Short communication: High frequency of β -lactam-resistant *Staphylococcus aureus* in artisanal coalho cheese made from goat milk produced in northeastern Brazil

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ABSTRACT

Reports of β -lactam-resistant *Staphylococcus aureus* in artisanal goat cheese are increasing, and this phenomenon is relevant to public health. The objective of the present study was to determine the prevalence of *S. aureus* strains carrying the *blaZ* and *mecA* resistance genes, as well as the genes encoding the staphylococcal enterotoxins SEA, SEB, SEC, SED, SEE, and TSST-1 in artisanal coalho cheese made from goat milk produced in northeastern Brazil. We used biochemical and molecular tests to characterize 54 *S. aureus* isolates found in artisanal coalho cheese collected from commercial establishments producing animal products in 11 municipalities of Pernambuco State, Brazil. A PCR analysis revealed that 42.6% (23/54) of the isolates were positive for the *blaZ* gene, and 7.4% (4/54) were resistant to methicillin by phenotypic testing. We did not detect *mecA* or any genes encoding enterotoxins. The presence of *S. aureus* carriers of the *blaZ* gene and the identification of methicillin-resistant *S. aureus* strains are of concern for the health of consumers of this type of cheese.

Key words: antimicrobial resistance, *blaZ* gene, goat cheese, enterotoxin gene

Short Communication

Goat farming is one of the most important agricultural activities in the northeast region of Brazil. The state of Pernambuco is the second-largest producer of goats in Brazil (IBGE, 2015). However, despite the economic importance of goat breeding, production systems have low adoption of technology and inadequate man-

agement, predisposing goat herds to diseases that cause economic losses. In addition to the economic impact of these factors, there is a risk to consumers of food of caprine origin, because pathogenic microorganisms, especially *Staphylococcus aureus*, can be transmitted from goat milk and its derivatives (Xing et al., 2016). This risk may result from direct secretion from the udder (Mørk et al., 2010) arise during one of the stages of obtaining and processing raw milk, or both (Argudin et al., 2010).

Staphylococcus aureus is one of the main pathogenic microorganisms that cause food poisoning and infections in humans and animals. It is an infectious agent of global importance to public health (Rahbar Saadat et al., 2014). Moreover, *S. aureus* has zoonotic potential, because it produces staphylococcal enterotoxins, contaminates food causing gastroenteritis (Xing et al., 2016), and is capable of forming a biofilm, which increases antimicrobial resistance and the risk of transmission and contamination of food (Akbas and Kokumer, 2015).

Several researchers have reported the significance of *S. aureus* in cheese made from goat's milk and the potential for staphylococcal enterotoxins to cause food poisoning in humans (Wieneke and Gilbert, 1987; Johler et al., 2015). In addition to the risk of food poisoning, certain strains of *S. aureus* carry resistance genes, including *blaZ*, which confer resistance to non-stable β -lactams (Zhang et al., 2001), and *mecA*, which causes resistance to certain β -lactam groups, such as cephalosporin (cefotaxime and ceftriaxone) and stable penicillins (oxacillin and methicillin; Rudkin et al., 2012).

Despite the importance of *S. aureus* in artisanal cheeses made with goat milk and its effect on public health, information on this subject in the national literature of Brazil is scarce. The objective of the present study was to determine the prevalence of *S. aureus* strains carrying *blaZ* and *mecA* resistance genes and staphylococcal enterotoxin genes encoding SEA, SEB,

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Figure 1. Distribution of samples by municipality in Pernambuco State, Brazil.

SEC, SED, SEE, and TSST-1 types in artisanal cheeses made from goat milk marketed in Pernambuco, Brazil.

Between March and August 2017, we collected 30 samples of artisanal coalho cheese made with goat milk from commercial establishments that produced animal products in 11 municipalities of Pernambuco, located in the regions of Sertão (municipalities A, B, C, D, and E) and Agreste (municipalities F, G, H, I, J, and K), as follows: 1 sample was collected in municipality A; 5 in municipality B; 2 in municipality C; 1 in municipality D; 2 in municipality E; 1 in municipality F; 3 in municipality G; 1 in municipality H; 9 in municipality I; 3 in municipality J; and 2 in municipality K (Figure 1). Each sample weighed approximately 500 g.

The isolation of *S. aureus* was performed according to the method recommended by Brazilian legislation (Brasil, 2003). We weighed 25 g portions from different parts of each cheese sample. We then transferred the fractions to sterile zipper storage bags containing 225 mL of sterile 0.85% saline solution. The samples were then homogenized for 60 s in a Stomacher blender (Seward, Bohemia, NY), and the resulting solutions were serially diluted to a factor of 10^5 . Aliquots of the diluted solutions (0.1 mL each) were inoculated onto plates containing Baird Parker agar (Difco Laboratories Inc., Detroit, MI) enriched with egg yolk emulsion and potassium telluride (HiMedia, Mumbai, India) using a Drigalski loop. The plates were incubated at 35 to 37°C for 48 h (Brasil, 2003) and then the colonies on the Baird Parker agar were classified as typical or atypical.

A total of 158 typical *S. aureus* colonies were selected and subjected to DNA extraction (Table 1) according to the method proposed by Fan et al. (1995). To confirm the *S. aureus* species, we amplified the specific region of the *nuc* gene according to the procedure of Kateete

et al. (2010). We used the *Staphylococcus aureus* ssp. *aureus* strain ATCC 43300 as a positive control for detection of the *nuc* gene, and DNA-free water (Qiagen, Hilden, Germany) as a negative control. To detect the *blaZ* gene, we used the method described by Sawant et al. (2009). We used *S. aureus* ssp. *aureus* strain ATCC 29213 as a positive control, and DNA-free water (Qiagen) as a negative control. To detect the *mecA* gene, we used the primers described by Nakagawa et al. (2005) and the thermal profile described by Paterson et al. (2012). We used *S. aureus* ssp. *aureus* strain ATCC 43300 as a positive control and DNA-free water (Qiagen) as a negative control.

To detect genes encoding the SEA, SEB, and SED enterotoxins, we followed the method described by Becker et al. (1998), and to detect genes encoding the SEC, SEE, and TSST-1 enterotoxins, we followed the method of Mehrotra et al. (2000). The following *S. aureus* strains were used as positive controls for the detection of enterotoxin-encoding genes: ATCC 13565 (*sea*), ATCC 14458 (*seb*), ATCC 19095 (*sec*), FRI 361 (*sed*), ATCC 27664 (*see*), and N315 (*tst1*). We used DNA-free water (Qiagen) as a negative control. The strain *S. aureus* N315 used as positive control was provided by the Laboratory of Microbiology of Animal Products, Departamento de Tecnologia e Inspeção de Produtos de Origem Animal, Universidade Federal de Minas Gerais. The other reference strains used as positive controls were provided by the Laboratory of Infectious Diseases of Domestic Animals, Departamento de Medicina Veterinária, Universidade Federal Rural de Pernambuco.

To determine antimicrobial resistance, we carried out MIC tests with oxacillin (≥ 4 $\mu\text{L/mL}$) according to the CLSI (2015) method. We used the *S. aureus* ssp. *aureus* strain ATCC 43300 as a positive control

for oxacillin resistance. The results of the qualitative diagnostic tests (PCR) are expressed as absolute and relative frequencies (Field, 2011).

Of the 158 typical colonies obtained in the microbiological analysis of artisanal coalho cheese samples made with goat milk, 54 (34.1%) contained the *S. aureus nuc* gene. Other studies have already highlighted the economic and health effects of *S. aureus* as a pathogen in cheeses manufactured from goat milk (Akineden et al., 2008; Ganai et al., 2016).

We detected the *blaZ* gene in 42.6% (23/54) of the isolates. Resistance to antibiotics can occur by 1 of 2 mechanisms: the first is related to the production of β -lactamase associated with the *blaZ* gene (Lowy, 2003), and the second involves the production of penicillin-binding protein 2a (PBP2a), a protein that binds to β -lactams and is associated with the *mecA* gene (Guignard et al., 2005). We did not detect *mecA* in any of the isolates. Therefore, we believe resistance was due to production of β -lactamase. Furthermore, according to our phenotypic tests, 7.4% (4/54) of the isolates were resistant to oxacillin, and must therefore have contained methicillin-resistant *S. aureus* (MRSA).

The results we obtained for the occurrence of oxacillin-resistant isolates corroborated those obtained by França et al. (2012), who applied phenotype and genotype analysis to 171 isolates of *Staphylococcus* spp. in milk from goats with subclinical mastitis in the states of Bahia and Pernambuco. Of those isolates, 15.8% (27/171) showed phenotypic resistance to oxacillin; the *mecA* gene was not detected, but the *blaZ* gene was present in 40.2% of the isolates. Peixoto et al. (2013) also carried out genotypic and phenotypic studies on isolates of *Staphylococcus* spp. in milk from goats with

subclinical mastitis in Bahia State. In that study, 54.5% of the isolates exhibited resistance; the *mecA* gene was not detected, but 33.3% of the *Staphylococcus* spp. isolates carried the *blaZ* gene.

Unlike the results obtained in the present study and those mentioned above, the authors of other studies have reported the *mecA* gene in *S. aureus* isolated from goat milk and its products, such as cheese (Basanisi et al., 2016; Ganai et al., 2016). Moreover, *mecA* has been related to oxacillin resistance, as demonstrated in a study by Obaidat et al. (2017), in which it was detected in 11.9% (5/42) of expansion tank samples and 11.5% (3/26) of milk samples. All the isolates carrying *mecA* were resistant to oxacillin, according to antimicrobial resistance tests.

Several hypotheses may explain the oxacillin resistance of 7.4% (4/54) of the *S. aureus* isolates observed in the present study, in the absence of *mecA*. The first hypothesis is related to the possible existence of oxacillin-resistant *S. aureus* strains that do not carry the *mecA* gene, which may be explained by the β -lactamase hyperproduction phenotype (Brown, 2001). Another hypothesis is that such isolates are carriers of a divergent form of the *mecA* gene called *mecC*, which also confers resistance to stable β -lactams (García-Álvarez et al., 2011). A third hypothesis depends on the existence of another oxacillin-resistant *S. aureus* strain such as MRSA ST130, which carries the homologous *mecA* gene but is not detected by conventional PCR assays for *mecA* because of its conformation (Pantosti, 2012).

There is major public health concern about MRSA strains, due to recent findings that such strains can colonize animals, especially cattle, and infect humans (Köck et al., 2011). They have also been identified in

Table 1. Primer sequences¹ used in the reactions and amplicon sizes observed in the PCR

Gene	Sequence (5'–3')	Predicted product size (bp)	Reference
<i>nuc</i>	F: GCGATTGATGGTGATACGGTT R: AGCCAAGCCTTGACGAACATAAAGC	279	Kateete et al. (2010)
<i>sea</i>	F: CCTTTGGAA ACGGTTAAAACG R: TCTGAACCTTCCCATCAAAAAC	127	Becker et al. (1998)
<i>seb</i>	F: TCGCATCAAACTGACAAAACG R: GCAGGTACTCTATAAGTGCCCTGC	477	Becker et al. (1998)
<i>sec</i>	F: AGATGAAGTAGTTGATGTGTATGG R: CACACTTTTAGAATCAACCG	451	Mehrotra et al. (2000)
<i>sed</i>	F: CTAGTTTGGTAATATCTCCTTTAAACG R: TTAATGCTATATCTTATAGGGTAAACATC	319	Becker et al. (1998)
<i>see</i>	F: GCTGGAGGCACACCAAATA R: CATAACTTACCGTGGACCCCTTC	301	Mehrotra et al. (2000)
<i>tst1</i>	F: ACCCCTGTTCCCTTATCATC R: TTTTCAGTATTTGTAACGCC	326	Mehrotra et al. (2000)
<i>blaZ</i>	F: AAGAGATTTGCCTATGCTTC R: GCTTGACCACTTTTATCAGC	517	Sawant et al. (2009)
<i>mecA</i>	F: TGGTATGTGGAAGTTAGATTGGGAT R: TAATCTCATATGTGTTCTGTATTGGC	155	Nakagawa et al. (2005)

¹F = forward; R = reverse.

other animal species (Loeffler et al., 2005), including horses (Cuny et al., 2008) and swine (Gómez-Sanz et al., 2010). Studies that evaluate the possibility of MRSA transmission from goats to humans remain scarce, but in a study conducted in Austria, Loncaric et al. (2013) observed the possible transmission of ST398 MRSA strains from goats to humans.

The risk of transmission of methicillin-resistant strains of *S. aureus* from goat milk and cheese has been treated as a serious public and animal health problem affecting food production and safety (Ganai et al., 2016; Obaidat et al., 2017). Further studies on MRSA are needed in animal foods with respect to the genetic relationship between clones isolated from food and those that cause infections in humans (Kwon et al., 2005; Pantosti, 2012).

In the present study, we did not detect any enterotoxin-encoding genes (*sea*, *seb*, *sec*, *sed*, *see*, or *tst1*). *Staphylococcus aureus* is the bacterium most frequently involved in outbreaks of food poisoning, and a strain can produce more than one enterotoxin (Omoe et al., 2005). In general, studies have revealed a high prevalence of classical *S. aureus* genes in goat milk products, with the *sec* gene being the most frequently detected (Akineden et al., 2008; Xing et al., 2016).

Despite the absence of enterotoxin genes in the samples of the present study, these samples may still pose a risk for food poisoning, because we did not investigate other staphylococcal enterotoxin-encoding genes. The presence of *S. aureus* in cheese may be a concern for public health authorities, because it is associated with inadequate hygiene in the production and modification of foods (Argudin et al., 2010). Moreover, *S. aureus* carries antimicrobial-resistant genes of the β -lactam class and exhibits resistance to oxacillin in MIC tests.

The high prevalence of *S. aureus* carriers of the *blaZ* gene and the identification of methicillin-resistant strains are a health concern for consumers of this type of cheese. These results should also alert public health surveillance agencies to the poor quality of artisanal cheeses made with goat milk in this region of north-eastern Brazil.

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