Staphylococcus aureus and their virulence genes from food contact articles in restaurants

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Background. *Staphylococcus aureus* is one of the most common pathogens responsible for food poisoning due to its ability to produce toxins known as staphylococcal enterotoxin (SEs). *S. aureus* can form biofilms on surfaces of food processing devices, allowing cross-contamination events to simply occur on foods. **Methods.** In this study, we investigated biofilm formation and identified virulence genes involved as well as enterotoxin genes in *S. aureus* isolated from food contact articles. **Results.** Two hundred *S. aureus* were isolated from 650 samples. The highest prevalence was found in the hands of food handlers. Although all of them were negative for the *mec*A gene, biofilm formation was detected using the CRA method. Forty-nine (24.5%) isolates produced biofilm, and biofilm-related adhesion genes (*ica*AD, *fnb*A, *cna*, and *bap*) were detected with the prevalence rates of 13.0, 14.5, 6.5 and 0.5%, respectively. Two classical enterotoxin genes (*sec* and *sed*) were also found in 4 and 6 of the *S. aureus* isolates. *S. aureus* with biofilm and enterotoxin production abilities discovered on food contact surfaces and food handlers imply significant sources of food contamination that can be harmful to consumers.

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Abstract

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Methods.

In this study, we investigated biofilm formation and identified virulence genes involved as well as enterotoxin genes in *S. aureus* isolated from food contact articles.

Results.

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and biofilm-related adhesion genes (*ica*AD, *fnb*A, *cna*, and *bap*) were detected with the prevalence rates of 13.0, 14.5, 6.5 and 0.5%, respectively. Two classical enterotoxin genes (*sec* and *sed*) were also found in 4 and 6 of the *S. aureus* isolates. *S. aureus* with biofilm and enterotoxin production abilities discovered on food contact surfaces and food handlers imply significant sources of food contamination that can be harmful to consumers.

Introduction

Staphylococcus aureus is an important pathogen generally found on human bodies, medical equipment, or even contact surfaces in hospital and community environments. It is the predominant species in surgical site infections (Al-Awaysheh, 2018), and was also found in subclinical mastitis in she-camels in Algeria that could directly contaminate their milk (Hadef, Aggad & Hamad, 2018). *S. aureus* can cause food poisoning. Food poisoning caused by staphylococcal enterotoxins (SEs) occurs on an annual basis (Wieneke, Roberts & Gilbert, 1993). The pathogenesis of *S. aureus* strain is due to the battery of virulence factors such as extracellular factors, toxins, adhesion, biofilm formation, and resistance to phagocytosis (; Mehrotra, Wang & Johnson, 2000; Arciola, Baldassarri & Montanaro, 2001; Khoramian et al., 2015). Although several enterotoxins have been reported, enterotoxins A and D are the most important as a result of their heat resistance (Hwang et al., 2007; Pelisser et al., 2009). The heating process during cooking may not be sufficient to destroy the toxins. Therefore, when *S. aureus* appears on food contact utensils or food handles, the chances of its contamination of food and causing disease are unavoidable (Ciccio et al., 2015; Castro et al., 2016).

S. aureus can produce biofilm on food processing surfaces such as polystyrene and stainless steel (Hamadi et al., 204; Ciccio et al., 2015). Biofilms have become a problem in the

food industry as they provide barriers to cleaning (including disinfection) of surfaces contaminated with bacteria and organic matter (Avila-Novoa et al., 2018). The presence of biofilm-related genes in *S. aureus* allows intramammary adherence and biofilm formation, leading to the inefficacy of antibiotic treatment and chronicity (Cucarella et al., 2001).

This study aims to detect *S. aureus* contamination from food handlers and food handling equipment. Detection of biofilm formation and genes involved (e.g., *ica*AD, *fnb*A, *cna* and *bap*) as well as classical enterotoxin genes (*sea*, *seb*, *sec*, *sed* and *see*) have been conducted. The results would help improve the prevention of *S. aureus* contamination, allowing restaurants to pay more attention to food hygiene and consumer safety.

Materials & Methods

Samples collection

This study was approved by the Institutional Review Board Naresuan University (IRB No. P10203/64). The consent form has been provided and signed by all participants prior to collecting samples for this study by swabbing from their hands. Six hundred fifty swab samples were collected from hands, plates, spoons, knives, chopping boards, and glasses from restaurants. For each sampling, the swab sticks were removed from their sterile wrappings and the tip was moistened by immersing them in 0.1% peptone water. The area to be swabbed was chosen, and the swab tips were pressed against the surface. After taking the swab and putting it back in the buffer, the microbiologist will examine it further in the laboratory. Swab samples were transferred to MSA agar. Cultures with yellow colonies were selected for further evaluation using Gram stain, catalase, and coagulase tests. All isolates were subsequently identified as staphylococci by PCR

reaction using 16S rRNA primers specific for staphylococci (Kohner et al., 1999). The methicillin-resistance gene (*mec*A gene) was determined by PCR based on research by Kitti et al., 2011.

Biofilm formation

A Congo red agar (CRA) test was performed to determine slime production. All *S. aureus* isolates were inoculated onto the CRA plate and incubated for 24–48 hours at 35°C. Slime production was distinguished by the color change of the colonies from red to black. Non-slime producers were identified as colonies that remained red (Freeman, Falkiner & Keane, 1989).

Molecular detection of biofilm-related adhesion genes

Table 1 shows the primers used to detect the *S. aureus* biofilm-related genes; *bap*, *fnb*A, *ica*AD, and *cna*. DNA templates were extracted from each sample by boiling method. The reaction was carried out with a total volume of 25 µl including a 2 µl DNA template, 20 pmol of each primer, and 12.5 µl of one PCR (GeneDireX, Inc., Taiwan Taoyuan), and the volume was adjusted with sterile distilled water. Amplification begins with a pre-denaturation step at 94 ^{o}C for 2 minutes, followed by 30 cycles of 94 ^{o}C for 20 sec, annealing for 30 sec at 58 ^{o}C for *ica*AD, at 55 ^{o}C for *fnb*A gene, at 52 ^{o}C for *cna* and at 50 ^{o}C for *bap* gene, extension at 72 ^{o}C for 1 minute and final extension at 72 ^{o}C for 5 minutes. The PCR products were analyzed by 1% agarose gel electrophoresis.

Molecular detection of enterotoxin genes

In this study, we used five primer sets for five classical enterotoxin genes (Table 1) followed by Mehrotra *et al*. with minor modifications to the given instructions (Mehrotra, Wang & Johnson, 2000). In a 1.5 ml microcentrifuge tube, prepare 25 µl of sterile deionized water. To

break a cell, suspend *S. aureus* in deionized water and heat it to 95°C for 2 minutes. Centrifuge at 6000 rpm for 2 minutes and use the supernatant as a template for PCR. The reaction was carried out with a total volume of 25 µl including a 2 µl DNA template, 20 pmol of each primer, and 12.5 µl of one PCR (GeneDireX, Inc., Taiwan Taoyuan), and the volume was adjusted with sterile distilled water. Amplification start with initial denaturation at 94°C for 5 min was followed by 35 cycles of amplification (denaturation at 94°C for 5 min, annealing at 50°C for 2 min, and extension at 72°C for 1 min), ending with a final extension at 72°C for 7 min. Amplicons were analyzed by 1% agarose gel-electrophoresis.

Results

Of the 650 swab samples, 200 isolates were identified as *S. aureus* (26.39%). The highest prevalence of *S. aureus* (58.5%) was detected on the hands of the food handlers, followed by the chopping board (13%), plates (11.5%), knives (8%), spoons (6.5%) and glasses (2.5%). All 200 isolates were found to be negative for the methicillin resistance (*mecA*) gene. Based on the CRA biofilm formation test, 49 isolates (24.5%) created biofilms as black colonies on Congo red agar, while 151 isolates (75.5%) grew as red colonies and were classified as non-biofilm producing *S. aureus*.

According to the PCR results of the 200 S. aureus identified, genes involved in biofilm formation, ica, fnbA and cna genes were found in 26, 29 and 13 isolates, respectively, whereas the bap gene was detected in only 1 isolate. Statistically, analyses were performed using Phi-coefficients, which is a measure of the strength of an association between two categorical variables. The sample size is 200 and the degree of freedom is 1. Phi-coefficients revealed that the *ica* gene was closely associated with the *fnb*A gene at the 0.01 significance level in the same direction,

followed by the relationships between *cna* and *fnb*A genes (0.01), and between *cna* and *ica* genes (0.05) in the same direction, while the *bap* gene was not related to other genes (Table 2).

Of the 5 SEs (*sea*, *seb, sec*, *sed*, and *see*), 2 SEs were detected in 200 *S. aureus* isolates. The *sec* gene was found in 4 isolates (2%) and *sed* gene in 6 isolates (3%).

Discussion

Among 650 swab samples collected from the surface of food contact articles (hands, plates, spoons, knives, chopping boards, and glasses) in restaurants, 200 isolates of *S. aureus* were found. Swab samples collected from the hands of food handlers were shown as the main source (58.5%) of *S. aureus* isolates. *S. aureus* is a symbiotic bacterium known to inhabit human skin, nostrils, and the gastrointestinal tract. A study of nasal carriers working in food production environments revealed that the *staphylococcus* carrier could contaminate food via contact with respiratory secretions, causing staphylococcal food poisoning (Bencardino et al., 2021). Ogidi *et al*. also reported the most occurrence of *S. aureus* (23.30%) found on street foods sold in Akure Metropolis, Nigeria, amongst the other bacterial contaminants (Ogidi, Ovetavo & Akinyele, 2016). This could be caused by unwashed hands and other poor hygiene practices of food vendors. Detection of *S. aureus* from the hands of food processors or other food contact containers is, therefore, an important source of staphylococcal contamination in foods, emphasizing the risks associated with cross-contamination.

Kim *et al*. reported the formation of biofilms affected the attachment of *S. aureus* to food contact vessels (Kim et al., 2017). Because of their low antibiotic sensitivity, Staphylococci in clinical isolation were difficult to treat clinically (Cramton, Gerke & Götz, 2001). In this study, we used the CRA method for the detection of *S. aureus* isolated from food contact articles. Forty-

149 nine biofilm-forming *S. aureus* (49/200), accounted for 24.5%, were found. Most of which (39/49)
150 (79.59%) were isolated from the hands of food handlers. In the Avila-Novoa *et al*. study, biofilm
151 formation of *S. aureus* isolated from food contact surfaces (FCS) of six dairy industries in the
152 Mexican state of Jalisco was found to produce 75% biofilm (63/84) (Avila-Novoa et al., 2018). In
153 the study of Ciccio *et al*., they found only one *S. aureus* strain from food handlers was a biofilm
154 producer at 12^{o}C (Ciccio et al., 2015).

155 PCR detection of 26 *S. aureus* isolates was positive for both phenotype and *ica* gene. This
156 is consistent with the study by Khoramian et al., 2015 and Arciola et al., 2001. They also
157 discovered the relationship between biofilm formation and the *ica* gene. The other 23 samples
158 were phenotypically positive but could not detect the *ica* locus gene, assuming that *S. aureus* might
159 use the other operon to produce biofilm (Marques et al., 2017). We didn't find the relationships in
160 biofilm formation between the other genes (*fnb*A, *cna*, and *bap*). However, the results of this
161 experiment are consistent with experiments by various researchers, suggesting that the presence
162 of the *cna*, *fnbA*, and *bap* genes is not consistent with biofilm formation (Płoneczka-Janeczko et
163 al., 2014; Tangchaisuriya et al., 2014; Seng et al., 2017).

164 The enterotoxin genes detected in this study were *sec* and *sed*. *S. aureus* with *sec* positive
165 came from kitchen utensils that usually have direct contact with meat, such as knives and cutting
166 boards. Previous reports showed that *sec* was most common in animals (Marr et al., 1993; Pinchuk,
167 Beswick & Reyes, 2010). Enterotoxin D is the second most common cause of food poisoning in
168 the world. A small amount of this toxic substance can cause the disease (Pinchuk, Beswick &
169 Reyes, 2010). Food poisoning has been caused by microbial contamination of kitchen utensils and
170 hands during cooking (Wit, Broekhuizen & Kampelmacher, 1979; Jong et al., 2008). Cleaning
171 utensils such as sponges are also a source of microbial contamination (Kusumaningrum et al.,

2003). The hands of food workers can be mutually contaminated with objects containing food (Jong et al., 2008).

Conclusions

In this study, *S. aureus* was detected from food contact articles, of which the most prevalence was found on the hands of food handlers. The isolated strains possess genes involved in biofilm formation and enterotoxin production, which increase the chance to deliver staphylococcal food poisoning to the consumers.

According to our food safety policy, zero tolerance for foodborne pathogens is currently of interest. The data from our results could be applied in restaurants, food shops, or even food stalls, allowing them to take more action and with more carefulness during the preparation of food. Especially, when it comes into contact with hands, or any other food-contact articles. Training of food hygiene and food safety regulations is urgently needed for the people involved in food preparation.

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Table 1(on next page)

primer list

Nucleotide sequences and sizes of PCR products for the *S. aureus* gene-specific oligonucleotide primers used in this study.

Table 1:

Nucleotide sequences and sizes of PCR products for the *S. aureus* gene-specific oligonucleotide primers used in this study.

Genes	Primer	Primer 5'->3'	Reference
16srRNA	16sF	CGAAAGCCTGACGGAGCAAC	Kohner et al., 1999
	16sR	AACCTTGCGGTCGTACTCCC	Kitti et al., 2011
*mec*A	mecAF	TGGCTATCGTGTCACAATCG	
	mecAR	CTGGAACTTGTTGAGCAGAG	Seng et al., 2018
*ica*AD	icaADF	AATGTGCTTGGATGCAGATACTATC	
	icaADR	GAATCGTCATCTGCATTTGCA	Arciola et al., 2005
cna	CnaF	AAAGCGTTGCCTAGTGGAGA	
	CnaR	AGTGCCTTCCCAAACCTTTT	Arciola et al., 2005
fnbA	FnaF	GATACAAACCCAGGTGGTGG	
	FnaR	TGTGCTTGACCATGCTCTTC	Cucarella et al., 2001
bap	Bap971F	CCCTATATCGAAGGTGTAGAATTGCAC	
	Bap971R	GCTGTTGAAGTTAATACTGTACCTGC	
Sea	SEAF	GGTTATCAATGTGCGGGTGG	Mehrotra et al., 2000
	SEAR	CGGCACTTTTTTCTCTTCGG	
Seb	SEBF	GTATGGTGGTGTAACTGAGC	
	SEBR	CCAAATAGTGACGAGTTAGG	
Sec	SECF	AGATGAAGTAGTTGATGTGTATGG	
	SECR	CACACTTTTAGAATCAACCG	
Sed	SEDF	CCAATAATAGGAGAAAATAAAAG	

	SEDR	ATTGGTATTTTTTTTCGTTC
See	SEEF	AGGTTTTTTCACAGGTCATCC
	SEER	CTTTTTTTTCTTCGGTCAATC

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Table 2(on next page)

biofilm related genes

The correlation between biofilm-related genes in this study.

Table 2:

The association between biofilm-related genes in this study.

		Ica	*fnb*A	*cna*	*bap*
Ica	Phi-coefficient		0.39**	0.14*	-0.03
	Sig. (2-tailed)		0.00	0.05	0.70
	N		200	200	200
*fnb*A	Phi-coefficient	0.39**		0.24**	-0.03
	Sig. (2-tailed)	0.00		0.00	0.68
	N	200		200	200
Can	Phi-coefficient	0.14*	0.24**		-0.02
	Sig. (2-tailed)	0.05	0.00		0.79
	N	200	200		200
Bap	Phi-coefficient	-0.03	-0.03	-0.02	
	Sig. (2-tailed)	0.70	0.68	0.79	
	N	200	200	200	

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).