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VIRULENCE AND RESISTANCE PROFILE OF *STAPHYLOCOCCUS AUREUS* ISOLATED FROM FOOD

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Staphylococcus aureus is considered a global community and health care pathogen responsible for staphylococcal food poisoning. The aim of this study was to characterize several isolates of *S. aureus* recovered from different food products concerning enterotoxin genes and other virulence factors including antimicrobial resistance. In 2009, a total of 78 coagulase-positive staphylococci from 1454 food samples were identified to species level; 73 were confirmed as *S. aureus*. Of the *S. aureus* isolates 5.5% were resistant to oxacillin, 52.0% showed resistance to erythromycin, and 45.2% to tetracycline. Multidrug resistance was observed in 33.3% of the isolates (resistance to three or more antibiotics of different classes). SCC*mec* types IV and V were detected among methicillin-resistant *S. aureus* (MRSA). One MRSA isolate was *pvl* positive. The 52.0% of food isolates were shown to be enterotoxigenic; *egc* (63.0%), *secbov* (44.7%) were the main detected SEs. *tst* gene was also detected in food isolates. The present work demonstrates the presence of virulent *S. aureus* collected in 2009 in foods.

Keywords: *Staphylococcus aureus* food isolates, MRSA, SEs, antibiotic resistance

Staphylococcus aureus is an extraordinarily versatile pathogen responsible for staphylococcal food poisoning, hospital- and community-acquired infections as well as for the toxic shock syndrome (Song et al., 2015).

Staphylococcus aureus can be present in different foods such as raw milk and dairy products (Jamali et al., 2015), fishery products (Vázquez-Sánchez et al., 2012), meat products (Bortolaia et al., 2016) among others. Staphylococcal food poisoning has been reported worldwide and is associated with oral intake of enterotoxins present in foods (Johler et al., 2015). Staphylococcal toxins were responsible for 7.5% of the total foodborne outbreaks reported to EFSA in 2014 (EFSA-ECDC, 2015a).

Staphylococcal enterotoxins are represented by a group of thermostable gastrointestinal protease-tolerant single chain exoproteins; at least 23 different SEs/SEls have been reported (Grumann et al., 2014). Another toxin, TSST-1, the toxic shock staphylococcal toxin lacks emetic activity and is known to be responsible for toxic shock syndrome (Otto, 2014). Other virulence factors, such as the presence of PVL (Panton–Valentine Leukocidin) and hemolysin-α, exfoliative toxins, thermonuclease, hyaluronidase, and lipases, are involved in tissue invasion of the host cells by *S. aureus* (Grumann et al., 2014).

Antibiotics are widely used not only in human but also in animal husbandry and other agricultural activities (Kluytmans, 2010). The occurrence of multi-resistant strains in foods has been increasing; contaminated food is considered an important vehicle for *S. aureus*

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antimicrobial resistance (EFSA-ECDC, 2015b). On the other hand, methicillin-resistant *S. aureus* (MRSA) strains are emerging in foods (Parisi et al., 2016).

The purpose of this study was to characterize *S. aureus* strains previously collected from several food products regarding their resistance to antibiotics and virulence factors.

1. Materials and methods

During 2009, seventy-eight presumptive colonies of coagulase-positive staphylococci were collected in routine analysis from several food companies (1454 food samples) in a microbiological lab (CINATE, Porto). Confirmation and presumptive identification of *S. aureus* were performed by Gram-staining, presence of catalase and coagulase, growth on Mannitol Salt Agar (Pronadisa, Spain). DNAse activity on DNase agar (Pronadisa) and thermostable DNAse activity were also detected according to Castro and co-workers (2016). Presumptive *S. aureus* isolates were thereafter stored in cryovials at –80 ºC in Tryptone Soy Broth (TSB, Pronadisa) plus 30% (v/v) of glycerol for further characterisation. All assays were performed as previously presented by Castro and co-workers (2016) including the identification to species level, namely multiplex PCR with 16S rRNA and *nuc* and the detection of MRSA strains with *mecA* gene. Antibiotic susceptibility testing and detection of enterotoxin genes were determined by agar dilution and PCR, respectively. Detection of Panton–Valentine leucocidin genes was performed only to MRSA strains by PCR. Finally, *SCCmec* typing of MRSA was performed as described by Boye and co-workers (2007). Control strains for five types of SCC*mec* were kindly supplied by Prof. Keiichi Hiramatsu (Juntendo University, Tokyo, Japan: Type I (NCTC 10442), Type II (N315), Type III (85/2082), Type IV (JCSC 4744) and Type V (Wis)).

2. Results and discussion

2.1. S. aureus *isolates and mecA gene*

Among the 1454 food samples, seventy-eight samples were positive for the presence of presumptive colonies of coagulase-positive staphylococci. Of those, 73 isolates were confirmed to be *S. aureus* (*nuc*+ and 16S rRNA+ detected simultaneously). *S. aureus* isolates were recovered from raw meat (n=3), raw fish (n=10), fermented and cured meat products (n=5), cheese (n=8), milk (n=14), pastry (n=7), bakery (n=2), seafood (n=3), ready-to-eat (n=19), and vegetables (n=2). *S. aureus* has already been isolated in similar products, namely: meat (Hadjirin et al., 2015), milk and raw-milk products (Jamali et al., 2015; Carfora et al., 2016), fish products (Vázquez-Sánchez et al., 2012), retail food products (Wang et al., 2014) and ready-to-eat food (Li et al., 2015). Of the food isolates 5.5% (4/73) were classified as MRSA as the gene *mecA* was detected; one strain recovered from a fermented meat product, two from ready-to-eat and one from a pastry product. The occurrence of MRSA strains in food samples varies between the food product and the place of isolation. Recently, Jamali and co-workers (2015) detected 16.2% of MRSA strains in milk and dairy products in Iran. In Italy, Carfora and co-workers (2016) demonstrated that 5.6% of MRSA strains were present in food from retail meat (pork and beef) in the USA. Of MRSA strains 1.4% were detected in ready-to-eat foods (Wang et al., 2014). No MRSA strains were reported on

powdered infant formula in China (WANG et al., 2014) and fishery products in Spain (VÁZQUEZ-SÁNCHEZ et al., 2012).

2.2. Antimicrobial resistance

High resistance to antibiotics was detected among the *S. aureus* food isolates: 83.6%, 90.4%, 52.0%, and 45.2% with respect to penicillin, ampicillin, erythromycin, and tetracycline (Table 1). All isolates were sensitive to vancomycin, gentamicin, and nitrofurantoin. It is globally accepted that antibiotic resistance in food isolates is due to the widespread usage of antibiotics (KLUYTMANS, 2010).

Table 1. Antimicrobial susceptibility of *S. aureus* isolates

Antibiotic	Sensitive	Intermediate	Resistant
	N (%)	N (%)	N (%)
Penicillin	12 (16.4)	*	61 (83.6)
Ampicillin	7 (9.6)	*	66 (90.4)
Oxacillin	69 (94.5)	*	4 (5.5)
Chloramphenicol	21 (28.8)	51 (69.8)	1 (1.4)
Ciprofloxacin	58 (79.5)	11 (15.0)	4 (5.5)
Erythromycin	25 (34.2)	10 (13.7)	38 (52.0)
Gentamicin	73 (100)	0 (0.0)	0 (0.0)
Rifampin	71 (97.3)	0 (0.0)	2 (2.7)
Vancomycin	73 (100)	0 (0.0)	0 (0.0)
Nitrofurantoin	73 (100)	0 (0.0)	0 (0.0)
Tetracycline	36 (49.3)	4 (5.5)	33 (45.2)

N: number of isolates; *: Antibiotic with no described value for the intermediate MIC

Of the *S. aureus* isolates resistant to oxacillin (5.5%), only one was *mecA* positive (isolate 709) showing a MIC of 32 μg ml^{-1}. The other three MRSA strains did not show oxacillin resistance beside the presence of gene *mecA*. Three other isolates (*mecA* negative strains) showed to be borderline oxacillin-resistant *S. aureus* strains (MICs varying from 1 to 8 μg ml^{-1}). These kinds of isolates have already been detected in foods (BYSTRÓN et al., 2010). Of *S. aureus* isolates 33.3% were multi-resistant (resistant to ≥3 antibiotics of different classes); 30.1% (22/73) and 3.2% (2/73) were resistant to three and four antibiotics, respectively. Among food isolates only one strain was susceptible to all the eleven antibiotics investigated (data not shown).

2.3. MRSA characterization

The detection of MRSA is based usually (with exception of *mecC*) on the presence of *mecA* gene in *S. aureus*. All MRSA were resistant to beta-lactams as an inherent characteristic due to presence of *mecA* gene (Table 2). The location of *mecA* gene is within the large chromosomal element known as the SCC*mec* (WATKINS et al., 2012). Only isolate 704 was nontypeable by SCC*mec* typing. The other MRSA isolates presented SCC*mec* types IV and V (Table 2).

Generally, these SCC*mec* types are the most prevalent types in food; MRSA isolates SCC*mec* type IV were found in hamburgers in Spain (ARGUDÍN et al., 2012) and type V in raw meats in the UK (HADJIRIN et al., 2015).

Table 2. MRSA strains collected from food samples

Isolate	Origin	Resistance profile	SEs genes	SCC*mec* type	*pvl*
301	Fermented meat product	Pen, Amp, Eri	*secbov*	IV	+
528	Ready-to-eat	Pen, Amp, Eri	*secbov*	V	–
704	Pastry	Pen, Amp	*seg, sei*	–	–
709	Ready-to-eat	Pen, Amp, Eri, Tetra, Oxa	–	V	–

Pen: Penicillin; Amp: Ampicillin; Eri: Erythromycin; Tetra: Tetracycline; Oxa: Oxacillin

2.4. Virulence factors

2.4.1. Enterotoxin genes detection. Staphylococcal food poisoning (SFP) results from the consumption of foods containing sufficient amounts of (one or more) enterotoxins (SE). Not all strains of *S. aureus* were enterotoxigenic; 52.0% presented at least one of the tested enterotoxin (38/73) and 13 SEs/TSST genes arrangements were found among food isolates 52.6% (20/38) that carried three or more SEs/*tst* genes (Table 3). Of methicillin-sensitive *S. aureus* (MSSA) 90.6% presented more than one SE gene (data not shown). The percentage of MSSA isolates harbouring two, three, four, and five SEs/*tst* genes were 34.4%, 12.5%, 31.3%, and 9.4%, respectively (data not shown). Moreover, one MSSA isolated from milk presented seven SEs genes. In contrast, MRSA strains showed lower presence of enterotoxin genes (Table 2). In agreement with previous findings (ARGUDÍN et al. 2012), *egc* was the most prevalent (63.0%). *secbov* was the most prevalent classical enterotoxin gene (44.7%). An outbreak due to *egc* has already been described (JOHLER et al., 2015). *tst* gene, the marker for TSST-1 (Staphylococcal Toxic Shock Syndrome) was detected (associated with others SEs) in 21.0% of the isolates. Similar results were obtained by ARGUDÍN and co-workers (2012) and ALIBAYOV and co-workers (2014), namely 25.8 and 30%, respectively. Absence of *tst* genes has already been reported by PU and co-workers (2011) for retail meats in the USA. Toxic shock syndrome toxin (TSST) is responsible for TSS, characterized by high fever, rash, desquamation, vomiting, diarrhoea, and hypotension, frequently resulting in multiple organ failure (GRUMANN et al., 2014). SEs, as superantigens (SAgs), selectively activate a vast number of T cells and interfere with intestine function and typically cause emesis and diarrhoea (OTTO, 2014). SEs possess extraordinary stability to denaturing conditions, such as heat and low pH, and resistance to most proteolytic enzymes, such as pepsin or trypsin (GRUMANN et al., 2014).

2.4.2. PVL genes detection and haemolysins, Another virulence determinant, PVL-encoding genes (*lukS-PV* and *lukF-PV*), was investigated only in the case of MRSA strains. PVL-like staphylococcal enterotoxins are *S. aureus* virulence determinants that are widely distributed in many European countries, and it is possible to recover *S. aureus* isolates with PVL from clinical, animal, and food sources (VERKADE & KLUYTMANS, 2014; HU et al., 2015). PVL positive MRSA isolates had been detected in some animals (pigs, poultry, cattle;

VERKADE & KLUYTMANS, 2014) and in foods (i.e. in meat in the United States and in raw and processed food commodities in Shanghai: HANSON and co-workers (2011) and SONG and co-workers (2015) respectively). The data in the literature concerning food isolates and pvl is scarce. Most of the studies concerning this virulence factor were performed with clinical isolates. In the present study, one MRSA isolated from a Portuguese traditional fermented meat product was detected. To author´s knowledge there are no reports concerning the detection of *pvl* genes in *S. aureus* collected from food products in Portugal. PVL is a cytotoxin that causes leukocyte destruction and tissue necrosis (WATKINS et al., 2012) associated with severe skin and soft tissue infection and necrotizing pneumonia (LINA et al., 1999). The presence of *pvl* genes represents an increment of virulence of MRSA food isolates.

Table 3. Distribution of enterotoxins among *Staphylococcus aureus*

Enterotoxin genes profile	N (%)
Secbov	5 (13.2)
seg, sei	9 (23.7)
secbov, seg, sei	4 (10.5)
secbov, seg, sei, tst	3 (7.9)
secbov, tst	4 (10.5)
seh, seg, sei	3 (7.9)
seh, sea, seg, sei	4 (10.5)
sea, sej, seb, seg, sei	1 (2.6)
sej, seb, sed, seg, sei	1 (2.6)
sec, sej, sed, seg, sei	1 (2.6)
seb, sed, seg, sei	1 (2.6)
secbov, seh, sea, sej, sed, seg, sei	1 (2.6)
sec, seg, sei, tst	1 (2.6)

N: number of isolates

Hla (α-haemolysin) is probably the best-known toxin of *S. aureus* with pore-forming and pro-inflammatory properties (OTTO, 2014). It is lytic to red blood cells and a series of leukocytes, but not neutrophils (OTTO, 2014). In the present study, Hla was present in 6.9% of *S. aureus* isolates. Hlg (ɣ-haemolysins) contrary to PVL is inflammatory but not necrotic in the rabbit skin model and is produced by more than 99% of *S. aureus* clinical strains (LINA et al., 1999). In contrast to our results (presence in 17.8% of *S. aureus* isolates) on retail foods in China none of *S. aureus* isolates had *hlg* gene (LI et al. 2015).

3. Conclusions

S. aureus is routinely detected and/or enumerated in a wide variety of ready-to-eat foods as part of preventive approach and microbiological safety checks based on hazard analysis and critical control point principles. In the present study, the characterization of *S. aureus* isolated

from food samples was evaluated. Globally, it was demonstrated that food might be an important source of dissemination of antibiotic resistant and virulent strains of *S. aureus*. Since the isolates presented here are from the year 2009, it would be interesting to collect and study more recent isolates to determine whether the occurrence of MRSA and the pathogenicity of *S. aureus* vary from year-to-year.

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