STAPHYLOCOCCUS AUREUS FROM READY-TO-EAT FOOD AS A SOURCE OF MULTIPLE ANTIBIOTIC RESISTANCE GENES

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Abstract: The emergence of antibiotic-resistant strains of *S. aureus* such as methicillin-resistant *S. aureus* (MRSA) is a worldwide problem. Ready-to-eat (RTE) food which does not need thermal processing before consumption could be a vehicle for the spread of antibiotic-resistant microorganisms. The present study evaluated the molecular genetic characteristics (RAPD) and pheno- and genotypical antimicrobial resistance profile of *S. aureus* isolated from 75 RTE food samples (sushi, hamburgers, salads). All of the isolates (n=32) were resistant to at least one class of antibiotic tested of which 75% strains were classified as multidrug resistant. Most of the isolates were resistant to cefoxitin (87,5%) followed by clindamycin (78,1%), tigecycline and quinupristin/dalfopristin (53,1%). All methicillin resistant staphylococci harbored *mec*A gene. Among tetracycline resistance isolates all of them harbored at least one gene: *tet*(M), *tet*(L) and/or *tet*(K) and 78,9% of them were positive for the Tn*916*/Tn*1545*-like integrase family gene. Our results indicated that retail RTE food could be considered an important route for transmission of antibiotic resistant staphylococci harboring multiple antibiotic resistance genes.

Introduction

Staphylococcus aureus is one of the leading etiologic agents of hospital infections (Le Loir et al., 2003). It is known to cause a number of pathological conditions in humans and animals like bacteremia, urinary system infections, systemic diseases, osteomyelitis (Lina et al., 1999; Hageman et al., 2006). Toxins produced by *S. aureus* are one of the most frequent causes of bacterial food poisonings (Hennekinne et al., 2012). Foodstuff contamination may result from poor hygiene during production processes or the retail and storage of food. The pathogenic nature of *S. aureus* is related to the high genotypic and phenotypic heterogeneity of its strains. This results mainly from the abilities of staphylococci to exchange genetic material through mobile genetic elements.

Growing antibiotic resistance in *S. aureus* strains is a worldwide problem. The most worrying are strains resistant to methicillin (Methicillin-resistant *Staphylococcus aureus* - MRSA). The presence of antibiotic resistant strains among food derived microorganisms suggests that it may play a much more important role in transferring the antibiotic resistance encoding genes than previously thought (Chajęcka-Wierzchowska et al., 2016). Antibiotics are used for prophylactic purposes in swine, cattle, rabbit and poultry production as well as in vegetable and fruit cultivation and beekeeping (Ding and He, 2010) and, thus, the meat of farm animals, fruit, vegetables and water may be a source of resistant strains. Food and feed safety is essential, and the presence of MRSA in the food chain may contribute to the increasing dissemination of MRSA worldwide (Oniciuc et al., 2017). The multi-drug resistant *S. aureus* strains may have an increased ability to spread. This does not only provide therapeutic challenges for clinicians but may be very detrimental to human health. The study was designed to determine the occurrence of *S. aureus* and MRSA strains in ready-to-eat food products as sushi, salads, and hamburgers in Poland. Moreover, the presence of genes which encode resistance to methicillin and tetracycline's was investigated.

Materials and methods

2.1. Isolation and biochemical identification of the strains

Staphylococci were isolated from samples obtained from sushi, hamburgers and salads purchases in fast-food bars and restaurants. Totally, 75 ready-to-eat samples were collected. These consisted of 25 sushi samples, 25 hamburgers and 25 salads. Samples were immediately transported to the laboratory under refrigerated conditions. All microbiological analyses were performed within the same day. Food samples (10 g) were homogenized in 90 ml buffered peptone water (Merck, Germany), incubated overnight at 37°C and streaked on selective plates containing Mannitol Salt Phenol-red Agar (Merck,

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Germany). Mannitol (+) colonies were differentiated into coagulase-positive and coagulase-negative with a test detecting the production of a clumping factor (CF) and production of coagulase on the RPF medium (Biomerieux) with rabbit blood plasma and fibrinogen. Isolates were Gram-stained using standard techniques. Gram positive, coagulase positive bacteria were presumptively identified as staphylococci and were selected for further biochemical identification. Prior to analysis, isolates were stored in Microbank at -80°C (Biocorp, Poland).

2.2. Bacterial strains and DNA extraction

For DNA extraction bacterial strains were cultivated on Brain Heart Infusion Broth with agar (BHI, Merck, Germany) at 37°C. Colony were suspended in TE buffer and lysed by Lizostaphin enzyme (0,6mg/ml). Total Genomic DNA of isolated and reference strains was extracted using the Genomic Mini DNA Purification Kit (A&A Biotechnology) according to the manufacturer's instructions.

2.3. Molecular identification of isolates to the genus level

RAPD-PCR reactions were performed with primer M13 according to protocol published by Andrighetto et al., (1996). For the identification of isolates to the genus level, part of the 16S rRNA *Staphylococcus* gene was amplified according to Morot-Bizot et al. (2004) Amplification was carried out in a Kyratec Super Cycler Trinity Triple Zone (Nippon Genetics Europe) PCR reaction mixture were analyzed by electrophoresis through a 2% high resolution agarose gel (Promega) in 1 x TBE buffer pH 8,3. The sizes of the amplification products were estimated by comparison with a 100-bp molecular size ladder (Thermo Scientific, Fermentas). Gels were stained with ethidium bromide. Images of the gels were visualized using the system for the documentation and analysis of fluorescently stained gels G-BOX F3 (Syngene) and analyzed using the program Gene Tools (Syngene). Each profile was visually compared with those obtained from the *S. aureus* ATCC® 25923 reference strain.

2.4. Antimicrobial susceptibility test

Antimicrobial susceptibility testing was performed on Mueller-Hinton agar (Biomérieux) using the disk diffusion method. Isolates were tested for their susceptibility to erythromycin (E, 15μg), clindamycin (DA, 2μg), gentamicin (CN, 120μg), cefoxitin (FOX, 30 μg), norfloxacin (NOR, 10μg), ciprofloxacin (CIP, 5μg), tetracycline (TE, 30μg), rifampicin (RD, 5μg), nitrofurantoin (F, 300μg), linezolid (LZD, 30μg), chloramphenicol (C, 30μg), trimethoprim (W, 5μg), and tigecycline (TGC, 1μg), trimethoprim/sulfamethoxazole (SXT, 25μg), quinupristin/dalfopristin (QDA,15μg) using antimicrobial disks (Oxoid). Plates were incubated at 37 °C for 20–24 h and results were interpreted according to the Clinical and Laboratory Standard Institute document M100-S20.

Table 1. Oligonucleotides used in PCR reactions.

Primer	Primer sequence (5'→ 3')	Amplicon size (bp)	Source
Staphylococcus spp.	TIACCATTTCAGTACCTTCTGGTAA	370	Morot-Bizot, 2005
	GGCCGTGTTGAACGTGGTCAAATCA		
S. aureus	CGTAATGAGATTTCAGTAGATAATACAACA	107	
	AATCTTTGTCGGTACACGATATTCTTCACG		
mecA	AAAATCGATGGTAAAGGTTGGC	533	Barski, 1996
	AGTTCTGGCACTACCGGATTTGC		
tet(L)	TGGTGGAATGATAGCCCATT	229	Rizzotti, 2005
	CAGGAATGACAGCACGCTAA		
tet(M)	GTGGACAAAGGTACAACGAG	406	
	CGGTAAAGT TCGTCACACAC		
tet(K)	TTATGGTGGTTGTAGCTAGAAA	348	Gevers, 2003
	AAAGGGGTTAGAAACTCTTGAAA		
int (Tn*916*/Tn*1545*)	GCGTGATTGTATCTCACT	1046	Macovei and Zurek, 2006
	GACGCTCCTGTTGCTTCT		

Source: Authors

2.5. PCR detection of antimicrobial resistance genes

All the strains phenotypically resistant to at least one antibiotic were examined for the presence of the resistance genes. Polymerase chain reaction for detection of genes *mec*A (533 bp) were carried out (Table 1). Amplification cycles for this two genes were done according to Barski et al. (1996). The

tetracycline efflux gene *tet*(K) amplification was performed according to Gevers et al. (2003). PCR detection of resistance to tetracyclines - *tet*(M), *tet*(L) was determined using the specific primers and the conditions reported by Rizzotti et al. (2005). For all the *tet*(M)-positive isolates the presence of conjugative transposons of the Tn*916*-Tn*1545* family was determined by using primers targeting the integrase gene *int* according to Macovei and Zurek (2006).

The amplicons were evaluated by 1,5% agarose gel electrophoresis followed by staining with ethidium bromide (0.5mg/mL). Images of the gels were visualized using the system for the documentation and analysis of fluorescently stained gels G-BOX F3 (Syngene) and analyzed using the program Gene Tools (Syngene).

Results and discussion

The present study evaluated the molecular genetic characteristics (RAPD) and pheno- and genotypical antimicrobial resistance profile of 32 *S. aureus* strains isolated from 75 RTE food samples (sushi, hamburgers, salads) (Figure 1).

Figure 1. Dendrogram derived from the RAPD-PCR profiles generated with primers M13.
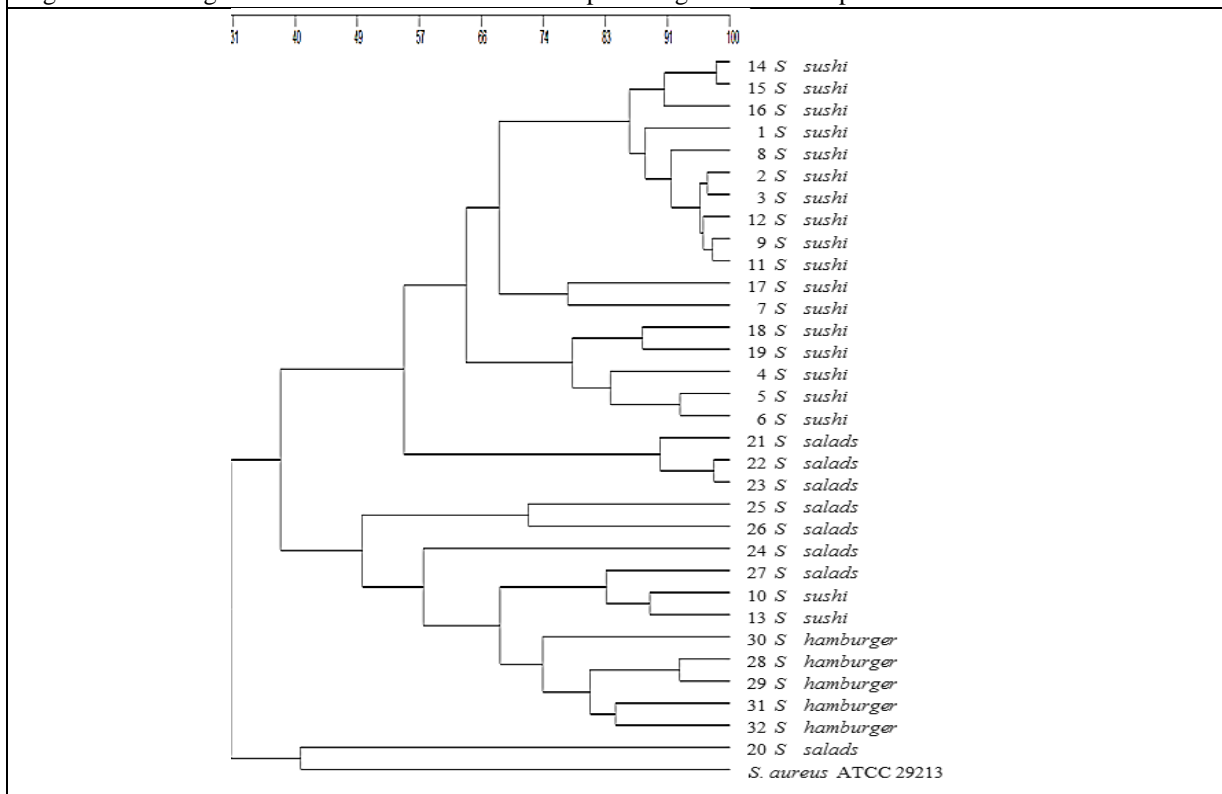
Source: Authors

All staphylococcal isolates were examined for their susceptibility to 15 antibiotics. The data obtained from the disc diffusion testing are summarized in Table 2. The overall percentages of antimicrobial resistant isolates were: 87,5% to cefoxitin, 78,1% to clindamycin, 53,1% to tigecycline and quinupristin/dalfopristin, 46,9% to tetracycline, 31,2% to rifampicin and gentamycin; 12,5% to trimetoprim/sulfametoxasol and nitrofurantoin; 9,4% to erythromycin and trimethoprim and 6,3% to ciprofloxacin and linezolid. All of the investigated strains (n=32) were resistant to at least one class of antibiotic of which 24 strains were classified as multidrug resistant –MDR (resistant to three or more classes of antibiotics). Most MDR strains (n=11) revealed simultaneous resistance to 4 classes of antibiotics, followed by 5 strains to 6 classes; 4 strains to 5 classes; 3 strains to 3 classes. One of the examined strains revealed simultaneous resistance to antibiotics of 7 various classes (Table 2). All MDR strains (n=24;100%) were resistant to cefoxitin (FOX) and the majority of them (n=20; 83,3%) were resistant to clindamycin (DA), 16 MDR strains (66,7%) were resistant to quinupristin/dalfopristin, 14 of them (58,3%) to tigecycline and 13 MRD strains (54,1%) were resistant to tetracycline (Table 2).

Table 2. Phenotypic and genotypic characterization of the *S. aureus* strains

Strain number	Source	Antibiotic resistance genes				Integrase	Antibiotic resistance phenotype
1 *S. aureus*	*sushi*	-	-	-	-	-	E, DA
2 *S. aureus*	*sushi*	*mec*(A)	*tet*(L)	*tet*(M)	-	*int*	FOX, TE, E
3 *S. aureus*	*sushi*	*mec*(A)	*tet*(L)	*tet*(M)	-	*int*	FOX, TGC, TE
4 *S. aureus*	*sushi*	*mec*(A)	-	-	-	-	TGC, QD
5 *S. aureus*	*sushi*	*mec*(A)	*tet*(L)	*tet*(M)	-	-	DA, FOX, TE, QD
6 *S. aureus*	*sushi*	*mec*(A)	-	*tet*(M)	-	*int*	DA, FOX, TE, CN, QD
7 *S. aureus*	*sushi*	*mec*(A)	-	-	-	-	DA, FOX, RD, QD
8 *S. aureus*	*sushi*	-	-	-	-	-	DA
9 *S. aureus*	*sushi*	-	*tet*(L)	*tet*(M)	-	*int*	DA, FOX, TGC, TE, RD, CN, QD
10 *S. aureus*	*sushi*	*mec*(A)	-	*tet*(M)	-	-	DA, FOX, TGC, SXT, RD, QD
11 *S. aureus*	*sushi*	*mec*(A)	-	*tet*(M)	-	*int*	DA, FOX, TGC, TE, CN, QD
12 *S. aureus*	*sushi*	*mec*(A)	*tet*(L)	*tet*(M)	-	*int*	DA, FOX, TGC, TE, RD, QD
13 *S. aureus*	*sushi*	*mec*(A)	-	-	-	-	DA, FOX, RD, QD
14 *S. aureus*	*sushi*	*mec*(A)	*tet*(L)	*tet*(M)	-	*int*	DA, FOX, TGC, CIP, RD, CN, QD
15 *S. aureus*	*sushi*	*mec*(A)	*tet*(L)	*tet*(M)	*tet*(K)	*int*	DA, FOX, TGC, TE, RD, CN, QD
16 *S. aureus*	*sushi*	*mec*(A)	*tet*(L)	*tet*(M)	-	-	DA, FOX, TGC, TE, RD, CN, QD
17 *S. aureus*	*sushi*	*mec*(A)	-	-	-	-	DA, FOX, TGC, TE, QD
18 *S. aureus*	*sushi*	*mec*(A)	*tet*(L)	*tet*(M)	*tet*(K)	*int*	DA, FOX, W, TE, SXT, CN, QD
19 *S. aureus*	*sushi*	*mec*(A)	*tet*(L)	*tet*(M)	*tet*(K)	*int*	DA, FOX, TGC, TE, QD
20 *S. aureus*	*salads*	*mec*(A)	*tet*(L)	*tet*(M)	-	-	FOX
21 *S. aureus*	*salads*	*mec*(A)	*tet*(L)	*tet*(M)	-	-	DA, FOX, TGC, CIP
22 *S. aureus*	*salads*	*mec*(A)	*tet*(L)	*tet*(M)	-	*int*	DA, FOX, TGC, TE,QD
23 *S. aureus*	*salads*	*mec*(A)	*tet*(L)	*tet*(M)	-	*int*	FOX, F, W, TGC, TE, SXT
24 *S. aureus*	*salads*	*mec*(A)	-	-	-	-	FOX, F, CN
25 *S. aureus*	*salads*	*mec*(A)	-	-	-	-	DA, FOX, F, CN
26 *S. aureus*	*salads*	*mec*(A)	-	-	-	-	DA, FOX, F, TGC
27 *S. aureus*	*salads*	*mec*(A)	*tet*(L)	*tet*(M)	-	*int*	DA, FOX, LZD, QD
28 *S. aureus*	*hamburger*	*mec*(A)	-	-	-	-	DA, FOX
29 *S. aureus*	*hamburger*	*mec*(A)	-	-	-	-	DA, FOX
30 *S. aureus*	*hamburger*	*mec*(A)	-	-	-	-	E, DA
31 *S. aureus*	*hamburger*	*mec*(A)	-	-	-	-	FOX, W, TGC, SXT, RD
32 *S. aureus*	*hamburger*	*mec*(A)	*tet*(L)	*tet*(M)	-	*int*	DA, FOX, TGC,TE, RD, CN, LZD

Abbreviations: E - erythromycin, DA - clindamycin, CN - gentamicin, FOX -cefoxitin, TE - tetracycline, TGC - tigecycline, RD - rifampicin, F - nitrofurantoin, LZD - linezolid, W - trimethoprim, QD - quinupristin/dalfopristin, SXT - trimethoprim/sulfametoxasol

Source: Authors

Genotyping analysis indicated that all of the isolates phenotypic resistant to tetracycline harbored at least one tetracycline resistance determinant on which *tet*(M) was most frequent. The *tet*(L) genes did also appear but were less common. Gene *tet*(K) was always associated with *tet*(M) and *tet*(L). All of the isolates positive for *tet*(M) genes were positive for the Tn*916*/Tn*1545*-like integrase family gene. All of the cefoxitin resistant strains harbored *mec*A gene. Most MRSA strains were resistant to at least three of the antibiotics tested, and 25 of 28 MRSA strains were classified as MDR.

In other recently reported studies, lower isolation frequencies for MRSA in foods were found. In an Italian survey of 160 *S. aureus* strains analyzed six strains (3.75%) harbored the *mec*A gene. In Japan, Hammad et al. (2012) examined 200 samples of retail ready-to-eat raw fish (sashimi) of which they recovered 10 MRSA/MR-CoNS isolated from 10 different samples (5%, 10/200). In our previous study we have examined 858 samples of RTE food (including cheeses, cured meats, sausages, smoked fishes) on which 6% of isolated strains were positive for *mec*A (35 MRSA and 17 MR-CoNS strains) (Chajęcka-Wierzchowska et al., 2014). Like as in these studies all MRSA strains carry *mec*A gene, in contrast to results published by other authors (Hammad et al., 2012; Lee et al., 2004)

Most of the studies on antibiotic resistance in *S. aureus* have concentrated on strains isolated from clinical samples. Recently, some researchers have suggested that environment and food could play a significant role in the transmission of resistance to humans (Wang et al., 2012; Chajęcka-

Wierzchowska et al., 2014, 2015). Furthermore, Gram-positive bacteria acquire and transfer resistance to antibiotics much more often and more easily than Gram-negative bacteria. The results obtained indicate the need for food monitoring for the presence of antibiotic-resistant *S. aureus* and the possibility of transferring and transmitting antibiotic resistance genes. *S. aureus* strains present in ready-to-eat food can pose a hazard for consumer health serving as a reservoir of resistance genes.

Conclusions

Our data indicate that *S. aureus* is widely present in retail sushi, but also hamburgers and salads. Many isolated strains are antibiotic resistant and carry transferable genes which represent a potential source of resistance transmission to bacteria in humans. The most disturbing fact is that most *S. aureus* strains were resistant to methicillin and had *mec*A gene. Our results indicated that retail RTE food could be considered as important route for transmission of antibiotic resistant staphylococci harboring multiple antibiotic resistance genes.

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