



Contamination level and characterization of *Staphylococcus aureus* associated with ready-to-eat foods, food handlers, and environmental factors

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ABSTRACT

Aims: Although several major food poisoning outbreaks caused by *Staphylococcus aureus* have been reported, monitoring of this pathogen is often neglected. The objectives of this study were to assess the contamination level of *S. aureus* and characterize the *S. aureus* isolated in ready-to-eat (RTE), food handlers, food contact surfaces, and table cleaning cloths (TCC).

Methodology and results: A total of 150 RTE foods, 59 food contact surfaces (FCS) and 34 table cleaning cloths (TCC) from food premises were examined. The contamination level of *S. aureus* in RTE foods was at acceptable level. However, more than 10% of the FCS and TCC were contaminated with high levels of *S. aureus* (>1.0 Log CFU/cm², >2.7 Log CFU/piece). Eighty-one isolated *S. aureus* including those isolated from hands of food handlers were further characterized by antimicrobial susceptibility testing, virulotyping and PFGE. Out of 81 isolates, only three were multidrug resistant. More than 96% (n = 78) of the *S. aureus* harboured at least one virulence gene. Almost half of the isolates carried at least one staphylococcal enterotoxin in which SEC was the most common enterotoxin detected.

Conclusion, significance and impact of study: The PFGE analysis showed that the *S. aureus* could be disseminated via the FCS, TCC and the hands of food handlers. Therefore, this study reiterates the importance of proper hand washing, sanitation of FCS and TCC, as well as continuous monitoring on *S. aureus* in food and the food handlers.

Keywords: genotyping, *Staphylococcus aureus*, ready-to-eat foods, virulence

INTRODUCTION

In Malaysia, a wide variety of ready-to-eat (RTE) foods are readily available at hawker stalls, cafeterias, and restaurants. Patronising street food stalls is a common habit amongst Malaysians since street foods are usually more affordable than restaurant foods and easily accessible. However, the food hygiene and safety of the RTE foods sold in these food premises are unknown. The Food and Agriculture Organization of the United Nations (FAO) has recognised street foods as one of the threats linked to foodborne diseases in many countries (FAO, 2016). Globally, many food poisoning cases are associated with consumption of RTE foods (CDC, 2013). In fact, most food poisoning cases in Malaysia are attributed to the consumption of RTE foods (Soon *et al.*, 2011).

According to the Centers for Disease Control and Prevention, USA, approximately 48 million people were sickened, and 3000 died because of foodborne diseases every year in USA (CDC, 2014). In Malaysia, food poisoning cases had increased from 44.93 cases per

100,000 population in 2013 to 50.23 cases per 100,000 population in 2015 (MOH, 2013; 2014a). Approximately 43% of the total food poisoning cases in the year 2014 occurred in academic institutions in Malaysia (MOH, 2014b). Numerous food surveys had been conducted by local researchers on the microbiological quality of RTE foods sold locally and frequently detected various types of foodborne pathogens such as *Salmonella* spp. (Modarressi *et al.*, 2010, Marian *et al.*, 2012), *Campylobacter* spp. (Chai *et al.*, 2007), *Vibrio parahaemolyticus* (Paydar *et al.*, 2013) and *Listeria monocytogenes* (Jamali *et al.*, 2013). However, there are limited reports on occurrence of *S. aureus* in food.

As food poisoning due to *S. aureus* enterotoxin is not a notifiable disease in Malaysia, the prevalence of this potential pathogen could be under-reported. However, staphylococcal food poisoning has become the sixth most prevalent causative agent in the USA (CDC, 2013). Most of the staphylococcal food poisoning incidents are caused by improper food handling (Le Loir *et al.*, 2003). *S. aureus* is an important foodborne pathogen and is difficult to eradicate because of the heat-resistant staphylococcal

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toxins. The combination of toxin-mediated virulence and antibiotic resistance causes a more severe staphylococcal food poisoning.

Staphylococcal enterotoxins are categorized into classical (SEA to SEE) and non-classical enterotoxins (SEG to SEU). The non-staphylococcal enterotoxins virulence genes like toxic shock toxin (TSST-1), Paton-Valentine Leucocidin (PVL) (Melles *et al.*, 2006) and fibronectin-binding protein (FnB) can cause toxic shock syndrome, leucocidal effect and skin tissue colonization, respectively (Arciola *et al.*, 2005).

Therefore, the objective of the study was to assess the contamination level of *S. aureus* in RTE foods, food contact surfaces (FCS) and the table cleaning cloths (TCC) in selected food premises. In addition, the isolated *S. aureus* were characterised by the virulence profiles, antibiotic resistance and DNA fingerprinting by PFGE.

MATERIALS AND METHODS

Sample collection

The food and environmental samples were collected from food premises located within the University and its vicinity in Malaysia. The University has a population of 17,312 students and 6,688 staff as recorded in 2014. Within the campus, there is a total of 25 food premises, but only 18 food premises serve lunch. Besides food premises within the campus, students and the staff also frequent the food establishments located in the vicinity of the campus. These food stalls, restaurants, and fast food franchises cater both the students and community.

A total of 150 RTE food samples were randomly purchased from food premises located within ($n = 115$) and at the periphery ($n = 35$) of the campus. Samples of RTE food were purchased during lunchtime (11.30 A.M. to 1.30 P.M.) and were collected and packed in plastic bags, plastic containers or polystyrene food containers by food handlers. The samples were then transported in an icebox to the laboratory and processed within one hour.

The environmental samples included swabs from the cutting boards, cutleries, plates, and kitchen countertops. These surfaces were swabbed using sterile cotton swabs and then, placed into sterile tubes containing 10 mL of maximum recovery diluent (MRD; Merck, Germany). Table cleaning cloth (TCC) used in the food premises were collected and immersed in a tube containing sterile 200 mL MRD. The samples were transported in ice to the laboratory for microbiological analysis within one hour.

Enumeration of *S. aureus*

Ten grams of food samples were mixed with 90 mL of MRD in a stomacher bag with filter (Interscience, France) and homogenised for 2 min. The food homogenate was diluted up to 10⁻⁵. An aliquot (1 mL) of the diluted samples including those from FCS and TCC samples were transferred onto the Petrifilm™ (3M™, US) Staph express count plate to enumerate *S. aureus*. The inoculated petrifilms were incubated and interpreted

according to the manufacturer's guidelines (3M, 2008). The colony forming unit (CFU) counts were compared to the published guidelines for FCS (Sneed *et al.*, 2004), RTE foods (Gilbert *et al.*, 2010) and TCC (Willis *et al.*, 2013)

Identification and detection of *S. aureus* using Polymerase Chain Reaction (PCR)

The isolates from the Petrifilm were picked and purified on Mannitol salt agar, followed by biochemical identification and Gram staining. All presumptive *S. aureus* were then confirmed by species-specific PCR using the primers which target the *nucA* (Brakstad *et al.*, 1992). *S. aureus* ATCC 25923 was used as the positive control strain in this study.

The PCR products were then subjected to gel electrophoresis with 1.5% LE agarose (Promega, USA) for 30 min at 100 V. Gel was visualised using Gel Doc XR (Bio-Rad, USA) after staining with GelRed Nucleic Acid Stain (Biotium, USA).

For a more comprehensive study of the characteristics of *S. aureus* in the food premises, an additional of 56 strains of *S. aureus* which were isolated at the same time from food handlers' hands as previously reported by Lee *et al.* (2017) were included in this study.

Antimicrobial susceptibility testing

The antimicrobial susceptibility testing of the *S. aureus* isolated was determined by the Kirby-Bauer Disc Diffusion Method according to the Clinical Laboratory Standards Institute guidelines (2015). The antibiotics tested were penicillin (10 U), teicoplanin (30 µg), gentamicin (10 µg), kanamycin (30 µg), erythromycin (15 µg), ciprofloxacin (5 µg), tetracycline (30 µg), clindamycin (2 µg), trimethoprim-sulfamethoxazole (25 µg), chloramphenicol (30 µg), rifampin (5 µg), and linezolid (30 µg). The antimicrobial susceptibility was interpreted based on CLSI guideline (2015).

Virulence gene profiling

Genomic DNA from each isolate was extracted by direct boiled cell lysate method. The PCR conditions and primers for 21 most common virulence genes that are associated with staphylococcal food poisoning and staphylococcal infections were as previously described: *sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, *sei*, *sej* (Colque-Navarro *et al.*, 2000, Kumar *et al.*, 2009), *cna*, *fnbA*, *fnbB* (Arciola *et al.*, 2005), *icaA*, *sdrE*, *hlg*, *tst*, *eta*, *etb*, *etd*, *efb* (Colque-Navarro *et al.*, 2000; Jarraud *et al.*, 2002), *pvl*, and *mecA* (Lina *et al.*, 1999).

Pulsed-field gel electrophoresis (PFGE)

Staphylococcus aureus isolated from RTE foods, FCS, TCC and food handlers' hands were further characterised by PFGE as described by previous study (Thong *et al.*,

2009). Chromosomal DNA was digested with 1.2 U of *Sma*I enzyme prior to PFGE.

BioNumerics 6.0 (Applied Math, Kortrijk, Belgium) was used to analyse the fingerprinting patterns generated by PFGE. The quantitative difference in the banding patterns was determined by the Dice coefficient, *F*. An *F* value of 0 means the pattern is dissimilar while *F* = 1 means the pattern is indistinguishable. Each strain was analysed twice to ensure reproducibility. The discriminatory index, DI of PFGE was obtained by using an online tool, Discriminatory Power Calculator.

RESULTS

Microbial load of *S. aureus* in ready-to-eat foods, food contact surfaces and table cleaning cloths

Microbial analysis of the 150 food samples showed that the level of *S. aureus* counts were within an acceptable range (<2.0 Log CFU/g (Table 1). However, the microbial loads of *S. aureus* were higher than the acceptable level (>1.0 Log CFU/cm²) in 10 % (n = 6) of FCS and 18 % (n = 6) of TCC.

A total of 25 *S. aureus* was isolated from RTE foods (n = 4), FCS (n = 9) and TCC (n = 12) samples. To have a more comprehensive analysis, additional 56 *S. aureus* strains that were previously isolated from hands of food handlers working at these premises (Lee *et al.*, 2017) were included for characterisation.

Antimicrobial susceptibility profile

A majority of the strains were pan-susceptible to the antibiotics tested (Table 2). Approximately 41 % the *S. aureus* strains (33/81) were resistant to penicillin (10 U), while 11% (n = 9) were resistant to tetracycline (30 µg). The resistant strains were mainly isolated from food handlers' hands (Table 2). Three *S. aureus* strains isolated from hands of food handlers were multidrug resistant (resistant to 4 - 7 classes of drugs). *Staphylococcus aureus* recovered from RTE foods, TCC

and FCS were susceptible to all antibiotics tested or resistant to < 2 classes of drugs.

Table 1: Microbial load of *S. aureus* and the number of strains recovered from different sources.

Sample type	Acceptable range	Satisfactory level, n (%)	Number of strains, n
RTE Foods (n = 150)	2.0 Log CFU/g ^a	150 (100.0)	4
FCS (n = 59)	1.0 Log CFU/cm ^{2b}	53 (89.8)	9
TCC (n = 36)	2.7 Log CFU/piece ^c	28 (82.3)	12
FHH (n = 85)	NA	NA	56

NA, not applicable; FCS, food contact surfaces; TCC, table cleaning cloths, FHH, food handlers' hands

^aThis standard is based on Gilbert *et al.*, 2010.

^bThis acceptable range is based on Sneed *et al.*, 2004

^cThis standard is adapted from Willis *et al.*, 2013.

Virulence genes profile

Out of 81 *S. aureus* strains, 78 (96.3%) carried at least one virulence gene (Table 3). About 48% of *S. aureus* (n = 39) harbored at least one staphylococcal enterotoxin gene (SE); *sea*, *sec* and *seh* were the three most common SEs detected among the *S. aureus*. The occurrence of SEs (*sea* - *seh*) was twice the occurrence of non-SEs in the isolated *S. aureus* (Table 3).

Two *S. aureus* recovered from RTE foods carried SE genes. On the other hand, almost all *S. aureus* (96.3%, n = 78) harbored at least one non-SE virulence gene, except for one strain originated from FCS and two from hands of food handlers. The *fnbA* was the most common gene found in 96.3% of *S. aureus*.

Table 1: Antibigrams of *S. aureus* isolated from food and non-food sources.

Antibiotics (µg)	Total n of resistant strains, (%)	Resistant strain from each source			
		RTE Foods (n = 4)	FCS (n = 9)	TCC (n = 12)	FHH (n = 56)
Penicillin G (10 U)	33 (40.7%)	-	5 (55.6%)	4 (33.3%)	24 (42.9%)
Teicoplanin (30)	2 (2.5%)	-	-	-	2 (3.6%)
Gentamycin (10)	0 (0.0%)	-	-	-	-
Kanamycin (30)	8 (9.9%)	1 (25.0%)	2 (22.2%)	-	5 (8.9%)
Erythromycin (15)	6 (7.4%)	-	-	2 (16.7%)	4 (7.1%)
Ciprofloxacin (5)	5 (6.2%)	1 (25.0%)	1 (11.1%)	-	3 (5.4%)
Tetracycline (30)	9 (11.1%)	1 (25.0%)	1 (11.1%)	-	7 (12.5%)
Clindamycin (2)	2 (2.5%)	-	-	-	2 (3.6%)
Trimethoprim-sulfomethoxazole (25)	0 (0.0%)	-	-	-	-
Chloramphenicol (30)	2 (2.5%)	1 (25.0%)	-	-	1 (1.8%)
Rifampicin (5)	2 (2.5%)	-	-	-	2 (3.6%)
Linezolid (30)	3 (3.7%)	-	-	-	3 (5.4%)

Table 2: The prevalence of virulence genes in the *S. aureus*.

Prevalence of virulence gene	Total n (%)	RTE Foods (n = 4)	FCS (n = 9)	TCC (n = 12)	FHH (n = 56)
<i>SE(s)</i>	39 (48.1%)	2 (50.0%)	5 (55.6%)	8 (66.7%)	24 (42.9%)
<i>Sea</i>	11 (13.6%)	n. d.	n. d.	4 (33.3%)	7 (12.5%)
<i>Seb</i>	6 (7.4%)	1 (25.0%)	n. d.	4 (33.3%)	1 (1.8%)
<i>Sec</i>	13 (16.0%)	1 (25.0%)	1 (11.1%)	2 (16.7%)	9 (16.1%)
<i>Sed</i>	2 (2.5%)	n. d.	n. d.	n. d.	2 (3.6%)
<i>See</i>	n. d.	n. d.	n. d.	n. d.	n. d.
<i>Seg</i>	4 (4.9%)	1 (25.0%)	n. d.	1 (8.3%)	2 (3.6%)
<i>She</i>	12 (18.5%)	n. d.	3 (33.3%)	4 (33.3%)	5 (8.9%)
<i>Sei</i>	10 (12.0%)	1 (25.0%)	1 (11.1%)	2 (16.7%)	6 (10.7%)
<i>Sej</i>	1 (1.2%)	n. d.	n. d.	n. d.	(66.7%)
Non-SE(s)	78 (96.3%)	4 (100.0%)	8 (88.9%)	12 (100.0%)	54 (96.4%)
<i>Efb</i>	36 (44.4%)	2 (50.0%)	5 (55.6%)	10 (83.3%)	19 (33.9%)
<i>Hlg</i>	32 (39.5%)	1 (25.0%)	4 (44.4%)	8 (66.7%)	19 (33.9%)
<i>Can</i>	n. d.	n. d.	n. d.	n. d.	n. d.
<i>Eta</i>	n. d.	n. d.	n. d.	n. d.	n. d.
<i>Etb</i>	n. d.	n. d.	n. d.	n. d.	n. d.
<i>Etd</i>	n. d.	n. d.	n. d.	n. d.	n. d.
<i>Tst</i>	n. d.	n. d.	n. d.	n. d.	n. d.
<i>Ica</i>	23 (28.4%)	n. d.	3 (33.3%)	2 (16.7%)	18 (32.1%)
<i>fnbA</i>	78 (96.3%)	4 (100.0%)	8 (88.9%)	12 (100.0%)	54 (96.4%)
<i>fnbB</i>	n. d.	n. d.	n. d.	n. d.	n. d.
<i>Pvl</i>	1 (1.2%)	n. d.	n. d.	1(8.3%)	n. d.
<i>sdrE</i>	n. d.	n. d.	n. d.	n. d.	n. d.
<i>mecA</i>	n. d.	n. d.	n. d.	n. d.	n. d.

n. d., not detected

Genetic diversity of *S. aureus* based on PFGE

A majority of the *S. aureus* were typeable by *Sma*I enzyme using PFGE, except for four strains (Figure 1). The genomic DNA were restricted to 9 – 14 bands which ranged from 30.3 kb to 700.0 kb. Fifty-three distinct reproducible banding patterns with D value = 0.9891 and F value = 0.36 to 1.00 were obtained. Among the reproducible banding patterns, 42 unique profiles were observed. Based on the arbitrary 80% similarity, the strains were subtyped into 15 clusters, with 2 to 6 strains in each cluster. There were five groups of indistinguishable strains annotated with A, B, C, D, and E in Figure 1. The virulotypes of the strains within each cluster were similar (SP1 - SP15). Moreover, the strains in SP6 and SP9 were genetically homogenous.

DISCUSSION

In this study, the TCC and FCS were highly contaminated with *S. aureus*. However, *S. aureus* were present in only 3% of the RTE foods. *S. aureus* is part of the microbiota of the human skin. The presence of virulent *S. aureus* in RTE, TCC and FCS indicates the poor sanitation and food handling practices and could poses a health risk to consumers. Microbiological analysis of the FCS and TCC in the food premises revealed their roles as potential vehicles for bacteria transmission.

Studies investigating the transmission of pathogens through FCS and TCC are limited. Cunningham *et al.* (2011) reported that 70.3% of the visually clean FCS samples failed in adenosine triphosphate bioluminescence assessment. Bacteria on the FCS are highly possible to be transferred onto the foods (Pérez-Rodríguez *et al.*, 2008). Willis *et al.* (2013) also demonstrated that one-third of the FCS samples that they tested were of an unsatisfactory level of hygiene while more than half of the cleaning cloth samples (56%, n = 98) failed in the microbiological quality assessment. Our results concurred with these reported cases. During sampling, we observed that some food handlers left their table cleaning cloths on the clean plates, and this could constitute a potential cross contamination and poses a potential health risk to consumers.

Cutting board is an important fomite in foodborne pathogen transmission. Wooden cutting boards are porous and suitable habitat for diverse bacteria (Cliver, 2006). The Malaysian Ministry of Health has banned the use of wooden cutting boards. Unfortunately, despite the ban, some of the food handlers in our study were still using wooden cutting boards. Furthermore, we observed that some table cleaning cloths were left on the cutting board after use. Therefore, sanitising cutting board is essential as proper sanitation of cutting boards can effectively decrease the rate of food borne illnesses (Cliver, 2006).

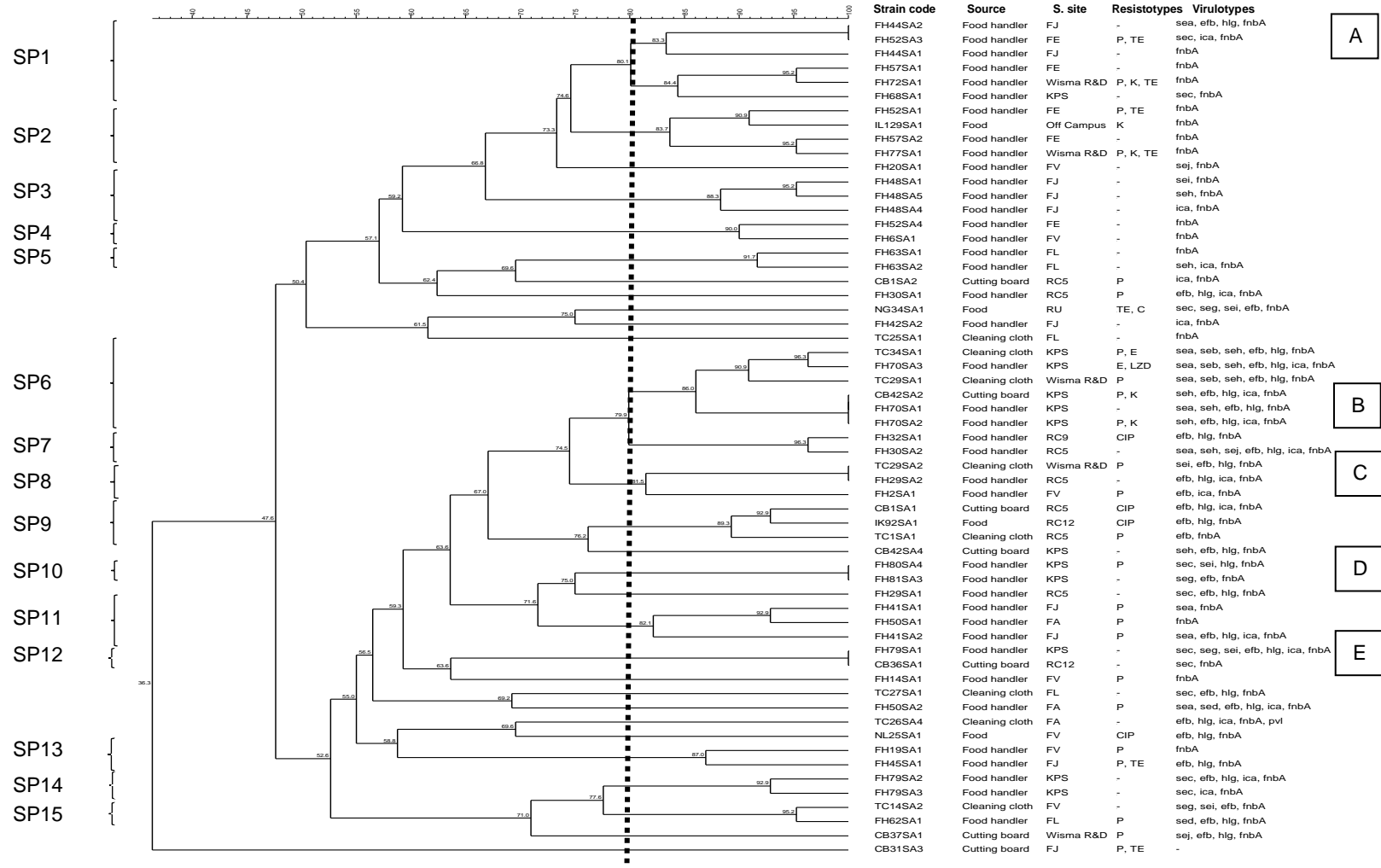


Figure 1: PFGE analysis of the *S. aureus* isolated from differences sources. A, B, C, D, and E represent the groups of *S. aureus* that share 100% similarity within the group.

About 41% of the *S. aureus* were resistant towards penicillin. A majority of the penicillin-resistant *S. aureus* were isolated from the food handlers. Almost 70% of the community-acquired *S. aureus* are penicillin resistant (Chambers, 2001) due to the *S. aureus* treatment introduced in mid-1940s for clinical practice (Appelbaum, 2007; Chambers and DeLeo, 2009). The extensive use of antibiotics has contributed to the exponential increase of multidrug-resistant (MDR) strains (Bogaard *et al.*, 2000), especially at the farms. A more worrying situation is that most of these antibiotics resistance genes are carried by mobile genetic elements. Thus, human skin inhabitants like *S. aureus* could be the potential reservoir for MDR strains.

Almost 50% of the *S. aureus* isolated harbored at least one SE gene, including two *S. aureus* isolated from food. Udo *et al.* (2006) reported a higher prevalence of SE genes, 71% in *S. aureus* isolated from food handlers' hands while Puah *et al.* (2016) reported 30.8% of the isolated *S. aureus* had at least one SE gene. In this study, 74.4% of the enterotoxigenic *S. aureus* carried only one SE gene. However, previous reports emphasized the co-existence of multiple SE genes in enterotoxigenic *S. aureus* (Holecková *et al.*, 2002; Cha *et al.*, 2006; Udo *et al.*, 2006). The enterotoxin gene, sea is recognised as the most common SE gene that causes staphylococcal food poisoning (Cha *et al.*, 2006; Argudin *et al.*, 2010; Ghaznavi-Rad *et al.*, 2010). It was the third most common SE gene (13.6 %) in this study. About 14% enterotoxigenic *S. aureus* carried seg which was the second prevalent SE after sec in this study. This is the only non-classical SE that is associated with staphylococcal food poisoning among the new types of SEs (Ikeda *et al.*, 2005; Argudin *et al.*, 2010). In this study, *S. aureus* isolated from food contained sec and seg. *Staphylococcus aureus* harbouring sea is not commonly isolated from food.

Apart from that, *fnbA* was detected in 96.3% of the strains (n = 78). This finding was further supported by Arciola *et al.* (2005) and Lim *et al.* (2012), in which *fnbA* was present in almost all human *S. aureus* strains. This gene is responsible for bacterial attachment to human epithelial cells. One *S. aureus* strain from TCC harboured *pvl* gene. In the study by Puah *et al.* (2016), *pvl*-positive *S. aureus* were isolated from both sushi and sashimi. Although the presence of *pvl* has always been associated with MRSA (Melles *et al.*, 2006), the *pvl*-positive *S. aureus* isolated in our study was not a MRSA. Aung *et al.* (2016) reported a higher occurrence of *pvl*-positive methicillin susceptible *S. aureus* from the asymptomatic food handlers in Myanmar. Since the *pvl* is encoded by bacteriophage, it could be easily spread to other strains (Jarraud *et al.*, 2002). Thus, it is relatively crucial to maintain good hygiene status, be it hands, food contact surfaces or the table cloths.

PFGE subtyping of *S. aureus* revealed that possible cross contamination could have occurred in these three scenarios: cleaning cloth and food handlers (group C); cutting board and food handler (group E); and among the food handlers (group A, B, and D) (Figure 1). This

scenario indicates poor food handling skill or poor sanitation practices among food handlers. Soon *et al.* (2011) reported that improper food handling accounted for 50% of the food poisoning episodes in Malaysia. This may be attributed to cross contamination and recontamination of RTE foods. Cross contamination will occur when proper sanitation is not followed especially during hand washing and the sanitization of food contact surfaces.

Furthermore, we observed the same group of food handlers working at several food premises in the University. These contract workers work at one food premise on one day and at another premise on another day. This could be the possible reason why certain strains from different cafeteria had 100% similarity in the PFGE profiles although the geographical locations of these strains are different (annotated with A, C and E in Figure 1).

In addition, it was noticed that several strains isolated from foods had > 80% similarity with *S. aureus* isolated from different non-food sources. The cutting board and table cleaning cloths could be a reservoir or transmission vehicle for *S. aureus* and eventually causes foodborne diseases. Inadequate sanitation could enhance dissemination of *S. aureus*.

CONCLUSION

In conclusion, the *S. aureus* contamination in RTE food was low. However, more than 10% of food contact surfaces and table cleaning cloth were contaminated with high microbial load of *S. aureus*. Thus, these fomites could be potential vehicles for transmission of *S. aureus*. A majority of *S. aureus* isolated carried more than one virulence gene. The use of disposable cleaning towel is encouraged. The poor microbiological quality of both food contact surfaces and table cleaning cloths necessitates close monitoring of the sanitation practices of food handlers.

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