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`Short communication

Bacterial content and characterization of antibiotic resistant *Staphylococcus aureus* in Danish sushi products and association with food inspector rankings

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ABSTRACT

This study examined the prevalence and phenotypic and genotypic antibiotic resistance patterns of *Staphylococcus aureus* in sushi from 20 Danish outlets. Microbial quality of sushi products and food inspector ranking of outlets were assessed and results for thirteen of the outlets were compared with findings from a previous study in 2012. Inspector rankings were similar in the two studies. The mesophilic aerobic counts were slightly lower ($p = 0.0296$) in 2017 than in 2012 with average values of the 13 shops of 5.2 log CFU/g and 5.7 log CFU/g, respectively. In both studies *E. coli* was only found in the products from outlets that did not have consistently superior rankings. On the other hand prevalence and average counts of *Staphylococcus* spp. were slightly higher in 2017 ($p = 0.0286$) but no methicillin-resistant *S. aureus* (MRSA) were observed in the present study. Methicillin-sensitive *S. aureus* (MSSA) were, however, isolated from 18.7% of sushi products with an average count below 2 log CFU/g. Based on *spa* and multilocus sequence typing (MLST), isolates belonged to clonal complex CC7 (t2016), CC20 (t7836), CC45 (t065, t127, t362), CC88 (t1998) and CC398 (t164, t331, t1451). The Panton-Valentine leukocidin (PVL)-encoding gene *lukF* was detected only in isolates of the t065 *spa*-type whereas the *scn* gene from the ΦSa3 prophage was detected in 76.5% of the isolates, supporting that the majority of isolates were of likely human origin. Thirty-six isolates (70.6%) were resistant to at least one of the antibiotic compounds tested. Antibiotic resistance genes that confer resistance to β-lactams (*blaZ*) and macrolides (*ermC*) were detected in 33.3% and 9.8% of isolates, respectively. The *tet*(K) gene that encode tetracycline resistance was only found in a t7836 strain. Overall, this study indicates that *S. aureus* in sushi products in Denmark do not represent a major food safety hazard due to, firstly, the low temperature and limited time of storage of product may prevent significant growth and production of toxic levels of enterotoxin of this species. Secondly, the *S. aureus* isolates obtained did not include MRSA variants and none of them encoded PVL that constitute one of the virulence factors in pathogenesis. Several MSSA isolates contained however genes encoding antibiotic resistance, which emphasize the potential role of foods as vehicles for transmission of such variants.

1. Introduction

Sushi is a traditional Japanese food, prepared from raw and cooked fish and soured rice. As this product is manually handled it may contain *Staphylococcus aureus* originating from the hands of workers with actual levels being determined by hygiene practices (Atanassova et al., 2008; Leisner et al., 2014). In recent years, there has been an increasing concern about antibiotic resistant bacteria, e.g. methicillin-resistant *S. aureus* (MRSA), in foods including ready-to-eat raw fish used in sushi. Several studies have described the presence of antibiotic resistant *S. aureus* in raw fish, including samples from Greece (Sengelidis et al., 2014), Japan (Hammad et al., 2012), Portugal (Moura et al., 2017) and

Switzerland (Boss et al., 2016) with detection of MRSA at most occasions. The presence of antibiotic resistant *S. aureus* in the food chain may present a health hazard and may also serve as an indication for how widely spread such variants are in the community.

Rankings of Danish food enterprises by public inspectors have taken the form of the so-called Smiley system since 2001 (http://www.findsmiley.dk; Leisner et al., 2014; Nielsen, 2006). This system include public records from unannounced visits by food inspectors conducting visual observations, measurements of temperatures in incubators and food products and inspection of data recorded under the shops own control programs. The public records include four levels of evaluations that result in different Smileys as follows: ☺ : the inspector

has no remarks; ☺: certain rules must be obeyed; ☺ an injunction or prohibitory order has been given; ☹: the restaurant or outlet has received an administrative fine, been reported to the police or approval has been withdraw. The public website (https://www.findsmiley.dk/English/Pages/FrontPage.aspx) of the National Food Agency lists the last four Smiley evaluations of a given food enterprise. Such rankings have in an American context been shown in some cases to indicate the risk for restaurant-associated food-borne outbreaks (Lee and Hedberg, 2016; Petran et al., 2012). Similarly, the rankings may in some but not all situations indicate the level of microbial quality (Kjeldgaard et al., 2010; Leisner et al., 2014). Previously the contents of total aerobic bacteria, *E. coli* and *Staphylococcus* spp. in Danish sushi products were compared with Smiley evaluation but in that study no *S. aureus* isolates were obtained for identification and determination of antibiotic resistant profiles (Leisner et al., 2014). The present study determined the prevalence, relatedness, antibiotic resistance and presence of virulence factors of *S. aureus* isolated from fresh sushi. The overall microbial quality of sushi products and food inspector rankings of outlets were also considered and assessed and for thirteen of the outlets compared with findings from the previous study in 2012 (Leisner et al., 2014).

2. Materials and methods

2.1. Evaluation of sushi restaurants with smiley rankings

A total of 20 sushi outlets with different Smiley rankings were examined between November and December 2017, thirteen of which were also included in a previous study conducted in 2012 (Leisner et al., 2014). The outlets were located in different geographical areas of Copenhagen, Denmark including the central business area ($n = 1$), Oesterbro ($n = 3$), Vesterbro ($n = 4$), Frederiksberg ($n = 9$), Noerrebro ($n = 1$) and Valby ($n = 2$). The outlets were divided into two groups with one group including the outlets with only positive Smileys in the last four rankings on display on the publicly accessible homepage (www.findsmiley.dk), and with the other group containing the outlets that received both negative and positive Smiley rankings among the last four rankings. Rankings were compared for the outlets visited both in 2012 and 2017 by averaging the last three rankings recorded for each outlet before microbiological sampling. In one case the 2012 average was based on two rankings as a third was not available. A positive ranking (happy Smiley) would give 4 points, a neutral ranking 3 points, a ranking recording minor non-compliances 2 points and a ranking recording major non-compliances (unhappy Smiley) 1 point. As an example, an outlet with two positive rankings and one neutral ranking would obtain an average of 3.67 points. Significance was determined through *t*-test and differences were considered significantly if $P < 0.05$.

2.2. Determination of bacterial counts

Packages of Maki salmon and Nigiri salmon were purchased on two separate occasions from each of the 20 outlets totaling 80 samples. Specifically, the mean weight of maki was 16 ± 0.6 g and consisted of roll ups with 5 ± 0.7 g raw salmon inside. Nigiri weighted 20 ± 1.8 g and consisted of rice balls with a top layer of 9.1 ± 0.1 g raw salmon. Products containing salmon were selected as this species is the most common raw fish used as an ingredient in Danish sushi. Samples were transported to the laboratory in ice bags within 1 h of purchase and processed immediately by homogenizing 10 g of products including rice and ingredients in 0.1% peptone saline (CM0982; Oxoid Ltd., United Kingdom) by use of a stomacher (Stomacher 400 Lab Blender, Seward Medical, London, United Kingdom) for 30 s. Numbers of mesophilic aerobic bacteria, *E. coli* and *Staphylococcus* spp. were determined after serial dilution in 0.1% peptone saline by pour plating (#3564024; Rapid *E. coli* 2 agar, Biorad) or surface plating on plate count agar (CM0325; PCA, Oxoid) or Baird Parker agar (CM0275; Oxoid),

respectively and incubated at 25 °C (PCA) or 37 °C (Rapid *E. coli* and Baird Parker agar) for three or two days, respectively.

2.3. Staphylococcus aureus isolation and identification

Ten grams of fresh sushi products were homogenized in 100 ml buffered peptone water (Merck, Darmstadt, Germany) and homogenized for 1 min by use of a Stomacher as described for the determination of overall bacterial counts. The direct inoculation and enrichment methods for detection of methicillin-sensitive *S. aureus* (MSSA) and MRSA were applied as previously described by Tang et al. (2017). Briefly, direct detection was performed by spreading 100 μl of sample broth onto SaSelect agar (Bio-Rad, Hercules, California, USA) and Brilliance MRSA 2 agar (PO1210; Oxoid) whereas the enrichment method was done by mixing 10 ml of sample broth with 90 ml Tryptone Soya Broth (CM0129; TSB, Oxoid) supplemented with 6.5% NaCl and incubated at 37 °C overnight. Thereafter, 10 μl aliquots of enriched broth were plated onto SaSelect agar and Brilliance MRSA 2 Agar. All plates were incubated at 37 °C for 18-24 h and suspected *S. aureus* colonies were transferred to Tryptone Soya Agar (CM0131; TSA, Oxoid) containing 5% bovine blood for 24 h at 37 °C and species identification was subsequently confirmed by MALDI-TOF MS (BioMérieux, France) analysis (Manukumar and Umesha, 2017; Seng et al., 2010).

2.4. Antimicrobial susceptibility testing for S. aureus

All *S. aureus* isolates were tested by disk diffusion assay for antibiotic susceptibility in accordance with the Clinical and Laboratory Standards Institute (CLSI) (2017) guidelines. A total of 17 antibiotics and synthetic antimicrobial compounds were used: ampicillin (10 μg), cefoxitin (30 μg), cefuroxime (30 μg), cephalothin (30 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), clindamycin (2 μg), erythromycin (15 μg), gentamicin (10 μg), kanamycin (30 μg), linezolid (30 μg), oxacillin (1 μg), penicillin (10 units), rifampicin (5 μg), sulphame-thoxazole (25 μg), tetracycline (30 μg), and trimethoprim (30 μg). Among these, cefoxitin and oxacillin were used to confirm potential methicillin-resistant strains, with *S. aureus* ATCC 25923 included as the control strain.

2.5. PCR and sequencing

All *S. aureus* isolates were screened by PCR for genes encoding penicillin (*blaZ*), methicillin (*mecA*) and aminoglycosides (*aadE* and *aacA-aphD*) (Clark et al., 1999; Leelaporn et al., 2008; Martineau et al., 2000).

Further, a total of five primer mixtures of multiplex PCRs for determining presence of genes encoding virulence and antibiotic resistance were also used (Suppl. Table 1). Primer mixture 1 included genes encoding protein A (*spa*), staphylococcal complement inhibitor (*scn*), Panton-Valentine leukocidin (*lukF*) (Tang et al., 2017). Primer mixture 2 included genes encoding resistance to macrolides and efflux pumps (*ermA*, *ermB*, *ermC*, *msrA/B*). Primer mixture 3 included genes encoding resistance to other macrolides (*ereA*, *ereB*, *mphA* and *mefA/E*) (Sutcliffe et al., 1996). If a strain was shown to contain an *erm* gene, it was subjected to a secondary PCR assay with individual primer set to resolve the determinant as *ermA*, *ermB*, or *ermC* (Suppl. Table 2). Primer mixture 4 included genes encoding resistance to vancomycin (*vanA*, *vanB*, *vanC1, C2, C3*) (Bell et al., 1998). Since no strain was shown to contain an *van* gene, it was subjected to a secondary PCR assay with individual primer set as *vanA*, *vanB*, or *vanC1, 2, 3* for confirmation (Suppl. Table 2). Primer mixture 5 included genes encoding resistance to tetracycline (*tet(L)*, *tet(K)* and *tet(W)*) (Gevers et al., 2003). Positive control strains obtained from Tang et al. (2017) were available for *ermA* (strain VRO2); *ermB* (strain VRO2); *ermC* (strain VSO1), *mrsA* (strain VR17), *tetL* (strain VRO8), *tetK* (strain VRO2), *blaZ* (strain VSO1) and *mecA* (strain RO2). Regarding primers set up without positive controls

and for confirmation of PCR results, isolates were subsequently whole genome sequenced and examined for presence of *ereA*, *ereB*, *mphA*, *mefA/E*, *aadE*, *aacA-aphD*, *vanA*, *vanB*, *vanC1*, *vanC2* or *vanC3* or *tet(W)* (unpublished data). Specifically, the database of those genes were downloaded from Center for Genomic Epidemiology (http://www.genomicepidemiology.org/) and BLAST searches of whole genome sequences were performed (https://blast.ncbi.nlm.nih.gov/Blast.cgi). All conditions and primers used in PCRs are shown in Suppl. Tables 1 and 2.

DNA from *spa* PCR products were purified by use of ExoSAP-IT (USB Corporation, USA), sequenced by Macrogen (http://dna.macrogen.com/eng/) and subsequently *spa*-typed using the Ridom StaphType software (www.spaserver.ridom.de). Ten strains representing all *spa*-types were selected for multilocus sequence typing (MLST). PCRs with seven pairs of primers were performed according to *S. aureus* MLST website (https://pubmlst.org/saureus/). Alleles and sequence types (STs) were obtained using the MLST database (http://saureus.mlst.net). Subsequently, eBURST v3 was used to allocate the STs to clonal complexes (CCs).

3. Results and discussion

Rankings of Danish food enterprises by public inspectors have since 2001 taken the form of the so-called Smiley system where the most recent hygiene inspection report must be visible to customers at the entrance to food enterprises (http://www.findsmiley.dk; Leisner et al., 2014; Nielsen, 2006). Such rankings may at some but not all instances indicate the risk for restaurant-associated food-borne outbreaks (Lee and Hedberg, 2016; Petran et al., 2012) and may in the case of sushi rankings not serve as an optimal indication of the microbial quality if based on aerobic mesophilic counts (Leisner et al., 2014).

Overall, the Smiley rankings for the 13 shops surveyed in 2012 (Leisner et al., 2014) and again in 2017 (this study) were nearly identical (difference not significant, $p = 0.337049$) with an average ranking score in 2017 of 3.79 as compared to 3.74 points in 2012. Six of the 13 outlets had a ranking average of the maximum value of 4.0 in 2012 whereas this was the case for 8 outlets in 2017. The overall total aerobic bacterial counts for the 13 shops were between 4.8 and 7.5 log CFU/g with an average of 5.7 log CFU/g in 2012 and in 2017 slightly less between 3.2 and 6.9 log CFU/g with an average of 5.2 log CFU/g ($p = 0.0296$).

Average total aerobic counts for all 20 shops analyzed in 2017 (this study) varied from 4.8 to 5.5 log CFU/g. Results were similar for shops that received only positive smileys in the last four visits before sampling (4.8 to 5.3 average log CFU/g) and for shops with a mixture of different smiley rankings (5.0 to 5.5 average log CFU/g; *p*-values of 0.5884 and 0.8245 for Maki and Nigiri products, respectively) (Table 1).

E. coli was only found among the outlets with mixed rankings, similar to what was observed in 2012 (Leisner et al., 2014). This result indicates that inspectors may notice shortcomings relevant to contamination with intestinal bacteria.

The average counts of *Staphylococcus* spp. in positive samples were between 3.3 and 3.8 log CFU/g in both ranking groups, slightly higher than 2.5–3 log CFU/g observed in 2012 (p-value 0.0286). *S. aureus* was in overall isolated from a minority of products with average counts below 2 log CFU/g in all cases (Table 1).

Staphylococcus spp. were present with a higher frequency in the present study than in 2012 (Leisner et al., 2014). The two ranking categories differed slightly regarding the frequencies of positive *S. aureus* samples. Thus, in the category with only positive smileys, 10% and 5% of Maki and Nigiri products, respectively, were positive for *S. aureus* whereas in the mixed smiley category this was the result for 15% and 7.5% of Maki and Nigiri products, respectively (Table 1).

S. aureus isolates with identical *spa*-types and isolated from the same sample were regarded as similar clones. The most common *spa* type among the outlets was t7836 (20%), followed by t1451 (15%), t065 (10%) and remaining types (each 5%). Only isolates with the t065 *spa*-type possessed the PVL gene (*lukF*), however the *scn* gene from the ΦSa3 prophage was detected in 76.5% of the isolates, supporting that the majority of isolates were of human origin (Ben Zakour et al., 2008; Price et al., 2012; Spoor et al., 2013). The *scn* gene was not found in isolates with the *spa* types t127, t164 and t362.

All 51 *S. aureus* isolates were screened for phenotypic antibiotic resistant profiles. Specifically, no isolates were determined as MRSA. Thirty-six isolates (70.6%) were, however, resistant to at least one of the antimicrobial compounds tested with 26 isolates resistant to penicillin, 21 isolates resistant to erythromycin, 16 isolates resistant to ampicillin and three isolates resistant to clindamycin and sulphamethoxazole. Kanamycin and tetracycline resistance were observed for 14 and 11 isolates. No isolates showed resistance to cefoxitin, cefuroxime, cephalothin, chloramphenicol, ciprofloxacin, gentamicin, linezolid, oxacillin, rifampin and trimethoprim. *S. aureus* isolates from Japanese sushi have been found to exhibit ciprofloxacin and gentamicin resistance; some were also resistant to oxacillin (MRSA) (Hammad et al., 2012). Moura et al. (2017) also reported a high frequency of antibiotic resistance including towards β-lactams, macrolides, and lincosamides for *S. aureus* isolates from Portuguese sashimi.

Among the 51 isolates tested by PCR 17 isolates were found to contain *blaZ*, a gene encoding beta-lactamase; the tetracycline resistance gene, *tetK*, was found in 11 isolates, and the erythromycin resistant gene, *ermC*, was found among t164, t127, t1451 and t7836 isolates ($n = 5$).

Ten strains representing the different *spa*-types were selected for MLST typing to identify the associated clonal complexes (CC) (Table 1). Strains belonging to CC398 can be either associated with livestock or with humans (Argudín et al., 2018; Tang et al., 2017). Human associated CC398 generally contain the *scn* gene (Price et al., 2012). This was also the case for the CC398 isolates in this study, which show that they most likely originate from human handling. CC7 is a common cause of bacteremia in Denmark (DANMAP, 2015). Similarly, CC45 represent the most prevalent MSSA lineage isolated from human nasal infections (DANMAP, 2015; Sarkar et al., 2016) and CC88 (including MRSA variants) has been isolated from humans (DANMAP, 2017, p. 123; references quoted in Monecke et al., 2016). The remaining CC20 and singletons do not indicate potential origin of the isolates.

In conclusion, this study gave results similar to the previous study regarding overall microbial quality of sushi and food inspector rankings of outlets in Copenhagen. However, fewer products purchased from outlets with only positive smiley rankings exceeded log 6 CFU/g, which has been suggested as a maximum value for satisfactory hygienic quality in sushi products according to Food Standards Australia New Zealand (FSANZ, 2016). Further, there was a correlation between rankings and the presence of *E. coli* similar to the previous study mentioned above (Leisner et al., 2014).

As expected, the majority of the *S. aureus* isolates belonged to human associated lineages. The examined products did not present major concerns regarding bacteriological safety in relation to *S. aureus* due to the low counts of this species which was much lower than the 100,000 to 1,000,000 CFU/g required for production of toxic levels of classical enterotoxin *SEA* to *SEE* (Food and Drug Administration [FDA], 2001; Schelin et al., 2011), absence of the *PVL* that constitute one of the virulence factors in pathogenesis, and the absence of MRSA. Several isolates showed, however, various phenotypic antibiotic resistant profiles that emphasize the potential role of foods as vehicles for transmission of such variants.

Table 1
Bacteriological contents of sushi and *S. aureus spa* type, CC lineage and antibiotic resistance patterns.

Smiley ranking	Product type	No. of samples[a]	Mean APC (log CFU/g)[b]	Mean *E. coli* (log CFU/g)[c]	Mean *Staphylococcus* spp. (log CFU/g)[c]	Mean *S. aureus* (log CFU/g)[d]	*spa* type	CC linage	Number of isolates	Antibiotic resistance patterns[e] Phenotype	Resistance genes
Only positive ☺	Maki	20 (10)	5.3 (3.6-6.8)	<1.0 (0%)	3.8 (27.5%)	<2.0 (10%)	t1451	CC398	8	PEN (5), ERY (6), AMP (1), KAN (2)	*blaZ* (1), *erm(C)* (1)
							t362	CC45	1	ND	ND
							t331	CC398	4	ERY (1), KAN (1)	ND
							t065	CC45	5	ND	ND
	Nigiri	20 (10)	4.8 (3.6-6.1)	<1.0 (0%)	3.5 (20%)	<2.0 (5%)	t236	Singleton	1	ERY (1)	ND
							t1451	CC398	7	PEN (1), ERY (7), AMP (3), DA (1)	*blaZ* (1)
Both positive and negative ☺☺ ☹☹	Maki	20 (10)	5.5 (3.2-6.9)	3.1 (12.5%)	3.7 (35%)	<2.0 (15%)	t164	CC398	3	PEN (3), ERY (3), KAN (1), AMP (3), RL (1)	*blaZ* (2), *erm(C)* (1)
							t127	CC45	1	PEN (1), ERY (1), AMP (1)	*blaZ* (1), *erm(C)* (1)
							t065	CC45	1	PEN (1), AMP (1)	*blaZ* (1)
							t7836	CC20	7	PEN (6), ERY (1), AMP (2), KAN (6), TET (7), DA (1)	*blaZ* (6), *tet(K)* (7), *erm(C)* (1)
							t2016	CC7	3	PEN (2), ERY (1), KAN (1)	ND
							t1998	CC88	1	ND	ND
	Nigiri	20 (10)	5.0 (3.3-6.7)	2.9 (7.5%)	3.3 (22.5%)	<2.0 (7.5%)	t7836	CC20	4	PEN (4), AMP (4), KAN (2), TET (4), RL (2)	*blaZ* (4), *tet(K)* (4), *erm(C)* (1)
							Not in database	ND	5	PEN (3), AMP (1), KAN (1), DA (1)	*blaZ* (1)

[a]Numbers in parentheses are numbers of outlets.

[b]Numbers in parentheses indicate the range.

[c]Numbers in parentheses are the percentage of positive samples for each product category (maki or nigiri).

[d]Numbers in parentheses are the percentage of positive samples for each product category (maki or nigiri). Positive samples were either found for enrichment cultures or if found by direct detection only in numbers < 2.0 log CFU/g.

[e]Numbers in parentheses indicate number of strains that possess the given phenotype or encode a specific gene.

Abbreviations: ERY, erythromycin; KAN, kanamycin; PEN, penicillin; AMP, ampicillin; TET, tetracycline; DA, clindamycin, RL, sulphame-thoxazole; ND: no detection. *blaZ, staphylococcal* beta-lactamase gene; *tet*(K), tetracycline K gene; *erm*(C): macrolide resistance gene; ND: no detection. APC: Aerobic Plate Count; *Spa*: *Staphylococcal* protein A; CC: Clonal Complex.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2019.108244.

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