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Virulence profile and genetic variability of *Staphylococcus aureus* isolated from artisanal cheese

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ABSTRACT

The objectives of this study were to characterize *Staphylococcus aureus* isolated from artisanal and industrialized Minas frescal cheeses, to determine their antimicrobial susceptibility profile as well as the genetic similarity among the isolates. The isolates were also tested for staphylococcal enterotoxin genes and other virulence factors. Fifty-six artisanal raw milk cheeses sold at street fairs and 10 industrialized cheeses commercialized in supermarkets of Goiânia, Goiás, were analyzed. *Staphylococcus aureus* was confirmed in 19 samples (33.9%) of artisanal cheese by detection of *femA* gene, in which 29 isolates were obtained. These isolates were submitted to the antimicrobial susceptibility test and classified into 9 different profiles (A–I). Thirteen isolates (44.8) were resistant to penicillin and 3 (10.3) to tetracycline, with 2 (7.4) resistant to both. The multiplex PCR technique was performed to detect virulence genes that code for the production of hemolysins (Hla and Hlb), toxic shock syndrome toxin (TSST-1), exfoliative toxins (ETa and ETb), and staphylococcal enterotoxins [SE; SEA–SEE, SEG–SEJ, SEM–SEO]. All the isolates amplified for the *hla* gene and 14 (48.3%) for the *hlb* gene. The *seh* gene was the most frequently detected (n = 11, 37.9%), followed by *seo* gene (n = 3; 10.3%). In one isolate (3.4%), 4 enterotoxins genes were detected, and in another, 6 (3.4%) were detected. The comparison performed by pulsed-field gel electrophoresis of the 29 isolates revealed 18 genotypic profiles, which were grouped into 5 clusters. The genotyping found high genetic similarity among the isolates. Identical isolates were obtained from different samples and one sample showed more than one genetically different isolate. The high prevalence of *S. aureus* in the Minas Frescal cheese samples, as well as the detection of toxin encoding genes identified in this study, warns of the necessity to reduce the contamination levels in this type of cheese through monitoring and controlling the production and trade of the product.
Key words: *Staphylococcus aureus*, cheese, antimicrobial resistance, staphylococcal enterotoxin

INTRODUCTION

Staphylococcus aureus is one of the most important human and animal pathogens. It has a broad set of virulence mechanisms associated with infectivity, production of toxins, and antimicrobial resistance (Argudin et al., 2010).

Some strains of *S. aureus* can produce toxins that are members of pyrogenic toxin superantigen family, such as the staphylococcal toxic shock syndrome toxin (**TSST-1**), which is responsible for generating an immune hyper-response in the host and has already been detected in *S. aureus* isolated from foods (Cha et al., 2007). These bacteria can produce exfoliative toxins, which promote the cleavage of the skin extract, causing severe cutaneous syndromes (Zschöck et al., 2005). Most strains secrete enzymes and cytotoxins that include coagulase and 4 hemolysins (α, β, gamma, and delta). Alpha and β hemolysins (**Hla** and **Hlb**) are dermonecrotic and neurotoxic, turn off the immune system by direct cytotoxic effect, and degrade soft tissues (Vandenesch et al., 2012).

Among the toxins produced by *S. aureus*, staphylococcal enterotoxins are a concern, because when produced in foods staphylococcal enterotoxins can cause food poisoning, which is one of the most prevalent foodborne diseases around the world (ECDC, 2015). These toxins present emetic activity and are called classic staphylococcal enterotoxins (**SE**; SEA–SEE), which occur more frequently in cases and outbreaks of food poisoning, along with other more recently identified (SEG, SEH, SEI, SER, and SET (Wallin-Carlquist et al., 2010; Xu and McCormick, 2012). In addition to these, other enterotoxins called staphylococcal enterotoxin-like proteins (**SE*l***) include the SE*l* J, K, L, M, N, O, P, Q, S, U, and V. Although the SE*l* toxins are homologous and structurally similar to the staphylococcal enterotoxins,

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emesis activity has not yet been confirmed in humans (Xu and McCormick, 2012).

Staphylococcus aureus was the second most common pathogen responsible for foodborne diseases in Brazil between 2000 and 2014. Milk and its derivatives were among the foods most frequently involved in outbreaks of foodborne diseases (ANVISA, 2015).

Minas frescal cheese is one of the most consumed cheeses in Brazil and presents high susceptibility to microbiological and proteolytic changes due to its high moisture content and handling (Chalita et al., 2009). According to Brazilian law, this type of cheese must be produced in dairy industry with pasteurized milk. However, this food is also manufactured from raw milk and marketed in the informal sector, without regulation and sanitary supervision. For these reasons, it is frequently involved in the transmission of pathogenic bacteria and staphylococcal food poisoning outbreaks (Dorigon, 2010).

Additionally, bacteria isolated from foods of animal origin may show resistance to several antimicrobial agents used to treat diseases, which may influence the effectiveness for treatment (Delsol et al., 2010; Araújo et al., 2011).

The aims of the study were to (1) evaluate the prevalence of *S. aureus* in artisanal and industrialized Minas frescal cheese sold in the municipality of Goiânia, Brazil; (2) determine the antimicrobial susceptibility profile of the isolates; (3) molecularly characterize the isolates for the presence of virulence genes and genetic similarity by macrorestriction analysis using pulsed-field gel electrophoresis (**PFGE**).

MATERIALS AND METHODS

Cheese Sampling

In this study, 56 artisanal and 10 industrialized samples of Minas frescal cheese were purchased from retail sale sites along the market streets and from supermarkets, respectively, in the municipality of Goiânia, Goiás, Brazil, from June to August 2012. All samples were immediately transported to the laboratory in a refrigerated box (4 to 8°C) and kept at 4°C until analysis.

Isolation of Coagulase-Positive Staphylococci

To enumerate coagulase-positive staphylococci (**CPS**), the samples were processed by surface plating on Baird-Parker agar with egg yolk tellurite emulsion and incubated at 37°C for 48 h under aerobic conditions. Up to 5 typical or atypical (or both) presumptive colonies were selected and tested using standard microbiological procedures such as Gram staining, catalase, and thermostable nuclease detection. The coagulase production was performed by tube test using rabbit serum (Bennett and Lancette, 2001). A pure culture of each isolate was kept frozen at −80°C for further analysis.

Antimicrobial Susceptibility Test–Disk Diffusion Method

All CPS isolates were subjected to an antimicrobial susceptibility test by the disc diffusion method on Müeller Hinton agar (CLSI, 2015). Penicillin (10 UI), cefoxitin (30 μg), trimethoprim/sulfamethoxazole (1.25/23.75 μg), rifampin (5 μg), ciprofloxacin (5 μg), tetracycline (30 μg), erythromycin (15 μg), quinupristin/dalfopristin (15 μg), and clindamycin (2 μg) were used as antimicrobial agents. *Staphylococcus aureus* ATCC 25923 was used as a reference strain for antimicrobial susceptibility testing.

DNA Extraction

The DNA extraction procedure was performed according to Aires-de-Sousa et al. (2007). The pure culture was thawed and streaked onto Tryptone Soya Agar culture and incubated at 35°C for 18 to 24 h. Three to 4 colonies were suspended in 50 μL of TE 1× buffer (10 m*M* Tris, 1 m*M* EDTA, pH 8.0) with 1 μL of lysostaphin (10 mg/mL) and incubated at 37°C. After 30 min, cell suspensions were placed in a boiling-water bath at 95°C for 15 min. Then, 150 μL of H_2O Milli-Q was added and the solution was centrifuged (13,000 × *g*) for 5 min at 4°C. The resulting supernatants were used as DNA templates in the PCR reactions.

Identification of Staphylococcus aureus

The CPS isolates were screened by PCR for the presence of *femA* gene, which is specific for *S. aureus*, according to Mehrotra et al. (2000). The primers used for gene amplification were *femA*-F: 5′AAAAAAGCACATAACAAGCG 3′ and *femA*-R: 5′GATAAAGAAGAAACCAGCAG 3′, to obtain a 132-bp amplicon.

The PCR reaction was performed in a reaction mixture with a final volume of 50 μL containing 10 μL of 5× reaction buffer [20 m*M* Tris-HCl (pH 8.4), 50 m*M* KCl], 1.5 m*M* $MgCl_2$, 100 μ*M* of each dNTP, 0.2 mM of *femA* primers, 2.5 U of *Taq* polymerase, and 20 ng of template DNA. The volume of this mixture was adjusted to 50 μL with sterile deionized water. As positive controls, PCR reactions were carried out in paral-

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