

Antimicrobial Resistance and Presence of Enterotoxins in *Staphylococcus* spp. Isolated from Cooked Meat and Desserts

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Abstract The aim of this study was to isolate *Staphylococcus* spp. from cooked meat and desserts from restaurants and test for the presence of enterotoxins A, B, C, D, E genes and antimicrobial resistance profile. Thirty-six samples of cooked meat and thirty-six samples of desserts were collected from nine restaurants from Pelotas, Brazil. *Staphylococcus* spp. isolates were tested for antimicrobial susceptibility profile by disc diffusion method. Multiplex Polymerase Chain Reaction was performed to identify the presence of enterotoxins. Seven out of the 38 isolates registered the presence of any gene of traditional enterotoxins and enterotoxin E is the most frequent, with 42.8%, and five different genotypes were reported. *Staphylococcus* spp. isolates registered a high percentage of antimicrobial resistance to penicillin and oxacillin.

Keywords: multiplex polymerase chain reaction, food safety, enterotoxins, restaurants, antimicrobial susceptibility

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1. Introduction

People's food habits are changing and have lunch outside the home has become a practical and fast alternative [1]. However, meals may also disseminate pathogens which may be passed to food during processing, especially due to inadequate handling. And foodborne diseases (FB) may be a consequence of contamination, characterizing safe food as challenging [2].

Coagulase-positive *Staphylococci* (CoPS) is the pathogenic bacteria mainly associated to desserts and cooked meat, and have been extant for more than ten years among the most frequently identified FB agent in Brazil [3]. CoPS may occur in food due to inadequate handling, and above high concentration (over 10⁵ CFU/g), they may produce enterotoxins and cause FB outbreaks [4]. The CoPS *Staphylococcus aureus* is the second most important pathogen involved in FB outbreaks in Brazil [3].

However, coagulase-negative *Staphylococci* (CoNS) are frequently isolated in food and may also produce enterotoxins [3,5,6]. When food is insufficiently cooked, the proliferation of microorganism and the production of *Staphylococcus* enterotoxins (SEs) may occur. The enterotoxin (SE) genes are encoded in mobile genetic elements, such as plasmids, prophages and *Staphylococcus* pathogenic islands, and

these mobile genetic elements are responsible for the horizontal transfer of virulence or antibiotic resistance genes between strains [7]. Molecular analysis techniques are widely employed to identify and verify the expression of SE genes [8], and multiplex Polymerase Chain Reaction (mPCR) may be underscored for SEs analysis since it amplifies simultaneously several genes.

Besides that, *Staphylococcus* spp. may be resistant to several classes of antimicrobial agents and may cause numberless infections, and normally it is resistant to β -lactam antimicrobial agents, such as penicillin and oxacillin [9,10]. *Staphylococcus* infections have been on the increase during the last few years and, consequently, the number of multi-resistant strains, making difficult and prolonging the treatment of such infections [11,12].

The aim of this study was isolate *Staphylococcus* spp. from cooked meat and desserts from restaurants and test for the presence of SE (A, B, C, D, E) genes and antimicrobial resistance profile.

2. Material and Methods

2.1. Isolation of *Staphylococcus* spp.

Thirty-six samples of cooked meat and 36 samples of desserts from restaurants of Pelotas city, Brazil were

analyzed for the county of *Staphylococcus* spp. Samples were collected as if one was actually buying the ready food which was placed in disposable thermal packing, at the restaurant counter. The package was duly closed and taken to the Food Microbiology Laboratory at School of Nutrition at the Federal University of Pelotas for analysis.

For the isolation of *Staphylococcus* spp., samples were plated on Agar Baird Parker (BP, Merck®), enriched with egg yolk emulsion and potassium tellurite 1% by surface spreading, and incubated at 37°C for 48h. Further, presumed typical and atypical colonies were counted and given as Colony-Forming Units per gram of food (CFU/g). Three typical and three atypical colonies were isolated and Gram-stained e confirmed colonies were tested for coagulase [13].

2.2. Resistance to Antimicrobial Factors

Staphylococcus spp. isolates were verified for resistance to antimicrobial agents by the disc diffusion technique, as recommended by the Clinical and Laboratory Standards Institute [14]. The isolates were first streaked in BHI broth (Merck®) at 36°C and inoculated in a saline solution 0.85% (Merck®) till 0.5 of Mac Farland scale. The culture was then spread on plates with Muller Hinton Agar (MH, Merck®) where the discs of antimicrobial agents for Gram-positive bacteria (Invitrogen®) were placed and incubated at 37°C for 24h. Zone of inhibition were measured and compared by CLSI resistance standard table [14]. The tested twelve antimicrobial agents that formed the antimicrobial disc were ampicillin (10µg), penicillin (10 units), oxacillin (1µg), clindamycin (2µg), sulfamethoxazole/trimethoprim (23.75/1.25µg), chloramphenicol (30µg), erythromycin (15µg), gentamicin (10µg), tetracycline (30µg), vancomycin (30µg), ciprofloxacin (5µg), cefepime (30µg) and rifampicin (5µg).

2.3. Verification of Enterotoxins *Staphylococcus* Genes

DNA chromosome was extracted from *Staphylococcus* spp. isolates with commercial kit (PureLink Genomic DNA – K1820-01, Invitrogen®). Extracted DNA underwent mPCR technique to verify the presence of SEs A, B, C, D and E genes. Primers to identify SEs genes were described by Mehrotra et al. [15] (Table 1).

Moreover, 1µl of each primer GSEAR-1/ GSEAR-2, GSECR-1/ GSECR-2, GSEDR-1/ GSEDR-2 was used in the first mPCR block; 2.5µl buffer solution for PCR (10x); 1.5µl magnesium chloride (MgCl₂) (5U/µl); 0.5µl of mix dNTPs (100nM), 0.3µl of Taq DNA polymerase (500U) and 1µl of DNA in 25µl of final volume. The second reaction block comprised the same reagents but primers were GSEBR-1/ GSEBR-2, GSEER-1/ GSEER-2. Further, mPCRs were done in thermal cycler (MJ Research, PTC-100, Peltier Thermal Cycler) at the following conditions: 95°C for 5min; 30 cycles (95°C – 30’’; 55°C – 1’ and 72°C – 1’) and final extension at 72°C for 7min.

All mPCR products were analyzed by agar gel electrophoresis 1.5% (Invitrogen) stained with ethyl bromide (Promega®), seen without transilluminator, and photographed (Kodak Digital Science TM DC120). A 100pb DNA Ladder (Ludwig Biotec®) was the molecular mass marker. Negative control of reactions had the same compositions as mPCR, although sterile Milli-Q-waterreplaced DNA. DNAs of *S. aureus* ATCCs 13565 (EEA), 14458 (EEB), 19095 (EEC), 23235 (EED) and 21664 (EEE) were used as positive controls.

3. Results and discussion

Table 2 shows results for the quantification of *Staphylococcus* spp. in cooked meat samples and dessert samples.

Of the seventy-two samples of cooked meat and desserts, five (6.9%) were contaminated with CoPS, but within standard limits permitted by Brazilian sanitary law, up to 10³ CFU/g [16], and CoNS counts were isolated in 33 samples (45.8%).

A study in Porto Alegre, Brazil evaluated 26 samples from several types of food, which included cooked meat and desserts, and reported 15 samples contaminated with CoNS and none with CoPS. [17] Other study evaluated 82 samples of desserts from university cafeterias in Greece, with 76.8% (n=63) of samples contaminated by *Staphylococcus* spp. [18].

Brazilian sanitary law only concentrates on the investigation of CoPS in food due to their pathogenicity [16], but CoNS may also produce enterotoxins. Seven out of the 38 isolates registered the presence of any gene of traditional SEs and SEE is the most frequent, with 42.8%. Five different genotypes were reported (Table 3).

Table 1. Primers used to detect gene of *Staphylococcus* spp. enterotoxins

Gene	Primer	oligonucleotide sequence (5' → 3')	Amplification product (bp)
EEA	GSEAR-1	GGTTATCAATGTGCGGGTGG	102
	GSEAR-2	CGGCACCTTTTTCTCTTCGG	
EEB	GSEBR-1	GTATGGTGGTGTAAGTACTGAGC	164
	GSEBR-2	CCAAATAGTGACGAGTTAGG	
EEC	GSECR-1	AGATGAAGTAGTTGATGTGTATGG	451
	GSECR-2	CACACTTTTAGAATCAACCG	
EED	GSEDR-1	CCAATAATAGGAGAAAATAAAAAG	278
	GSEDR-2	ATTGGTATTTTTTTTCGTC	
EEE	GSEER-1	AGGTTTTTTCACAGGTCATCC	209
	GSEER-2	CTTTTTTTCTTCGGTCAATC	

Table 2. Quantification of *Staphylococcus* spp., in cooked meat and in desserts

Bacteria	Cooked meat n (%)	Desserts n (%)	Total
Total	36 (100)	36 (100)	72 (100)
CoPS (CFU/g)	1 (2.8)	4 (11.1)	5 (6.9)
CoNS (CFU/g)	15 (41.6)	18 (50.0)	33 (45.8)

CoPS: coagulase-positive *Staphylococcus* CoNS: coagulase-negative *Staphylococcus* CFU/g: Colony- Forming Units per gram of food.

Table 3. Genotype of *Staphylococcus* spp. isolates from food with regard gene of traditional enterotoxins.

Genotype	Source (number of isolates)	Coagulase	SE	%
1	Dessert (1)/ cooked meat (1)	-	SEE	25.0
2	Dessert (1)	+	SEE	12.5
3	Dessert (1)	-	SEB	25.0
4	Dessert (1)	-	SED	12.5
5	Dessert (2)	-	SEC	25.0

Legend: - negative coagulase; + positive coagulase; SEB, SEC, SED and SEE, gene of *Staphylococcus* enterotoxin B, C, D and E; %, percentage.

Moreover, 85.7% of the 7 positive isolates of SE genes were CoNS. Only one of the five CoPS isolates, from a dessert sample, had SE genes (SE E). SEE is not the most frequent SE in food or in outbreaks [19,20]. Xing *et al.* [21] evaluated 128 *S. aureus* isolates from ready-to-eat food and reported SED in 28.5% of food, whereas SEE represented only 2.3% (n=3). Mello *et al.* [17] evaluated isolates of *Staphylococcus* spp. in 26 samples of ready-to-eat food and registered SEB in 42.1% and SE only associated with another SE (SEB and SED).

SEA seems to be the most frequent gene in food and the most common in food *Staphylococcus* intoxication. However, SE E gene has already been registered in food intoxication [4,22]. Ostyn *et al.* [23] reported the first food intoxication by SEE in France due to common cheese made from non-pasteurized milk. Cheese analysis registered *S. aureus* counts of < 10² UFC/g and quantification of 0.45 ng of SEE. SEE is 79 – 90% structurally similar to SEA and may be potentially pathogenic. Further, the thermo-stability of SE provides selective resistance [24]. Consequently, processes in food production that reduce vegetative cells are effective for food safety.

Table 4. Results of resistance test to antimicrobial agents of *Staphylococcus* spp. isolated from cooked meat and desserts

Antimicrobial agents	Sensitive (%)	Intermediate (%)	Resistant (%)
Clindamycin	94.7	0	5.3
Vancomycin	94.7	0	5.3
Erythromycin	84.2	5.3	10.5
Chloramphenicol	100	0	0
Rifampicin	94.8	2.6	2.6
Cefepime	94.7	0	5.3
Oxacillin	65.8	0	34.2
Penicillin	60.5	0	39.5
Ciprofloxacin	97.4	0	2.6
Gentamycin	100	0	0
Tetracyclin	100	0	0
Sulfamethoxazole/Trimethoprim	100	0	0

All *Staphylococcus* spp. isolates (n=38) were tested for antimicrobial resistance. They are greatly resistant to penicillin (39.5%) and oxacillin (34.2%), intermediate resistance to erythromycin (5.3%) and rifampicin (2.6%). Further, all isolates were sensitive to the antimicrobial agents chloramphenicol, gentamicin, tetracycline and sulfamethoxazole/trimethoprim and 5.3% (n=2) of isolates were multi-resistant (Table 4).

Further, 68.7% and 66.6% of *Staphylococcus* spp. isolates respectively from meat (n=16) and dessert (n=22) were resistant to at least one antimicrobial agent. Only 12% (n=25) of all isolates resistant to any antimicrobial agent were CoPS. One isolate from a dessert (CoNS) was resistant to six antimicrobial agents (clindamycin, vancomycin, erythromycin, chloramphenicol, rifampicin, cefepime, oxacillin and penicillin).

Similar studies evaluating the antimicrobial resistance of *Staphylococcus* spp. isolates registered 53.8% (n=80) which were resistant to penicillin [25]; 23.1% (n=78) to oxacillin [26] and approximately 100% (n=16) sensitive to chloramphenicol, gentamycin, tetracycline and sulfamethoxazole/trimethoprim [27] Penicillin and oxacillin intervene in the synthesis and remodeling of bacterial peptidoglycans. Penicillin-binding proteins (PBPs) have a low affinity for antimicrobial agents. When they replace other PBPs, the survival of bacteria occurs in high concentrations. Although penicillin was the first antibiotic to combat *S. aureus*-caused infections, its excessive and inadequate use favored the bacterium's resistance capacity to the antimicrobial agents [26,28,29].

4. Conclusion

Staphylococcus spp. isolates registered a high percentage of antimicrobial resistance to penicillin and oxacillin. SE E gene was the most frequent among *Staphylococcus* spp. isolates. Results demonstrate the importance of quality procedures in food production because they control important pathogens and, consequently, the enterotoxins in food, mainly in eat-to-eat products produced by restaurants.

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