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Detection of *mecA* and *seh* genes from *Staphylococcus sp.* isolated from samples of food, surfaces and utensils of an industrial kitchen in Rio de Janeiro

Detecção dos genes *mecA* e *seh* de *Staphylococcus sp.* isolados de amostras de alimentos, superfícies e utensílios de uma cozinha industrial do Rio de Janeiro

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Abstract

Staphylococci are microorganisms increasingly associated with food poisoning. This work aimed to determine the prevalence, and to detect factors of virulence and resistance, of *Staphylococcus spp.* isolated from samples of food, utensils and handling surfaces in an industrial kitchen of a supermarket chain in Rio de Janeiro; and to analyze the presence of genes encoding staphylococcal enterotoxin H (*seh*) and antimicrobial resistance (*mecA*). A total of 50 samples were collected between January and March 2016. After isolation, microbial species were identified by MALDI-TOF MS, as well as *seh* and *mecA* genes, which were detected by polymerase chain reaction (PCR). Of the 50 samples analyzed, 45 (90%) were positive for *Staphylococcus sp.* Presence; 41 samples were of the genus, 37 of which were identified at the species level, 40 samples coagulase-negative Staphylococcus (CNS), and one coagulase-positive (CPS). The identification of the species by the MALDI-TOF MS technique proved to be accurate. The *seh* gene was detected in only one CNS sample (*S. saprophyticus*), isolated from a cold slicer. When the PCR technique was performed for the detection of the *mecA* gene in the 41 strains obtained from *Staphylococcus sp.*, 6 food samples and 7 surface samples were positive. It is concluded that CNSs are widespread in the environment and need to be given more attention with regard to their detection in food.

Keywords: *Staphylococcus*. Food Contamination. Mass Spectrometry. Methicillin resistance. Bacterial Toxins.

Resumo

Os *Staphylococcus* são micro-organismos cada vez mais associados a intoxicações alimentares. Este trabalho teve como objetivo determinar a prevalência e detectar fatores de virulência e resistência de *Staphylococcus spp.* isolados de amostras de alimentos, dos utensílios e superfícies de manipulação de uma cozinha industrial de uma rede de supermercados do Rio de Janeiro, e analisar a presença de genes codificadores da enterotoxina estafilocócica H (*seh*) e de resistência a antimicrobianos (*mecA*). Foram coletadas 50 amostras entre janeiro e março de 2016. Após o isolamento, foram realizadas a caracterização das amostras através de identificação em nível de espécie dos isolados por MALDI-TOF MS, a identificação dos genes *seh* e *mecA*, pelo método de reação em cadeia da polimerase (PCR). Das 50 amostras analisadas, 45 (90%) foram positivas para presença de *Staphylococcus sp.* 41 amostras eram pertencentes ao gênero, 37 das quais foram identificadas em nível de espécie, sendo 40 amostras *Staphylococcus* coagulase-negativas (SCN) e uma coagulase-positiva (SCP). A identificação das espécies pela técnica de MALDI-TOF MS demonstrou ser acurada. O gene *seh* fora detectado em uma amostra de SCN (S. *saprophyticus*) isolada de fatiadora de frios. Quando realizada a técnica de PCR para a detecção do gene *mecA* nas 41 estirpes obtidas de *Staphylococcus sp.*, 6 amostras de alimentos e 7 amostras de superfícies foram positivas. Conclui-se que os SCN se encontram disseminados no ambiente e precisam ter maior atenção no que diz respeito a sua detecção em alimentos.

Palavras–chave: *Staphylococcus*. Contaminação de Alimentos. Espectrometria de Massas. Resistência à Meticilina. Toxinas Bacterianas.

Introduction

The genus *Staphylococcus* comprises gram-positive, facultative anaerobic, immobile and catalase positive cocci,[1] being divided into two groups based on the production of coagulase, which are: Coagulase-positive Staphylococci (CPS) and coagulase-negative Staphylococci (CNS). Within the CPS group, we find the *S. aureus*, the most described pathogen and with high virulent potential, besides others, such as *S. delphini*, *S. intermedius*, *S. coagulans* and some strains of *S. hyicus*.[2] Among the CNS, it may be cited the *S. epidermidis, S. saprophyticus* and species most associated with infections, such as *S. haemolyticus* and *S. lugdunensis*, considered as the main nosocomial pathogens.

CNS have been considered a risk group for food contamination, since they are found in large quantities in foods and many of them are capable of producing toxins, causing food poisoning.[3]

Staphylococcal food poisoning is one of the most common foodborne illnesses in the world and occurs after ingestion of Staphylococcal Enterotoxins (SE), which are produced by enterotoxigenic strains of *Staphylococcus*, mainly *S. aureus,* and very occasionally by other species of staphylococci, such as *S. intermedius*.[2] Staphylococcal Enterotoxins are the major bacterial intoxication agents in man and have been reported in several outbreaks of foodborne diseases. Toxins are thermostable[4]) and cause symptoms such as vomiting and diarrhea, among others.[5]

Food poisoning caused by toxins produced by *Staphylococcus* is a risk in food handled and improperly stored. However, in addition to the toxins produced and their adverse effects on the body, there is also the risk of food becoming reservoirs for micro-organisms with antibiotic resistance. [4]

Staphylococcus aureus resistant to methicillin, generally referred to by the acronym MRSA (*Methicillin-resistant Staphylococcus aureus*) or ORSA (*Oxacillin-resistant Staphylococcus aureus*), are pathogens that have become resistant to various antimicrobials – first, to penicillin in 1947, and soon thereafter to methicillin.[6] Symptoms of a MRSA infection can progress substantially over the 24-48 hour period.[7]

Resistance to methicillin and other β-lactam antibiotics without MRSA strains is caused by the expression of a gene contained in the genome thereof, called *mecA*.[7] This gene is contained in a chromosome cassette of 21 to 60 kb called SCC*mec,* a mobile genetic element that may also contain genetic structures, such as *Tn*554, *pUB110*, *pT181.*[8] After the acquisition of gene *mecA*, it must be integrated and located on the chromosome of *S. aureus*, and it encodes an altered penicillin binding protein, termed PBP2a, which differs from other penicillin binding proteins.[9] According to data from International Working Group on the Staphylococcal Cassette Chromosome elements[10], 11 types of *SCCmec* have already been described presenting different combinations of six classes of the *mec* gene complex (A, B, C1, C2, D, E) and eight types of *ccr*[9] complex.

Given the possible inefficiency during the hygiene stage of the utensils used in food preparation, and possible failures during cooking and maintenance of adequate temperatures during the portioning stage, a more detailed study is necessary that can identify strains of *Staphylococcus sp.* isolated from the foods and utensils in question.

The objective of this study was to identify strains of *Staphylococcus* isolated from food samples produced in an industrial kitchen of a supermarket chain in Rio de Janeiro and their respective utensils, to detect genes associated with antimicrobial resistance (*mecA*) and production of enterotoxins *(seh*).

Methodology

Fifty samples of food, surfaces and utensils used in an industrial kitchen of a supermarket chain in Rio de Janeiro were collected from January to March, 2016. Twenty-one food and protein samples were considered, and 29 samples were obtained from utensils and / or surfaces used for pre-preparation and food preparation. For the collection of food, sterile bags of type *stomacher* were used, and to investigate contamination of the utensils used in food preparation, sterile *swabs* was used for collection. The samples were conditioned in thermal bag with blocks of ice and transported to the Laboratory of Microbiology, where the analyzes were performed.

For isolation of *Staphylococcus* in food, 25 grams of the food in question were suspended in 225 mL of sterile peptone water, and then homogenized in *stomacher*. After this procedure, 0.1 mL of the suspension was inoculated into Baird-Parker agar (Himedia) for isolation of typical colonies of *Staphylococcus*.[11] Subsequent dilution plating was not performed due to the qualitative nature of the analysis. Samples with more than 30 typical colonies grown on plaque were taken into account (which entails the minimum of 3.0 x 10^3 UFC/g) as determinants for the sample to be considered positive and with a maximum of 200 colonies, following the Normative Instruction No. 62.[11]

For analysis of surfaces and utensils, after collection of samples using sterile *swab* soaked in saline solution, these were seeded in Mannitol Salt Agar, using the depletion technique (qualitative method), and after seeding, these were incubated at 36 ± 2°C / 18-24 h for subsequent visual analysis, where the samples with Staphylococcus characteristic colonies were seeded onto TSA-containing plaques (Tryptic Soya Agar, Himedia) and incubated in an oven at 36 ± 2°C / 18-24 h for growth and isolation. Samples were stored in cryotubes containing 0.8 mL of TSB (Tryptone Soya Broth, Himedia) and 0.2 mL Glycerin at -20 °C.

As recommended by the Normative Instruction No. 62, which regulates the Official Analytical Methods for Microbiological Analysis for Control of Animal and Water Products,[11] for the characterization of the genus *Staphylococcus,* Gram staining and catalase testing were performed. After this analysis, the coagulase test was applied to identify the strains, such as CPS or CNS.

For identification of species of *Staphylococcus* isolated, these were inoculated in TSA for 24 hours and transferred to a 96-well plate to be subjected to Mass Spectrometry (MALDI-TOF MS – Bruker, Microflex LT model), where the samples were applied in duplicate.

For research on genes *mecA* and *seh*, the release of the bacterial DNA was done by thermal lysis.

Detection of the gene *seh*, which encodes staphylococcal enterotoxin H, was performed by the PCR technique, as described by Sila and contributors,[12] while the detection of the gene *mecA* was performed as described by Santos and contributors.[13] As controls, strains of *S. aureus* ATCC 33591 were used, positive for the gene *mecA* and negative for *seh*[12] and *S. aureus* 633a, positive for the gene *seh* and negative for *mecA*.[11]

The amplified products were analyzed by 1.0% agarose gel electrophoresis in TBE (0.89 M Tris [Sigma], 0.89 M boric acid [Madison, WI, EUA], 2.5 mM EDTA [Sigma], pH 8.2), at 80 V for 1:30 h. The gel of 1 µL / mL of GelRed® solution, previously added, was analyzed under ultraviolet light. As a DNA standard for the electrophoretic run, we used 100 bp DNA ladder (Biotools).

Results and Discussion

Of the isolated food samples, 19 (90%) were positive for *Staphylococcus sp.* Isolated samples of utensils or surface were seeded in Mannitol Salt Agar, where 26 samples (89%) were positive for *Staphylococcus sp.*, being 3 of these mannitol-fermenting. Of the 45 samples positive for *Staphylococcus sp.*, two were catalase-negative (identified by the MALDI-TOF MS technique as *Enterococcus faecium* and *Enterococcus faecalis*). Four samples were identified only at the gender level, these being not belonging to *Staphylococcus group.*

Of the 41 samples of *Staphylococcus* analyzed, 16 (39%) were identified as *S. saprophyticus*, 14 (34%) as *S. sciuri*, 3 (7%) as *S. vitulinus*, 2 (5%) as *S. xylosus*, 1 (2%) as *S. carnosus*, 1 (2%) as *S. aureus* and 4 (11%) as *Staphylococcus sp.* Although it is not frequently associated with food samples, the high incidence of *S. saprophyticus*, a CNS associated with community-based urinary tract infections, is observed, which leads to contamination from manipulators, as well as *S. sciuri*from microbiota of domestic animals.[13,14]

Paim et al.[15] demonstrated that the methodology of identification of microorganisms using MALDI-TOF MS produced a concordant identification in 440 (97.8%) of the 450 isolates of gram-positive cocci identified by the conventional phenotypic method. The identification of species by the MALDI-TOF MS technique proved to be accurate and rapid. Of the 45 samples, only 4 (9%) were identified at the gender level. The technique identifies microorganisms in a very short time (<10 min.), with minimal sample preparation and low cost in terms of reagents. A conventional biochemical identification would take 48 to 72 hours to be performed, or would use commercially available kits of high cost.[16] In addition, it is a technique that presents high precision in species level and that, in this study, presented 90% accuracy for identification of the species with reliability for confirmation of the *Staphylococcus* genus.

Of the 41 samples of *Staphylococcus sp.*, 1 (2.4%) was CPS (identified as *S. aureus* by MALDI-TOF MS) and 40 (97.6%), CNS. The sample of *S. aureus* found was isolated from food sample (isolated sample from chicken wing), and the genes *seh* and *mecA* were not detected in this sample. However, no positive CPS samples isolated from surfaces and utensils were found, while Silva[17] (2006) obtained high CPS counts in a microbiological quality assessment study of utensils and food handling surfaces of a feed unit. The large percentage of positive samples of *Staphylococcus*

spp. in food preparation surfaces demonstrates the importance of these sites as potential sources of dissemination of microorganisms, mainly through cross-contamination of food. It should be noted that cross-contamination has frequently been reported as a factor responsible for the occurrence of food-borne diseases.[18] However, it is noticed that more and more CNSs have been described associated with samples related to food. In a recent study of Santos,[19] of a total of 60 isolated surface samples in a dairy industry, 41 belonged to the genus *Staphylococcus*, 16 (39.0%) of them being CPS and 25 (61.0%) CNS, showing that these findings are of concern, especially considering the enterotoxigenic capacity of some strains of coagulase-positive staphylococci and coagulase-negative staphylococci, a fact that may mean the occurrence of risks to public health. In our study, a high rate of CNS isolation was also observed.

When the PCR technique was performed for the detection of the gene *mecA* in the 41 strains obtained from *Staphylococcus sp.*, 6 food samples and 7 surface samples were positive for the gene (Table 1). The species bearing the genes are also listed in Table 1. In this study, we found samples of *S. saprophyticus*, *S. sciuri, S. vitulinus* and *S. xylosus* carriers of the gene *mecA*. Samples of these multiresistant CNSs have already been described in the literature.[20,21,22] For confirmation of gene *mecA* expression, which encodes resistance to oxacillin and other antimicrobials, the samples were subjected to agar screening containing 4 µg / mL oxacillin, according to Ferreira et al.[23] All samples grew confluently, showing that they all expressed the gene. The gene *mecA* is highly conserved among the species of *Staphylococcus* and its origin is not yet defined. However, some studies have shown that there is similarity between the amino acid sequences of the PBPs of *S. sciuri* and *S. aureus,* which suggests that the gene *mecA* may have been originated from coagulase-negative *Staphylococcus*.[24]

Table 1. Characteristics of the 41 strains obtained from *Staphylococcus sp.* found in samples of food, surface and utensils collected in a supermarket chain in the State of Rio de Janeiro.

Samples	Origin	mecA	seh
CPS (1)	Food (1)	Positive (0)	Positive (0)
CNS (40)	Food (13)	*Staphylococcus sp.* (2) *S. saprophyticus* (2) *S. vitulinus* (1) *S. xylosus* (1)	Positive (0)
	Surface / Utensils (27)	*Staphylococcus sp.* (2) *S. saprophyticus*(3) *S. vitulinus* (1) *S. sciuri* (1)	*S. saprophyticus* (1)*

*Isolated sample of cold slicer.

The CNS presented an expressive percentage for *mecA* gene positivity, mainly in surface samples, and this type of gene can be transferred between species of the genus *Staphylococcus* and favor cross-contamination between surface x manipulator x food, which may contribute to food contamination and the possibility of occurrence of DTAs.

Gene *seh* was detected in a CNS sample (*S. saprophyticus*) isolated from cold slicer. This result is important because the Brazilian legislation does not recommend CNS screening (only coagulase detection), so that this toxigenic sample would not be detected if only the current parameters were used. For confirmation purposes, the 336 bp amplicons found in the control sample (633a) and the sample isolated in the present study were sequenced and confirmed as belonging to a fragment of said gene. For a long time, *S. aureus* was considered the only pathogen species among the species of *Staphylococcus*, while CNSs were classified as contaminants. However, molecular biology techniques have now shown that these microorganisms also have genes encoding enterotoxins and other virulence factors..[25] Borges et al.[26] recommended that the presence of CNS species should not be ignored in investigations of suspected cases of staphylococcal intoxication, since this group of pathogens, present in the food, presents a risk of causing intoxication to the consumer.

The production of coagulase is a characteristic used in the identification of *S. aureus*.[27] Brazilian legislation, through Resolution RDC No. 12,[28] indicates the coagulase-positive staphylococcus search as indicative of *S. aureus*. Although the Brazilian legislation does not require the investigation of the presence of (CNS), studies show that these are enterotoxin producers, as well as CPS, in particular *S. aureus*, associated with outbreaks of foodborne diseases.[29] In the present study, only one sample presented enterotoxin H and this was CNS. Due to this fact, they have received more attention by governments and industries in establishing microbiological standards, methods or specifications.[30] Therefore, it is suggested that the Brazilian legislation includes the CNS research as a new category of evaluation.[28]

Conclusion

According to the results found in the present study, it is concluded that there is a high percentage of contamination of food products, and especially of the surfaces that come into direct contact with food, suggesting a deficiency from a hygienic-sanitary point of view, being necessary the implementation of techniques that may decrease or cure the occurrence of bacterial multiplication in foods and surfaces. The significant number of CNS strains reinforces their growth in cases of food contamination and it is suggested that Brazilian legislation should include CNS research as a new category of evaluation.

Collaborators

PL Nascimento worked on all steps, from designing the study to reviewing the final version of the article. JPO Martinez contributed in the stage of MALDI-TOF and PCR and in the final conclusion of the work.

Conflict of Interests: The authors declare no conflict of interest.

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