Short communication: Diversity of species and transmission of antimicrobial resistance among *Staphylococcus* spp. isolated from goat milk

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ABSTRACT

The increasing production of goat milk and its derivatives is affected by the occurrence of intramammary infections, which are highly associated with the presence of *Staphylococcus* species, including some with zoonotic potential. Staphylococci in general can exchange mobile genetic elements, a process that may be facilitated by the isolate's capacity of forming biofilms. In this study we identified, to the species level, *Staphylococcus* isolated from goat milk samples by MALDI-TOF and confirmed the identification by sequencing housekeeping genes (*rrs* and *tuf*). Eight species were identified, more than half being either *Staphylococcus epidermidis* or *Staphylococcus lugdunensis*. The isolates were shown by pulsed-field gel electrophoresis to be genetically diverse between the studied herds. Resistance to ampicillin and penicillin was widespread, and 2 *Staph. epidermidis* isolates contained the methicillin-resistance gene *mecA*. Most of the isolates that were resistant to at least 1 of the 13 antimicrobials tested harbored plasmids, one of which was demonstrated to be conjugative, being transferred from a *Staph. epidermidis* to a *Staphylococcus aureus* strain. Biofilm formation was observed in almost every isolate, which may contribute to their capacity of exchanging antimicrobial resistance genes in addition to acting as a physical barrier to the access of drugs. Our results showed that antimicrobial resistance among goat staphylococci may be emerging in a process facilitated by the exchange of mobile genetic elements between the bacteria and the establishment of biofilms, which calls for careful monitoring and more effective control therapies.

Key words: goat intramammary infection, antimicrobial resistance, horizontal gene transfer, biofilm, staphylococci

Short Communication

The consumption of goat milk and its derivatives is boosted by their greater digestibility and lower allergenicity compared with cow milk products (García et al., 2014). Although goat milk accounts for only about 3% of global milk production, the aforementioned benefits of goat milk and the selection of highly productive dairy breeds are responsible for the growth of the industry, which goes through expressive 4.1% annual growth in developing countries (FAO, 2013; OECD-FAO, 2017). In Brazil, the annual 3.5% growth of the goat dairy industry is driven by the support and subsidy of federal government programs (Jung and Júnior, 2016).

Several factors can affect milk productivity, such as the animals' genetic potential, nutrition, and feeding practices, but proper management to avoid IMI is crucial to ensure higher yield and quality of the final product (Goetsch et al., 2011). Intramammary infections may lead to mastitis, one of the most frequent diseases in dairy herds, which is usually accompanied by abnormal changes in the physical, chemical, and bacteriological features of milk. The extensive losses due to this disease in herds are associated with a decrease in milk quality and production, expenses with drugs and veterinary care, and culling of animals (Peixoto et al., 2010).

Bacteria of the *Staphylococcus* genus can account for 60 to 96% of the microorganisms causing goat mastitis, *Staphylococcus epidermidis* being the predominant species. Given the zoonotic potential of *Staph. epidermidis* and *Staphylococcus aureus*, with the focus on these species, other species isolated from goats with mastitis may be overlooked or not properly identified (Peixoto et al., 2010; Goetsch et al., 2011). However, studies have shown that different species of *Staphylococcus*, considered to be less pathogenic, are able to exchange genetic material among themselves, and the transfer of antimicrobial resistance genes from harmless species to potentially pathogenic ones represents a threat to their hosts (Otto, 2013a; Rossi et al., 2017). This transfer is facilitated by the strains' capacity of forming biofilms, which provide an environment of high cellular density,

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availability of mobile DNA, and increased natural capacity to exchange genetic elements (Madsen et al., 2012). Thus, this work aimed to identify *Staphylococcus* species from goat milk samples collected from 3 herds as well as evaluate their genetic diversity, the profile of antimicrobial resistance, biofilm formation, presence of mobile genetic elements, and their transfer potential.

Milk samples were aseptically collected from both halves (composite milk) of lactating goats of Toggenburg breed (1 herd, $n = 131$) and Saanen breed (2 herds, $n = 45$ and 20). All animals were considered to be clinically healthy as they did not present signs of clinical mastitis or udder inflammation, they were not under treatment with antibiotics and the initial streams of foremilk (which were discarded) did not present any organoleptic alterations. After predipping disinfection, teat ends were cleaned with ethanol 70%. Goats were hand milked and produced an average of 2.0 to 2.5 L of milk/d. Approximately 5 mL of milk was collected from each udder half and pooled in a sterile tube. Samples were kept at 4°C and taken to the laboratory for processing, and the microbiological culture was performed within 24 h of sampling according to National Mastitis Council (2004) recommendations. A volume of 10 μ L of each sample was spread on blood agar plates. Plates were examined after 24 and 48 h of aerobic incubation at 37°C. The following criteria were evaluated: colony morphology, type of hemolysis, Gram stain, catalase activity, and coagulase activity on rabbit plasma (Bio-Rad, Hercules, CA). The gram-positive, catalase-positive, and coagulase-negative cocci were identified as coagulase-negative staphylococci, whereas the gram-positive, catalase-positive, and coagulase-positive cocci were identified as coagulase-positive staphylococci. *Staphylococcus* colonies were isolated on 51 of the 196 goat milk samples. All isolates were inoculated in brain heart infusion (BD, Franklin Lakes, NJ) and incubated at 37°C for 18 h before every test. Staphylococci were considered to be agents of IMI if 5 or more colonies were growing in pure culture on the blood agar plates (National Mastitis Council, 2004).

Isolates were identified to species level by the combination of 3 analytical approaches to ensure proper identification. First, the differentiation of protein profiles of the bacterial cultures was analyzed in triplicate by MALDI-TOF/MS. Extraction was performed as suggested by Sauer et al. (2008), with modifications: pure colonies were spotted in triplicate on a polished steel target plate, dried, and overlaid with 1 μ L of formic acid (Sigma-Aldrich, St. Louis, MO). After drying again, all spots were overlaid with 1 μ L of matrix, a solution of α -cyano-4-hydroxycinnamic acid (Sigma-Aldrich) prepared in 50% acetonitrile (Sigma-Aldrich) and 2.5%

trifluoroacetic acid (Sigma-Aldrich). After complete drying, the plate was inserted into the Bruker Microflex LT MALDI-TOF system (Bruker, Billerica, MA). Mass spectral data were collected within the mass/charge ratio (m/z) range of 2,000 to 20,000, and data were acquired and analyzed using FlexControl software 3.3 and the Biotyper database version 3.1.2. (Bruker). As suggested by the software's manufacturer, the score cutoff of ≥ 2.000 was considered a secure genus identification and probable species identification. The MALDI-TOF results were confirmed by sequencing the *rrs* (16S rRNA) and *tuf* genes. Genomic DNA was isolated from the bacterial isolates by the guanidinium thiocyanate extraction method (Pitcher et al., 1989). The *rrs* gene was amplified with the universal primers 9f/1492r (Weisburg et al., 1991), and the *tuf* gene was amplified with the primers *tufF*/*tufR* (Heikens et al., 2005) with the GoTaq G2 Green Master Mix (Promega, Madison, WI) following the manufacturer's protocols and PCR conditions according to the cited reference. The PCR fragments were purified with the Wizard SV Gel and PCR Clean-Up System (Promega) and sequenced on the Illumina MiSeq platform (Illumina, San Diego, CA). Eight *Staphylococcus* species were identified (Table 1). Most isolates (15/51, 29%) were *Staph. epidermidis*, followed by *Staph. lugdunensis* (11/51, 22%), *Staph. aureus* (7/51, 14%), *Staph. simulans* (6/51, 12%), *Staph. xylosus* (4/51, 8%), *Staph. caprae* (3/51, 6%), *Staph. capitis* (2/51, 4%), and *Staph. pausteri* (1/51, 2%). Only 2 isolates (3%) did not produce reliable MALDI-TOF or coincident sequencing results, being identified as *Staphylococcus* spp. (Table 1).

Isolates of the same species were shown to be genetically diverse by their chromosomal restriction patterns produced by the *Sma*I enzyme (Appendix Figure A1), separated by pulsed-field gel electrophoresis, following the protocols suggested by Nunes et al. (2005). Clustering analysis of the pulsed-field gel electrophoresis products was performed using Bionumerics 6.0 software (Applied Maths, Sint-Martens-Latem, Belgium) with the unweighted pair-group method with arithmetic mean (UPGMA) algorithm and the Jaccard coefficient. Isolates were clustered in several pulsotypes (Appendix Figure A1). The genetic variability among the isolates of different farms is most likely due to the nature of the goat milk production in Brazil, which is typically performed by small independent properties (Marcondes et al., 2017), which avoids the exchange of bacteria between them. On the other hand, the occurrence of similar pulsotypes on the same farm indicates transmission between the animals.

The antimicrobial susceptibility of all isolates was tested by the disk diffusion method against the follow-

ing antimicrobials (Cecon, São Paulo, Brazil): ampicillin (10 µg), cefoxitin (30 µg), ciprofloxacin (5 µg), clindamycin (2 µg), erythromycin (15 µg), gentamicin (10 µg), mupirocin (5 and 200 µg), oxacillin (1 µg), penicillin G (10 UI), rifampicin (5 µg), trimethoprim-sulfamethoxazole (1.25/23.75 µg), tetracycline (30 µg), and vancomycin (30 µg). All tests were performed using Müller-Hinton agar (Oxoid, Basingstoke, UK) according to the Clinical and Laboratory Standards Institute guidelines (CLSI, 2016a). *Staphylococcus aureus* ATCC 25923 was included in each batch of test as a quality control. Inhibition zone interpretative criteria for anti-

microbials were determined using human data (CLSI, 2016b) when not available for veterinary practice.

More than half (28/51, 55%) of the isolates were resistant to at least 1 of the 13 antimicrobials evaluated (Table 1). Resistance to penicillin and ampicillin was observed in 39% (20/51) of isolates, which is consistent with the fact that these antimicrobials have been widely used in veterinary practice, including to treat mastitis caused by gram-positive bacteria (Silva et al., 2012). All the isolates were sensitive to rifampicin, vancomycin, oxacillin, and mupirocin. Two *Staph. epidermidis* isolates (8389 and 8398) were resistant to

Table 1. Characterization of the *Staphylococcus* spp. isolated from the milk of goats with mastitis used in this work¹

Isolate	Antimicrobial resistance ²	Plasmid	<i>mecA</i>	Biofilm	<i>icaA</i>	<i>bap</i>
<i>Staph. epidermidis</i>						
13	amp, pen, ery	+	—	Strong	—	+
8388	amp, pen	—	—	Strong	—	—
8389	amp, pen, gen, tet, cip, cfo	+	+	Moderate	—	—
8398	amp, pen, gen, tet, cip, cfo, sul	+	+	Moderate	—	—
8, 6050	amp, pen	+	—	Weak	—	—
6059, 7054	amp, pen	+	—	Strong	—	—
7034, 8064, 8067	amp, pen	+	—	Strong	—	+
3083, 8054, 8061, 8380	amp, pen	+	—	Moderate	—	—
<i>Staph. lugdunensis</i>						
3	—	—	—	Moderate	—	+
6	—	—	—	Strong	—	+
10	—	+	—	Moderate	—	+
19	—	—	—	Moderate	—	+
8001	—	—	—	Moderate	+	+
8383	—	—	—	Strong	—	+
4016, 6062, 8029, 8384, 8386	—	—	—	Strong	+	+
<i>Staph. aureus</i>						
8387	—	—	—	Moderate	+	—
8391, 8397, 9057	—	—	—	Strong	—	—
8396	ery	—	—	Weak	—	—
9004	ery	+	—	Moderate	—	—
9009	ery	—	—	Moderate	—	—
<i>Staph. simulans</i>						
6034, 8005, 8382, 9045	—	—	—	Moderate	—	—
8390	—	—	—	Strong	—	—
9060	—	—	—	Weak	—	—
<i>Staph. xylosus</i>						
8371	amp, pen, ery	—	—	Strong	+	—
8381	amp, pen	—	—	Strong	+	—
9008	amp, pen, ery	—	—	Moderate	—	—
9048	tet	+	—	Strong	—	—
<i>Staph. caprae</i>						
8392	amp, pen	—	—	Moderate	+	—
9056	—	—	—	Strong	+	—
9057cb	ery	—	—	Strong	+	—
<i>Staph. capitis</i>						
7008	—	—	—	Moderate	+	—
8379	amp, pen	+	—	None	+	—
<i>Staph. pasteurii</i>						
8374	—	—	—	Moderate	—	—
<i>Staph. sp.</i>						
8372	—	—	—	Moderate	—	—
8376	cli	—	—	Moderate	—	—

¹+ denotes the presence of plasmids or genes investigated; — denotes their absence as well as no resistance to the antimicrobials tested.

²Antimicrobials: ampicillin (amp), cefoxitin (cfo), ciprofloxacin (cip), clindamycin (cli), erythromycin (ery), gentamicin (gen), penicillin G (pen), trimethoprim-sulfamethoxazole (sul), tetracycline (tet).

Figure 1. Characterization of methicillin resistance in the 2 ceftiofur-resistant isolates identified in this work. (A) Detection of the *mecA* gene by PCR and (B) classification of the SCC*mec* type by multiplex PCR in the *Staphylococcus epidermidis* 8389 and 8398 isolates. M = molecular weight marker; C+ = positive control, *Staphylococcus haemolyticus* isolate MD2.

multiple drugs and capable of growing in the presence of penicillin, ampicillin, gentamicin, tetracycline, ciprofloxacin, and ceftiofur. Isolate 8398 was also resistant to trimethoprim–sulfamethoxazole.

All isolates were assessed for the presence of the methicillin-resistance gene *mecA* by PCR with the primers MRS1/MRS2 (Del Vecchio et al., 1995) using the GoTaq G2 Green Master Mix (Promega) following the manufacturer's protocols and PCR conditions according to the cited reference. The *Staphylococcus haemolyticus* isolate MD2 (Rossi et al., 2016) was used as a positive control for the reaction. The *mecA* gene was detected in *Staph. epidermidis* 8398 and 8389 (Table 1 and Figure 1A). These were the only isolates resistant to ceftiofur, which is indicative of the presence of *mecA* (CLSI, 2016b).

The determination of the type of staphylococcal cassette chromosome *mec* (SCC*mec*) present in isolates 8398 and 8389 was performed by the multiplex PCR suggested by Milheirico et al. (2007). Although both presented the same pattern of 4-fragments amplification (Figure 1B), the profile obtained did not match any of the previously characterized (Milheirico et al., 2007). Although methicillin-resistant *Staphylococcus aureus* has been isolated from goats with mastitis (Obaidat et al., 2018), this is, as we are aware, the first report of methicillin-resistant *Staph. epidermidis* obtained from goat milk samples.

To correlate antimicrobial resistance profiles with the presence of mobile genetic elements, all isolates were submitted to plasmid extraction using a lysis buffer containing lysostaphin (Sigma-Aldrich, 10 µg/mL) and pronase (Sigma-Aldrich, 1 mg/mL), followed by

treatment with SDS 2% at 65°C for 5 min, protein precipitation with 0.4 M KCl, and DNA precipitation with ethanol, as previously described (Giambiagi-Marval et al., 1990).

Most (18/26, 69%) of the isolates that presented resistance to at least 1 antimicrobial tested harbored plasmids (Table 1). On the other hand, only 1 (*Staph. xylosus* 9048) from the 25 isolates that did not present any resistance showed plasmidial forms. It is then very likely that much of the antimicrobial resistance observed is due to the presence of genes located within mobile genetic elements.

The multidrug-resistant isolates *Staph. epidermidis* 8389 and 8398 were selected to evaluate the transferability of the plasmid through conjugation experiments using *Staph. aureus* RN2677 as the recipient strain. We selected the resistance to gentamicin, which is well documented as a transferrable resistance among staphylococci (Ramsay et al., 2016), as the molecular marker of the transfer. Conjugations were performed using overnight filter matings with 10^9 cfu of both donor and recipient cells (Projan and Archer, 1989). Transconjugants were selected on brain heart infusion agar containing gentamicin (10 µg/mL), novobiocin (5 µg/mL), and rifampicin (5 µg/mL). Only *Staph. epidermidis* 8398 successfully transferred a copy of its plasmid to *Staph. aureus* RN2677, which acquired resistance to gentamicin, with a conjugation frequency of 2.7×10^8 per donor cells.

The transfer of plasmids has been demonstrated for different species of staphylococci (Otto, 2013a; Rossi et al., 2016), including between staphylococci isolated from domestic animals and isolates from human beings

(Rossi et al., 2017). Our results highlight the fact that bacteria of the *Staphylococcus* genus act as antimicrobial resistance gene reservoirs and are able to transfer mobile genetic elements among themselves, thus increasing their capacity to resist antimicrobial therapy. Even though the plasmid from isolate *Staph. epidermidis* 8389 was not transferred to *Staph. aureus*, it was reported that plasmids characterized as nonmobilizable may be horizontally transferred in biofilms (Madsen et al., 2012).

All isolates were then tested for biofilm production in 96-well microplates, following the crystal violet staining method. Isolates were classified as nonproducers or weak, moderate, or strong biofilm producers depending on how strong their optical density at 570 nm reading was (equal or lower, up to twice, up to 4 times, or higher than 4 times, respectively) compared with the negative control (Stepanović et al., 2007). The detection of the genes *icaA* and *bap*, related to biofilm production, was performed by PCR with the primers ICAAF/ICAAR and BAP2F/BAP2R (Potter et al., 2009) with the Go-Taq G2 Green Master Mix (Promega), following the manufacturer's protocols and PCR parameters according to the cited reference.

Biofilm production was an almost universal feature among the *Staphylococcus* isolates studied in this work (Table 1), as only 1 isolate was not capable of adherence. Therefore, 98% (50/51) of the isolates formed biofilms, of which 8% (4/50) were weakly adhered to the surface tested, 46% (23/50) were moderately adhered, and the other 46% formed strongly attached biofilms. There did not seem to be a direct correlation between the occurrence of the *icaA* and *bap* genes and how strong the biofilm was attached to the surface in the isolates tested (Table 1). Although isolates such as *Staph. lugdunensis* 4016, 6062, 8029, 8384, and 8386 formed strong biofilms and had both *icaA* and *bap* genes, these genes were not detected in the also strong biofilm producers *Staph. aureus* 8391, 8397, and 9057. In addition the non-biofilm-producing *Staph. capitis* 8379 was positive for the *icaA* gene. Overall, 27% (14/51) of the isolates were positive for *icaA* and 29% (15/51) were positive for *bap*. It is probable that other factors may be involved with biofilm formation in these isolates, such as different surface-anchored proteins that bind to host matrix proteins, collectively called microbial surface components recognizing adhesive matrix molecules, accumulation-associated proteins, and polysaccharide intercellular adhesin (Otto, 2013b). The importance of biofilms in the exchange of antimicrobial resistance genes and as physical barriers to the access of drugs to the target cells is well known (Madsen et al., 2012), making it an important target for therapies

aiming to control the occurrence and dissemination of goat mastitis.

Our work shows that several species of the *Staphylococcus* genus can be isolated from goat milk, potentially leading to infectious processes such as mastitis. The antimicrobial resistance phenotypes presented by them seem to be associated with mobile genetic elements, which can be transferred between different staphylococcal species, a process that is likely facilitated by the isolate's capacity to form biofilms.

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APPENDIX

Figure A1. Dendrograms built with the clustering displayed by the *Sma*I restriction fragments separated by pulsed-field gel electrophoresis of the DNA isolated from *Staphylococcus* causing goat mastitis. To allow clearer observation of genetic diversity, strains were separately analyzed according to their species (A–F).