

Research Paper

Sanitary quality, occurrence and identification of *Staphylococcus* sp. in food services

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Abstract

Sanitary conditions are essential for the production of meals and control of the presence of pathogens important to guarantee the health of customers. The aim of this study was to evaluate the sanitary quality of food services by checking the presence of thermotolerant coliforms, *Staphylococcus* sp. and evaluate the toxigenic potential from the latter. The analysis was performed on water, surfaces, equipment, ready-to-eat foods, hands and nasal cavity of handlers in seven food services. The water used in food services proved to be suitable for the production of meals. Most food, equipment and surfaces showed poor sanitary conditions due to the presence of thermotolerant coliforms (60.6%). Twenty-six *Staphylococcus* species were identified from the 121 *Staphylococcus* isolates tested. *Staphylococci* coagulase-negative species were predominant in the foods, equipment and surfaces. In food handlers and foods, the predominant species was *Staphylococcus epidermidis*. Twelve different genotypes were found after PCR for the classical enterotoxin genes. The *seb* gene (19.8%) was the most prevalent among all *Staphylococcus* sp. Both coagulase-positive and coagulase-negative *Staphylococci* showed some of the genes of the enterotoxins tested. We conclude that there are hygienic and sanitary deficiencies in the food services analyzed. Although coagulase-positive *Staphylococci* have not been present in foods there is a wide dispersion of enterotoxigenic coagulase-negative *Staphylococci* in the environment and in the foods analyzed, indicating a risk to consumer health.

Key words: food service, sanitary quality, *Staphylococcus* sp., Staphylococcal enterotoxin.

Introduction

The major objectives of food services are the production and distribution of foods with nutritional and sanitary quality. To achieve this quality, the World Health Organization recommends the adoption of good hygienic practices (Sambrook and Russel, 2001; WHO, 2012). Most outbreaks are caused by the ingestion of contaminated food after inadequate hygiene practices, production, storage and/or distribution (Losasso *et al.*, 2012). The food services

occupy the second position as a source of food intoxications/infections in Brazil, with *Salmonella* sp., *Staphylococcus aureus* and *Escherichia coli* being the main causative agents (USFDA, 2004; Brasil, 2011).

Outbreaks of *Staphylococcus* sp. are related to the production of one or more enterotoxins (SE), and SEA, SEB, SEC, SED and SEE are together responsible for 95% of the cases (Aragon *et al.*, 2007; Pereira *et al.*, 2009). Among these, SEB is the SE which has the highest thermo-

stability and toxicity (Balaban and Rasooly, 2000). Although *Staphylococcus aureus* is the most evident species in food-borne outbreaks, coagulase-negative *Staphylococci* (CoNS) can also be producers of SE (André *et al.*, 2008; Veras *et al.*, 2008; Rall *et al.*, 2010a; Oliveira *et al.*, 2011).

Knowing and controlling the factors that can lead to the contamination of foods produced in a food service can minimize risks to the health of customers. This study aimed to evaluate the sanitary quality of food services, verify the presence of thermotolerant coliforms, *Staphylococcus* sp. and assessing the toxigenic potential from the latter.

Materials and Methods

Food services and samples

This study was conducted in seven large-scale food services (500 or more meals per day) and active in the city of Porto Alegre/Rio Grande do Sul - Brazil. Analyses were performed for thermotolerant coliforms and *Staphylococcus* sp. in: (I) ready-to-eat foods (raw salad, processed salad produced by processing or cooking, hot meal and dessert - total of 26 samples); (II) equipment (refrigerator, cutting board, gastronomical tank, blender, cutter and vegetable processor - total of 33 samples); and (III) surfaces (stainless steel bench - total of 7 samples). Before distribution of lunch in each food service, we collected aseptically 25 g of each food in sterile plastic bag and stored under refrigeration until the time of analysis. Samples of equipment and surfaces were collected by swab smearing (50 cm²), moistened in saline (0.85%). At the time of sampling, all points had been cleaned/sanitized in accordance with the parameters of each food service. In the absence of any of these items, the collection point was deleted. Material from the hands and nasal cavities of 21 food handlers (3 handlers from each food service) was collected using a swab that was moistened with saline (0.85%), transported in Stuart medium (Laborclin) and used for *Staphylococcus* sp. enumeration. We analyzed the drinkability of the water used for the sanitization of salads. This research was conducted according to ethical principles and was approved by the Ethics Council from the Federal University of Rio Grande do Sul (Brasil, 1996).

Microbiological analyses

The dilution and homogenization of the samples as well as the analysis and identification of thermotolerant coliforms and *Staphylococcus* sp. were performed according to the Food and Drug Administration guidelines (USFDA, 2012). The species of *Staphylococcus* isolates were identified by Gram staining, catalase testing, coagulase testing, growth on mannitol salt agar, anaerobic growth on mannitol, hemolysis, pigment, Voges-Proskauer test, nitrate, fermentation of maltose and mannitol, urease, oxidase, growth at 15 °C, 45 °C and in the presence of 15% NaCl (Cohen, 1968; Macfaddin, 2000). Water (100 mL)

was analyzed by cultivation in Hicoliforme broth (Himedia), with prior inactivation of chlorine by adding sodium thiosulfate (10%). All isolates were maintained in brain heart infusion broth (Himedia) with 25% glycerol and stored at -20 °C.

Parameters for the microbiological analysis

The results of the analyses were compared to the criteria described in the Technical Regulation on Microbiological Standards for Foods of the National Agency for Sanitary Vigilance Committee (Brasil, 2001). The study considered item 22, specific to ready-to-eat meals, produced by food services or similar, where the presence of coagulase-positive *Staphylococci* (CoPS) is considered non-compliant. The evaluation of water quality was guided by Ordinance No. 2914/11 of the Brazilian Ministry of Health that determines the absence of thermotolerant coliforms and *E. coli* in 100 mL of water (Brasil, 2011). For the analysis of surfaces and equipment, the standards of the Pan American Health Organization (PAHO) were considered, which state that up to 49 colony-forming units per square centimeter is considered as a regular hygiene condition (Moreno, 1982).

Detection of enterotoxin genes in the *Staphylococcus* sp. strains

All strains of *Staphylococcus* sp. were subjected to polymerase chain reaction (PCR) for the presence of genes encoding the classical SE (*sea*, *seb*, *sec*, *sed*, and *see*). PCR reactions were performed in a final volume of 25 µL: 1.5 mM MgCl₂, 0.2 mM each dNTP, 0.2 mM of each primer, 1 U Taq polymerase (Promega), 10 ng of DNA in a thermocycler Master Cycler Personal. The reaction was incubated for: 5 min at 94 °C followed by 30 cycles of 1 min at 94 °C, 1 min at the annealing temperature according to Table 1, 1 min at 72 °C and a final cycle of 5 min at 72 °C. To confirm the absence of inhibitors in the PCR reaction, all negative reactions for some SE genes were subjected to a new PCR reaction with primers detecting the 16S rRNA-prokaryote gene (8f and 925r) described by Liu *et al.* (1997). The PCR products were visualized as described elsewhere (Sambrook and Russel, 2001). Sterile ultrapure water (Milli-Q) was used instead of DNA as a negative control. *S. aureus* ATCCs 13565 (*sea*), 14458 (*seb*), 19095 (*sec*), 23235 (*sed*) and 21664 (*see*) were used as positive controls.

Results

Water analysis

The water samples showed no thermotolerant coliforms or *E. coli*. The seven food services evaluated in this study used only water that was treated and distributed by the Municipal Water and Sewer Systems of Porto Alegre.

Table 1 - Nucleotide sequences, annealing temperature and expected size of the PCR products for *Staphylococci* enterotoxins.

Gene	Primer	Nucleotide sequence (5'-3')*	Annealing temperature (°C)	Amplicon (bp)
<i>sea</i>	<i>sea</i> ₁	GGTTATCAATGTGCGGGTGG	56	102
	<i>sea</i> ₂	CGGCACTTTTTTCTCTTCGG		
<i>seb</i>	<i>seb</i> ₁	GTATGGTGGTGTAAGTACTGAGC	54	164
	<i>seb</i> ₂	CCAAATAGTGACGAGTTAGG		
<i>sec</i>	<i>sec</i> ₁	AGATGAAGTAGTTGATGTGTATGG	58.5	451
	<i>sec</i> ₂	CACACTTTTAGAATCAACCG		
<i>sed</i>	<i>sed</i> ₁	CCAATAATAGGAGAAAATAAAAAG	49	278
	<i>sed</i> ₂	ATTGGTATTTTTTTTCGTTC		
<i>see</i>	<i>see</i> ₁	AGGTTTTTTCACAGGTCATCC	53	209
	<i>see</i> ₂	CTTTTTTTTCTTCGGTCAATC		

Legend: 1, primer forward; 2, primer reverse; bp, base pairs; °C, Celsius degrees; *, nucleotide sequences described by Mehrotra *et al.* (2000).

Analysis of thermotolerant coliforms and *Staphylococcus* sp.

In an overall evaluation of foods, equipment and surfaces, it was observed that 60.6% of the points analyzed showed thermotolerant coliforms counts above the recommended (Table 2).

All foods analyzed showed minimum number of CoPs, and these parameters are within of the standards set by Brazilian legislation. Of the 108 sampling points analyzed, 121 strains were distributed between 26 different species of *Staphylococcus* sp. (15 strains from foods, 36 from equipment/surfaces, 37 and 33 from hands and nasal cavities of handlers, respectively). Among these strains, 105 were CoNS and 16 were CoPS. All of the CoPS were isolated from food handlers. All species isolated from foods (Table 3) were identified as CoNS. *S. epidermidis* was the most common species in foods, although it was not found in any of the equipment or surfaces analyzed. Thirty-six CoNS were isolated from equipment and surfaces, and 13

different species were identified. The species that was most frequently isolated from hands (9 of 37 isolates) and from the nasal cavities (14 of 33) was also *S. epidermidis*. Among all of the isolates from hands, only four were CoPS, represented by *Staphylococcus hyicus* (1 strain), *S. lutrae* (1 strain) and *S. schleiferi* (2 strains). Among the *Staphylococcus* sp. isolated from the nasal cavities, 17 were CoPS, and the second most common species was *S. schleiferi* (10 out of 33 isolates). Among all handlers analyzed, nine were carriers of *Staphylococcus* sp. on their hands, and six harbored two or three different species. While twelve of the handlers showed *Staphylococcus* sp. in the nasal cavity, only three handlers were carriers of two different species in the same sampling.

Analysis of Staphylococcal enterotoxin

The gene *seb* was the most prevalent, either alone (19.8%) or concomitant with other toxin(s) gene(s) (Table 4).

Table 2 - Compliance of food services regarding the microbiological limits for thermotolerant coliforms.

FS	Salad		Hot meal	Dessert	S. bench	V. cutter	Blender	G.T.	C. board	V. processor	Refrig.
	A	B									
1	n	+	-	+	-	-	-	n	-	-	-
2	+	+	+	-	-	-	-	n	-	-	+
3	+	+	+	+	-	n	-	+	-	+	-
4	-	-	+	-	-	-	n	-	-	n	+
5	+	-	+	n	-	n	n	+	+	-	+
6	-	+	-	+	+	+	n	-	-	-	-
7	+	+	-	-	-	-	-	n	-	-	+

Legend: FS, food service; Salad A, raw salad; Salad B, processed salad; S. bench, stainless steel bench; V. cutter, vegetable cutter; G.T., gastronomic tank; C. board, cutting board; V. processor, vegetable processor; Refrig., refrigerator; n, inexistent equipment or food in food service; +, compliant; -, non-compliant.

Table 3 - *Staphylococcus* sp. isolated from foods, equipment, surfaces and food handlers of food services.

Species	Coag.1	Source (n. of isolates)	n. of isolates
<i>S. arlatae</i>	-	Food (3); Food handler - hand (2)	5
<i>S. aureus</i>	+	Food handler - nose (1)	1
<i>S. auricularis</i>	-	Food (1); Food handler - hand (2), nose (1)	4
<i>S. capitis</i>	-	Equipment (1)	1
<i>S. caprae</i>	-	Food handler - hand (5)	5
<i>S. carnosus</i>	-	Food (1); Equipment (1); Food handler - hand (1)	3
<i>S. caseolyticus</i>	-	Equipment/surface (4)	4
<i>S. chromogenes</i>	-	Food (1); Equipment (2)	3
<i>S. cohnii</i> sub. <i>urealyticus</i>	-	Food handler - hand (1)	1
<i>S. epidermidis</i>	-	Food (5); Food handler - hand (9), nose (14)	28
<i>S. equorum</i> sub. <i>equorum</i>	-	Equipment (1)	1
<i>S. gallinarum</i>	-	Food (1); Food handler - hand (1)	2
<i>S. haemolyticus</i>	-	Food (1); Equipment/surface (5)	6
<i>S. hominis</i>	-	Equipment (5); Food handler - hand (4)	9
<i>S. hyicus</i>	-	Food handler - hand (1), nose (4)	5
<i>S. hyicus-chromogenes</i>	-	Food handler - hand (1)	1
<i>S. intermedius</i>	+	Food handler - nose (2)	2
<i>S. lutrae</i>	+	Food handler - hand (1)	1
<i>S. pasteurii</i>	-	Food (1); Equipment (1); Food handler - hand (2), nose (1)	5
<i>S. saccharolyticus</i>	-	Equipment (5)	5
<i>S. saprophyticus</i>	-	Equipment/ surface (3); Food handler - hand (4)	7
<i>S. schleiferi</i>	+	Food handler - hand (2), nose (10)	12
<i>S. simiae</i>	-	Food handler - hand (1)	1
<i>S. simulans</i>	-	Food (1); Equipment (4)	5
<i>S. vitulinus</i>	-	Equipment (3)	3
<i>S. warneri</i>	-	Equipment (1)	1
Total			121

Legend: 1, coagulase; -, coagulase-negative *Staphylococci*; +, coagulase-positive *Staphylococci*; n., number.

The gene encoding *seb* was commonly found in both CoPS (29.2%) and CoNS (70.8%). This study revealed twelve different genotypes, consisting of four genotypes with single genes (*sea*, *seb*, *sec* e *sed*), six genotypes with combinations of two genes (*sea+seb*; *sea+sec*; *seb+sec*; *seb+sed*; *seb+see*; *sed+see*) and two genotypes with three genes (*sea+seb+sed*; *seb+sec+sed*). The enterotoxin E gene was present only in combination with another enterotoxin, rather than forming an individual genotype. Among the 121 *Staphylococcus* sp. tested in this study, 57 (47.1%) were positive for any of the classical SE genes. Eighteen of the 26 species identified were positive for any of the SE genes. The species *S. aureus* (1), *S. capitis* (1), *S. cohnii* subspecies *urealyticus* (1), *S. equorum* subspecies *equorum* (1), *S. lutrae* (1), *S. intermedius* (2), *S. simiae* (1) and *S. vitulinus*

(3) were negative for the presence of the genes tested. The genotype *sea+seb* are present in two strains of *Staphylococcus* sp. isolated from foods (Table 5). In contrast, among isolates of equipment, surfaces and handlers, the *seb* genotype was predominant.

Discussion

As no thermotolerant coliforms were found after the microbiological analysis of the water used by the food services, it was classified as satisfactory. This is a very important point since it can act as a vehicle for the transmission of pathogens and spoilage agents (WHO, 2005). Foods that presented results from non-compliance due to high count thermotolerant coliforms were those who had added some

Table 4 - Genotypic profile of the enterotoxins genes in *Staphylococcus* sp. isolates from the food services.

Genotype	Number of isolates positive for genotypes (%)
<i>sea</i>	4 (7.0%)
<i>seb</i>	24 (42.1%)
<i>sec</i>	1 (1.7%)
<i>sed</i>	6 (10.5%)
<i>sea+seb</i>	5 (8.8%)
<i>sea+sec</i>	2 (3.5%)
<i>seb+sec</i>	5 (8.8%)
<i>seb+sed</i>	4 (7.0%)
<i>seb+see</i>	1 (1.7%)
<i>sed+see</i>	1 (1.7%)
<i>sea+seb+sed</i>	1 (1.7%)
<i>seb+sec+sed</i>	3 (5.3%)
Total	57 (100%)

Legend: (%) percentage; *sea*, *seb*, *sec*, *sed*, *see*, gene of *Staphylococcus* enterotoxin A, B, C, D and E, respectively.

ingredients after cooking, suggesting post-processing contamination. This suggests a failure in the use of good hygienic practices and in the quality control of raw materials. The high percentage of poor sanitary conditions of the surfaces and equipment of food services is alarming. It is known that equipment and countertops can provide conditions for the growth of microorganisms on their surfaces forming biofilms, as well as the possibility for further cross

contamination (Legnani *et al.*, 2004; Meira *et al.*, 2012; Trinetta *et al.*, 2012). In addition, the cross-contamination, post-processing, could be the explanation for at least 30% of the food analyzed in this study having levels of thermotolerant coliforms above the acceptable levels.

Considering only the analysis of *Staphylococcus* sp. and the Brazilian legislation, the results of this study showed that all foods produced by food services were in agreement with that legislation (Brasil, 2011). This legislation determines only the investigation of CoPS in foods because they are producers of toxins that are of considerable risk for human health. CoNS possessing SE genes have been isolated from foods by other authors, although they have not yet been identified as causative agents of food poisoning (Rode *et al.*, 2007; Zell *et al.*, 2008). So far, in Brazil, studies searching for the presence of *Staphylococcus* sp. in foods produced by food services were not described. However, some studies showed the presence of *Staphylococcus* sp. in many foods (Aragon *et al.*, 2007; André *et al.*, 2008; Rall *et al.*, 2008, 2010a; Borelli *et al.*, 2011; Oliveira *et al.*, 2011).

Equipment and surfaces can accommodate a diverse microbiota. In the food services this can be influenced by contact with food (raw or processed), food handlers and by the processes of cleaning. Equipment and poorly sanitized environments may contain organic matter, which, combined with convenient extrinsic factors, can provide favorable conditions for the growth of microorganisms (Rode *et al.*, 2007). Also, the contaminated equipment could be the

Table 5 - Enterotoxin genes found in *Staphylococcus* sp. isolates from the food services.

Gene of enterotoxins ¹	Source			
	Food (26) ²	Equipment/surface (40) ²	Food handler (21) ²	
			Hand	Nasal cavity
<i>sea</i>	-	2 (5%)	1 (4.8%)	1 (4.8%)
<i>seb</i>	1 (3.8%)	5 (12.5%)	5 (23%)	13 (62%)
<i>sec</i>	1 (3.8%)	-	-	-
<i>sed</i>	-	2 (5%)	4 (19%)	-
<i>see</i>	-	-	-	-
<i>sea+seb</i>	2 (7.7%)	2 (5%)	1 (4.8%)	-
<i>sea+sec</i>	-	1 (2.5%)	1 (4.8%)	-
<i>seb+sec</i>	1 (3.8%)	1 (2.5%)	3 (14%)	-
<i>seb+sed</i>	-	1 (2.5%)	3 (14%)	-
<i>seb+see</i>	-	1 (2.5%)	-	-
<i>sed+see</i>	-	1 (2.5%)	-	-
<i>sea+seb+sed</i>	-	1 (2.5%)	-	-
<i>seb+sec+sed</i>	-	-	2 (9.5%)	1 (4.8%)

Legend: 1, *sea*, *seb*, *sec*, *sed*, *see*, gene of *Staphylococcus* enterotoxin A, B, C, D and E, respectively; 2, Number of samples; (), percentage of presence of the gene; -: absence.

source of microorganisms for foods prepared with them (Meldrum *et al.*, 2009; Meira *et al.*, 2012). With regard to the *Staphylococcus* sp. isolated in this study, except for *S. caseolyticus*, which is commonly found in dairy products, all other isolated species from equipment and surfaces may be part of human microbiota (Cohen, 1986; Bes and Brun, 2012). The handlers' hands can be vehicles of contamination, so care with personal hygiene is essential in the production of meals (Shojaei *et al.*, 2006; Rodriguez *et al.*, 2011). In this study it was also observed that the same handler could accommodate more than one species in their microbiota, as was also observed by other authors (Acco *et al.*, 2003; André *et al.*, 2008).

The most common gene in *Staphylococcus* sp. identified from food services and foods was *seb*. SEB is one of the most potent toxins and the expression of that gene in food can be a danger to the health of the customers (Ahanotu *et al.*, 2006; Sospedra *et al.*, 2012). The prevalence of SE genes in foods varies and depends on regional and human factors. Their frequency in foods has been described in other studies and the rate was variable between them (Rall *et al.*, 2008, 2010b; Pelisser *et al.*, 2009; Borelli *et al.*, 2011; Wang *et al.*, 2013). In this study, 33% of the *Staphylococcus* sp. isolated from foods harbored one or two SE genes.

Thus, it was observed that 61.5% and 100% of the food produced by food services in Porto Alegre showed counts of thermotolerant coliforms and *Staphylococcus* sp., respectively, which are within the limits defined by Brazilian legislation (Brasil, 2001). This result of compliance does not consider the wider dissemination of CoNS in food services, or the toxigenic potential of these isolates. It was observed that the sanitary conditions of equipment and surfaces were inadequate (75%) for the production of meals; the enterotoxin B gene was the most prevalent among the *Staphylococcus* sp. evaluated. These results showed a risk to the customers of food services when exposed to food produced with inadequate hygiene practices and possessing thermotolerant coliforms and *Staphylococcus* sp. isolates with toxigenic potential. Thus, it is evident the necessity of the implementation and constant monitoring of programs of quality control in food services.

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