materials of animal origin. In addition, knowledge concerning antibiotic resistance in *S. aureus* strains from food samples may be beneficial for the control of antibiotic usage in local communities or on livestock farms.

Material and Methods

***S. aureus* isolation from food samples.** A total of 60 Thai fermented pork sausage samples were collected from local retail markets in the Amnatcharoen municipal area, which is located in northeastern Thailand. For *S. aureus* isolation, 10 g of Thai fermented pork sausage was transferred to 90 mL of tryptic soy broth (TSB) (Himedia, Mumbai, India) containing 10% NaCl and blended for 10 min by laboratory flapping homogenizer (HG400VW, Wiggens, Straubenhardt, Germany). Ten millilitres of suspension were inoculated into 90 mL of TSB (containing 10% NaCl), and the solution was incubated at 37°C for 24 h. Then, one loopful of enrichment broth was streaked on Baird-Parker agar (BPA) (Himedia) containing egg yolk tellurite emulsion (FD046, Himedia); this was incubated at 37°C for 48 h. Typical black shiny colonies with an opaque zone were selected and subsequently subjected to Gram staining and biochemical determination with coagulase, phenol red (PR) glucose, and PR mannitol tests.

The anterior nares of 30 healthy volunteers were swabbed for *S. aureus* isolation. The swabs were streaked on BPA (supplemented with egg yolk tellurite emulsion) and incubated at 37°C for 48 h. Typical colonies were identified by Gram staining and biochemical testing.

Clinical strains of *S. aureus* isolated from 54 hospitalised patients were provided by the Clinical Microbiology Laboratory at Amnatcharoen Hospital, Amnatcharoen, Thailand. All *S. aureus* isolates were previously confirmed by biochemical testing.

Detection of classical SE genes (*sea–see*) and toxic shock syndrome toxin-1 (*tsst-1*). Genomic DNA of *S. aureus* was extracted using a Genomic DNA Extraction Kit (Bio-Helix, Keelung, Taiwan) according to the manufacturer's instructions. DNA pellets were dissolved in TE buffer and stored at −20°C until use. The presence of a gene encoding thermostable nuclease (*nuc*) was confirmed in all isolates by a PCR assay. Classical enterotoxin-associated genes (*sea*, *seb*, *sec*, *sed*, and *see*) and *tsst-1* (*sef*) were detected by a PCR assay using primers with sequences shown in Table 1 and under conditions as given in previously published reports (10, 12, 20, 28). PCR solutions consisted of One PCR Plus reaction mixture (GeneDireX, Keelung, Taiwan) and 0.5 µM of each primer. Amplification was performed in a T100 Thermal Cycler (Bio Rad Laboratories, Hercules, CA, USA). The resulting PCR products were evaluated by 1.5% agarose gel electrophoresis and visualised with a UV illuminator. The reference *S. aureus* strains DMST 19376 (*sea*), DMST 19377 (*seb*), DMST 19378 (*sec*), and DMST 19379 (*sed*) were provided by the Department of Medical Sciences Thailand Culture Collection and were used as positive controls (*sea−sed*). In addition, the in-house *S. aureus* clinical strains SP1709 and P950 were used as positive controls for *see* and *tsst-1*, respectively.

The toxin production of all *S. aureus* isolates positive for classical enterotoxin genes was determined using reversed passive latex agglutination (RPLA) against staphylococcal enterotoxins A, B, C, and D, according to the manufacturer's instructions (Oxoid, Wesel, Germany).

Table 1. Primer sequences used for detection of classical SE genes (*sea–see*) and the gene encoding toxic shock syndrome *tsst-1*

Gene	Primer sequences	Product size (bp)	Reference
nuc	GCGATTGATGGTGATACGGTT	279	(12)
	AGCCAAGCCTTGACGAACTAAAGC		
sea	ACCGTTTCCAAAGGTACTGTA	135	(28)
	TGGTACACCAAACAAAACAGC		
seb	CCTAAACCAGATGAGTTGCAC	592	(28)
	CAGGCATCATGTCATACCAAA		
sec	AGATGAAGTAGTTGATGTGTATGG	454	(20)
	CTTCACACTTTTAGAATCAACCG		
sed	GCTTGTACATATGGAGGTGTCA	263	(28)
	GACCCATCAGAAGAATCAAACT		
see	CAGTACCTATAGATAAAGTTAAAACAAGC	178	(10)
	TAACTTACCGTGGACCCTTC		
tsst-1	GGCAGCATCAGCCTTATAATTT	371	(28)
	GTGGATCCGTCATTCATTGTT		

Determination of extracellular enzyme and haemolysin production in *S. aureus* isolates. *S. aureus* isolates were cultured in TSB at 37°C for 24 h. Subsequently, the bacterial suspensions were diluted to 0.5 McFarland (~1 × 10^8 cells/mL) and used to determine the production of extracellular enzymes and haemolysin by dropping 10 µL of bacterial suspension onto skim milk agar (for protease production), tributyrin agar (for lipase production), DNase agar (for DNase production), or blood agar base containing 5% human blood (for haemolysin production) (all from Himedia). All culture plates were incubated at 37°C for 48 h. The production of extracellular enzymes and haemolysin was evaluated by measuring clear zone diameters in mm in triplicate independent experiments. To determine the production of DNase, 15 mL of 1 N HCl was poured onto agar plates, and excess HCl was aspirated; clear zones around colonies indicated DNase activity.

Determination of biofilm formation by *S. aureus* isolates. *S. aureus* isolates were inoculated into Luria–Bertani broth (LB) (Himedia) and incubated at 37°C for 24 h in a shaking incubator (180 rpm). Each inoculum was adjusted to a bacterial turbidity of 0.5 McFarland (~1 × 10^8 CFU/mL). To evaluate biofilm formation, 200 µL was added into each well of a polystyrene 96-well microtiter plate and incubated at 37°C for 48 h, as described in a previous protocol, with slight modification (30). After the incubation, non-attached bacteria were removed from the wells, and the plates were washed three times with PBS. Afterwards, 200 µL of 0.1% crystal violet solution was added into each well, and the plate was incubated at room temperature for 30 min. Next, the suspension of crystal violet was removed, and all wells were washed three times with PBS for 5 min with shaking. The stained samples were air dried for 1 h and then solubilised by incubation with 200 µL of absolute ethanol for 10 min. The absorbance of solubilised crystal violet was measured at 570 nm using a microplate reader (SPECTROstar NANO, BMG Labtech, Ortenberg, Germany). Sterile LB was used as a blank negative control. Biofilm production was quantified based on triplicate independent experiments. The criteria used for categorising isolates in terms of biofilm production were those in the previous report of Singh *et al*. (25).

OD_{cutoff} = average OD of negative control + 3 × standard deviation (SD) of ODs of negative control;

< OD_{cutoff} = biofilm non-former;

≥ OD_{cutoff} and < 2×OD_{cutoff} = weak biofilm former;

≥ 2×OD_{cutoff} and < 4×OD_{cutoff} = moderate biofilm former;

≥ 4×OD_{cutoff} = strong biofilm former.

Antibiotic susceptibility testing. All strains of *S. aureus* (0.5 McFarland) were tested for antibiotic susceptibility using the Kirby–Bauer protocol, which was conducted on Mueller–Hinton agar (MHA) (Himedia). A panel of 13 antibiotics was used to test drug susceptibility based on the Clinical and Laboratory Standard Institute guidelines (4). This panel (Oxoid, Basingstoke, UK) included: (1) β-lactams, consisting of penicillin (10 U), ampicillin (10 µg), amoxicillin/clavulanic acid (30 µg), ceftriaxone (30 µg), cephazolin (30 µg), ceftazidime (30 µg), and cefoxitin (30 µg); (2) lincosamides, consisting of clindamycin (10 µg); (3) macrolides, consisting of erythromycin (30 µg); (4) aminoglycosides, consisting of gentamicin (10 µg); (5) phenicols, consisting of chloramphenicol (30 µg); (6) glycopeptides, consisting of vancomycin (30 µg); and (7) sulphonamides, consisting of trimethoprim/sulfamethoxazole (25 µg). Notably, cefoxitin has been suggested for the detection of methicillin resistance in *S. aureus* (4).

Data analysis. The chi-squared test was used to analyse differences in the prevalence of SEs among the three experimental groups. Differences in clear zones and biofilm formation among the various groups were determined by Student's *t*-test. P values less than 0.05 were considered to be statistically significant.

Results

Isolation of *S. aureus* from Thai fermented pork sausages. *S. aureus* strains were detected in 36 (60%) samples out of the 60 samples tested. Among the

30 healthy volunteers tested, this study found *S. aureus* in 10 (33.33%). PR glucose, PR mannitol, and coagulase tests were positive for all *S. aureus* isolates. Additionally, positive results with *S. aureus*-specific primers (*nuc*) were obtained (Fig. 1).
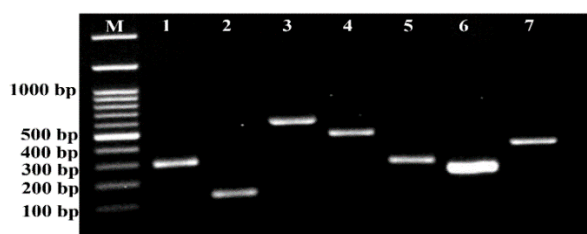
Fig. 1. PCR amplification of a *S. aureus* specific gene (*nuc*), classical SE genes (*sea–see*), and the *tsst-1* gene. Lane M – 100 bp DNA marker; Lanes 1–7 – *nuc*, *sea*, *seb*, *sec*, *sed*, *see*, and *tsst-1*, respectively

Prevalence of classical SE (*sea–see*) and toxic shock syndrome toxin-1 (*tsst-1*) genes. PCR assays (Fig. 1) were used to determine the presence of classical SE (*sea*, *seb*, *sec*, *sed*, and *see*) and *tsst-1* genes in the 36 *S. aureus* isolates derived from Thai fermented pork sausages. This was compared to their prevalence in strains isolated from clinical specimens (n = 54) and healthy carriers (n = 10), as summarised in Table 2. The SE genes were detected in 47% (*sea*), 11% (*seb*), 0% (*sec*), 0% (*sed*), and 3% (*see*) of Thai fermented pork sausage samples. Additionally, two or three SE genes co-occurred in several samples: *sea* and *seb* (5%), *seb* and *sec* (17%), and *sea*, *seb* and *sec* (11%). However, *tsst-1* was not detected either alone or in combination with other genes. Among hospitalised patients, SE genes were respectively detected in 13% (*sea*), 33% (*seb*), 2% (*sec*), 0% (*sed*), and 2% (*see*) of *S. aureus* isolates. These strains also exhibited genes in combination, including *sea* and *seb* (2%), *seb* and *sec* (17%), *sea*, *seb* and *sec* (4%), and *seb*, *sec* and *tsst-1* (7%). Ten isolates from healthy carriers were positive for *sea* (40%), *seb* (10%), *sea* and *seb* (10%), and *sea*, *seb* and *sec* (10%).

Production of classical enterotoxins (SEA–SED) by PCR-positive isolates was confirmed by RPLA testing. SEA-positive strains were highly frequent in isolates from Thai fermented pork sausages (61%) and healthy carriers (60%), and had low frequency in isolates from hospitalised patients (7%) (P < 0.05).

Production of extracellular enzymes and haemolysin by *S. aureus* isolates. All *S. aureus* isolates from the three different groups were positive for DNase, lipase, protease, and haemolysin production. For strains from Thai fermented pork sausages, the average clear zones for DNase, lipase, protease, and haemolysin were 20.6, 18.2, 13, and 12.5 mm, respectively, and were similar to the average clear zones for strains from healthy carriers which were 21.6 mm, 18 mm, 13.9 mm, and 12.9 mm (Fig. 2). Additionally, significantly higher production was observed for strains from hospitalised patients (DNase 29.8 mm, lipase 20.4 mm, protease 15.3 mm, haemolysin 15.1 mm) relative to other tested strains (P < 0.05).

Biofilm formation by *S. aureus* isolates. We also determined the biofilm formation capability of isolated *S. aureus* strains and categorised them accordingly as biofilm non-formers, weak biofilm formers, moderate biofilm formers, and strong biofilm formers (Table 3). Most isolates were determined to be strong biofilm formers, but two strains detected in Thai fermented pork sausages (6%) were classified as moderate biofilm formers.

Although most strains were strong biofilm formers, we found that the production of biofilm in *S. aureus* isolated from hospitalised patients (mean OD 2.73 ± 0.37) was significantly higher than that of strains isolated from Thai fermented pork sausages (mean OD 1.62 ± 0.36) or healthy carriers (mean OD 1.72 ± 0.32), as shown in Fig. 3.

Table 2. Detection of classical SE and *tsst-1* genes by PCR and of classical SE production by RPLA

S. aureus enterotoxin genotype	Thai fermented pork sausages (n = 36)		Hospitalised patients (n = 54)		Healthy carriers (n = 10)	
	PCR	RPLA	PCR	RPLA	PCR	RPLA
sea	17 (47%)	22 (61%)[a]	7 (13%)	4 (7%)	4 (40%)	6 (60%)[b]
seb	4 (11%)	15 (42%)	18 (33%)	15 (28%)	1 (10%)	3 (30%)
sec	0 (0%)	10 (28%)	1 (2%)	6 (11%)	0 (0%)	1 (10%)
sed	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
see	1 (3%)	ND	1 (2%)	ND	0 (0%)	ND
tsst-1	0 (0%)	-	0 (0%)	-	0 (0%)	-
sea/seb	2 (5%)	-	1 (2%)	-	1 (10%)	-
seb/sec	6 (17%)	-	9 (17%)	-	0 (0%)	-
sea/seb/sec	4 (11%)	-	2 (4%)	-	1 (10%)	-
seb/sec/tsst-1	0 (0%)	-	4 (7%)	-	0 (0%)	-
Total	34 (94%)		43 (80%)		7 (70%)	

[a] – more frequent in Thai fermented pork sausages (P< 0.00001, compared to the hospitalised patient group)
[b] – more frequent in healthy carriers (P = 0.000189, compared to the hospitalised patient group)
RPLA – reversed passive latex agglutination testing of staphylococcal enterotoxins A, B, C, and D used to determine toxin production in isolate positive by PCR for *sea–sed* genes either alone or in combination
ND – not determined due to lack of a commercial RPLA test kit

Determination of antibiotic susceptibility in S. aureus from different sources. Susceptibility was examined for seven groups of antibiotics (Table 4). The 36 strains of *S. aureus* isolated from Thai fermented pork sausage samples mainly exhibited resistance against the β-lactam group. Specifically, resistance to penicillin, ampicillin, and amoxicillin/clavulanic acid was detected in 83%, 72%, and 8% of those isolates respectively, but all were susceptible to ceftriaxone, cephazolin, ceftazidime, and cefoxitin. All isolates from sausage were also found to be susceptible to other antibiotic groups, including lincosamides (clindamycin), macrolides (erythromycin), aminoglycosides (gentamicin), phenicols (chloramphenicol), glycopeptides (vancomycin), and sulphonamides (trimethoprim/sulfamethoxazole).

Similar resistance patterns were found in *S. aureus* isolates from hospitalised patients. These isolates were resistant to the β-lactam group, with 87%, 87%, and 2% of isolates being resistant to penicillin, ampicillin, and amoxicillin/clavulanic acid, respectively, and susceptible to ceftriaxone, cephazolin, ceftazidime, and cefoxitin. Fewer than 10% of patient isolates were resistant to the lincosamides (clindamycin), macrolides (erythromycin), or phenicols (chloramphenicol) groups. All patient isolates were susceptible to the aminoglycosides (gentamicin), glycopeptides (vancomycin), and sulphonamides (trimethoprim/ sulfamethoxazole).

S. aureus strains isolated from healthy carriers were also resistant to the β-lactam group, including penicillin (80%), ampicillin (70%), and amoxicillin/ clavulanic acid (10%), but were susceptible to ceftriaxone, cephazolin, ceftazidime, and cefoxitin. Ten percent of these isolates exhibited resistance to the lincosamide and macrolide groups, including clindamycin and erythromycin. All isolates were also found to be susceptible to the aminoglycosides (gentamicin), phenicols (chloramphenicol), glycopeptides (vancomycin), and sulphonamides (trimethoprim/sulfamethoxazole).
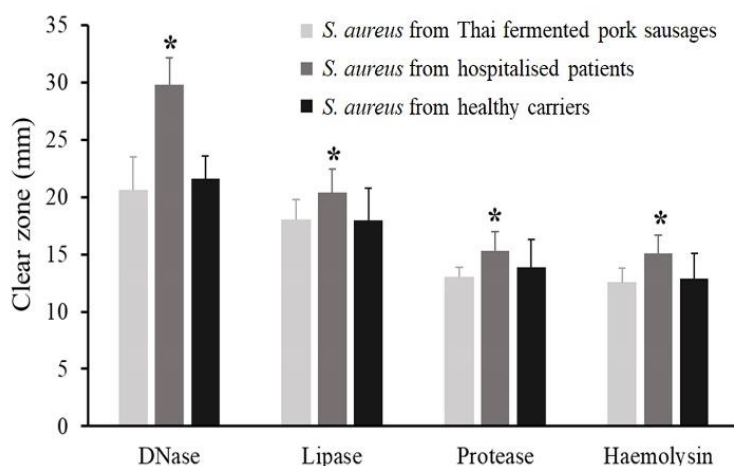

Fig. 2. Production of extracellular enzymes (DNase, lipase, protease) and haemolysin by *S. aureus* strains isolated from three different sources. Data are presented as the mean diameter of clear zones surrounding colonies (mm), determined from triplicate independent experiments * P < 0.05 is considered to be statistically significant in between-group comparisons

Table 3. Grading of biofilm formation in *S. aureus* strains isolated from three different sources

OD ranges (570 nm)	Biofilm quantity grade	*S. aureus* isolates		
		Thai fermented pork sausages (n = 36)	Hospitalised patients (n = 54)	Healthy carriers (n = 10)
<0.19	Biofilm non-former	0 (0%)	0 (0%)	0 (0%)
≥0.19 and <0.38	Weak biofilm former	0 (0%)	0 (0%)	0 (0%)
≥0.38 and <0.76	Moderate biofilm former	2 (6%)	0 (0%)	0 (0%)
≥0.76	Strong biofilm former	34 (94%)	54 (100%)	10 (100%)

The OD $_{cutoff}$ was determined using the average OD of negative control + (3 × standard deviation (SD) of ODs of negative control)
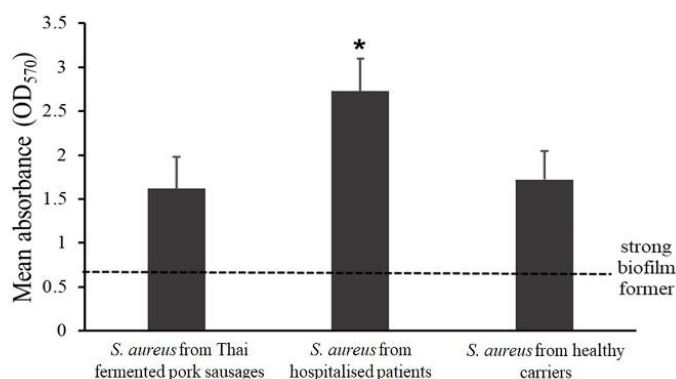
Fig. 3. Biofilm formation was evaluated by crystal violet staining in triplicate independent experiments. Absorbance at 570 nm was measured with a microplate reader. Isolates with OD_{570} values ≥ 0.76 were considered to be strong biofilm formers. * $P < 0.05$ was considered to be significantly different in between-group comparisons

Table 4. Antibiotic resistance in *S. aureus* strains isolated from samples of Thai fermented pork sausage, hospitalised patients, and healthy carriers

Antibiotic group	Antibiotic	Antibiotic resistance in *S. aureus* isolates		
		Thai fermented pork sausages (n = 36)	Hospitalised patients (n = 54)	Healthy carriers (n = 10)
β-lactams	penicillin	30 (83%)	47 (87%)	8 (80%)
	ampicillin	26 (72%)	47 (87%)	7 (70%)
	amoxicillin/clavulanic acid	3 (8%)	1 (2%)	1 (10%)
	ceftriaxone	0 (0%)	0 (0%)	0 (0%)
	cephazolin	0 (0%)	0 (0%)	0 (0%)
	ceftazidime	0 (0%)	0 (0%)	0 (0%)
	cefoxitin	0 (0%)	0 (0%)	0 (0%)
Lincosamides	clindamycin	0 (0%)	4 (7%)	1 (10%)
Macrolides	erythromycin	0 (0%)	4 (7%)	1 (10%)
Aminoglycosides	gentamicin	0 (0%)	0 (0%)	0 (0%)
Phenicols	chloramphenicol	0 (0%)	2 (4%)	0 (0%)
Glycopeptides	vancomycin	0 (0%)	0 (0%)	0 (0%)
Sulphonamides	trimethoprim/sulfamethoxazole	0 (0%)	0 (0%)	0 (0%)

Discussion

Due to the ability of *S. aureus* to colonise the nares and skin of animals, several foods produced from raw materials originating from animals such as poultry, cattle, and swine have been assumed to be reservoirs for *S. aureus* contamination. There are many risk factors associated with food produced from pathogen-contaminated animals, such as poor sanitary conditions in slaughterhouses, unclean conditions in retail markets, and poor personal hygiene of food producers. In this study, we evaluated Thai fermented pork sausages sold in local retail markets of the Amnatcharoen municipal area, northeastern Thailand, and identified a high incidence of *S. aureus* contamination (60%) that may cause food poisoning in consumers. The main ingredient of this local food consists of raw minced pork, and it is usually sold at open retail markets, creating a risk of contamination by various pathogens, including *S. aureus*. Furthermore, food producers usually touch raw minced pork with bare hands during food production, which increases the risk of *S. aureus* contamination.

Overall, classical SE genes were detected in 94% of strains from Thai fermented pork sausages, a frequency that is close to the prevalence in hospitalised patients (80%) and in healthy carriers (70%). However, detection of enterotoxin genes may not be indicative of enterotoxin production. Using RPLA assays, it was found that isolates from Thai fermented pork sausages and healthy carriers predominantly produced SEA, while clinical strains exhibited very low production of the same toxin. It is well-recognised that SEA-producing *S. aureus* strains are the most common toxin producers associated with food poisoning (17) and are responsible for food poisoning outbreaks worldwide, including in the USA (1).

Different distributions of SE genes in contaminated foods have been reported worldwide. In Thailand, previous studies conducted in Khon Kaen province revealed a high prevalence of SEA-positive strains in food from different sources (Thai local food, seafood, fruit juice, and beverages) and in healthy carriers (28, 29). In China, the *seb* genotype was the most frequent enterotoxin gene in *S. aureus* isolated from pork products sampled from a slaughterhouse and terminal market (31). Meanwhile, in South Korea, *S. aureus* isolates derived from milk products produced two or more types of toxin (mainly SEA, SEB, and/or SEC), whereas isolates from raw meat and vegetables primarily produced SEA alone (22). We expect that Thai fermented pork sausages may become contaminated with SE-producing strains from carriers among slaughterhouse workers or food producers. However, Momoh *et al.* (21) reported that *sea* was the most common enterotoxin gene in *S. aureus* isolated from nasal swabs of pigs. Therefore, contamination by *S. aureus* flora that normally colonise pigs should also be considered. The varied prevalence of genes encoding SEs in strains colonising different foods, animals, humans, or geographical environments may be partially explained by the effect of mobile genetic elements (MGEs), which mediate horizontal gene transfer among staphylococci strains (19). The gene encoding *tsst-1* or *sef*, which can cause serious morbidity and mortality, was found only in four isolates from hospitalised patients (where it occurred alongside *seb* and *sec*). The small number of *tsst-1*–positive strains in this study may be due to this gene only being carried by a limited number of *S. aureus* strains overall (15). Furthermore, the marked predominance of *tsst-1* positivity in clinical strains indicates that hospitals may be an important reservoir of *S. aureus*-associated toxic shock syndrome. We did not determine the prevalence of new SEs in this study, both because their emetic activity is still not clear and because of limitations of the diagnostic RPLA kit.

Along with haemolysin, *S. aureus* can produce a large number of extracellular enzymes (such as DNase, lipase, and protease) which allow the bacteria to acquire nutrients necessary for survival, to propagate, and to evade the immune system (27). Preliminary screening of extracellular enzymes and haemolysin production was performed on the *S. aureus* isolates in this study. All *S. aureus* isolates secreted the investigated enzymes and haemolysin, but the clinical isolates derived from hospitalised patients collectively exhibited the highest levels of DNase, lipase, protease, and haemolysin production, while strains derived from food and healthy carriers showed equal expression levels. The significant production of extracellular enzymes and haemolysin by strains from hospitalised patients may indicate an adaptive response to stressful environments inside the human body (6).

Biofilm formation ability was also examined in all *S. aureus* isolates. Almost all were classified as strong biofilm producers, with only two strains being moderate producers. However, between-group comparisons showed significantly greater biofilm formation ability for *S. aureus* strains from hospitalised patients. The particularly strong biofilm formation of clinical strains may be a consequence of bacterial exposure to various immune factors and cytokines during infection. It is conceivable that exposure of *S. aureus* to inflammatory cytokines such as IL-1β and IFN-ϒ promotes biofilm formation ability. Such exposure has been previously reported to induce significant stimulation of bacterial growth of planktonically- and biofilm-growing *S. aureus* strains both *in vitro* and *in vivo* (5, 9). However, the overall strong biofilm formation of isolates from food, patients, and general healthy carriers alike is an important concern; in accordance with previous work (23), we suggest that this capability may promote multidrug resistance and that awareness of this trait should be increased.

The antimicrobial susceptibility of all *S. aureus* isolates was also investigated in this study. We found a high rate of resistance against the β-lactam group antibiotics, especially penicillin and ampicillin. A few isolates were resistant to amoxicillin/clavulanic acid. Most *S. aureus* isolates were susceptible to lincosamides, macrolides, aminoglycosides, phenicols, glycopeptides, and sulphonamides. However, we identified six clinical isolates that exhibited multidrug resistance to β-lactams, lincosamides, macrolides, and phenicols. Fortunately, methicillin- or vancomycin-resistant *S. aureus* (MRSA or VRSA) was not detected among the isolates in this study. Nevertheless, the existence of penicillin and ampicillin resistance in isolates derived from all three sources is of concern and may be due to the abundant usage of penicillin for production of swine, cattle, and poultry, especially in low- to middle- income countries. In Thailand, amoxicillin and ampicillin are widely sold in local grocery stores and general retail shops (13); furthermore, most antibiotics are easily available for home or farm use from retail pharmacies without a prescription (26). Thai adults were reported to have a low level of knowledge of antibiotics generally when this was tested by eliciting judgments of the truth or falsehood of prepared statements. This may indicate a large knowledge gap in the Thai population regarding the appropriate use of antibiotics (3). These previous observations cohere with the increasing antibiotic resistance in local Thai communities. Although MRSA strains were not found in this study, long term surveillance and frequent monitoring are necessary.

In summary, high levels of *S. aureus* contamination were detected in Thai fermented pork sausages, which are a local food of northeastern Thailand. We suggest that this local food may be a high-risk reservoir for staphylococcal food poisoning. SEA appeared to be the major SE in these sausages, similar to strains isolated from healthy carriers. This

study may help us to better understand the distribution of the types of *S. aureus* in local foods produced from animals in northeastern Thailand, and may be useful as a basic database for further epidemiological surveillance and prevention of food poisoning. To reduce foodborne pathogen contamination in local foods, dissemination to local communities of knowledge of better sanitary and hygiene practices in slaughterhouses or food production operations, along with knowledge of antibiotic usage, should be accorded great importance.

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