Molecular typing and antimicrobial susceptibility profile of *Staphylococcus aureus* isolates recovered from bovine mastitis and nasal samples

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Abstract

In the present study, we aimed to determine the antimicrobial resistance and genetic structure of a population of *S. aureus* recovered from transient and persistent intramammary infections and nares/muzzles. We investigated the antimicrobial resistance of 189 *S. aureus* strains using a broad antimicrobial susceptibility profile. Furthermore, 107 *S. aureus* isolates were strain-typed using staphylococcal protein-A (*spa*) typing. Here, a great proportion of strains exhibited multidrug resistance to antimicrobials, including resistance to critically important antimicrobials, although no methicillin-

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resistant *S. aureus* strains were found. Our study did not strengthen the idea that extramammary niches (i.e., nares/muzzles) are an important source for *S. aureus*. A discrepancy in the antimicrobial resistance between *S. aureus* strains isolated from nasal/muzzles and milk samples was observed. Furthermore, *S. aureus* isolates from transient and persistent IMIs did not differ by *spa* typing, suggesting that the persistence of bovine IMIs was determined by cow factors. Thus, the high level of multidrug-resistant *S. aureus* found in the two herds studied together with the predominance of a well udder-adapted *S. aureus* strain may contribute to the history of the high prevalence of mastitis caused by *S. aureus*, leading to great animal and public health concerns.

Keywords: intramammary infection, *spa* typing, antimicrobial susceptibility, dairy cow.

Introduction

Bovine mastitis is the most prevalent and expensive disease that affects dairy farming. Moreover, it has great implications for milk production, quality of milk and dairy products, antimicrobial usage, animal welfare, the environment, and the image of the dairy sector. Among the mastitis pathogens, *Staphylococcus aureus* is a major prevalent mastitis pathogen representing a real issue for bovine udder health with unquestionable importance to human and veterinary medicine (Cunha et al. 2020). Although antibiotic treatment is widely used to fight bovine mastitis, *S. aureus* resistance to antimicrobials not only complicates antimicrobial treatment but also represents a huge challenge for public health and food security, as cows are the major reservoir for the emergence of *S. aureus* human epidemic clones (Richardson et al. 2018).

Furthermore, understanding the relationships among *S. aureus* strains is crucial for tracking epidemiology, understanding the pathogenesis of *S. aureus* infection and determining its likely origin. A variety of molecular methods have been extensively used for typing *S. aureus* isolates, of which staphylococcal protein-A (*spa*) typing is one of the most common. *spa* typing is adequate for epidemiological studies and gives reproducible, unambiguous and easily interpreted results (Stepan et al. 2004). Currently, the growing *spa* typing database developed by Harmsen et al. (2003) is the largest database for typing *S. aureus*, surpassing multilocus sequence typing.

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Thus, the objective of the present study was to determine the antimicrobial resistance and genetic structure of a population of *S. aureus* recovered from transient and persistent intramammary infections and nares/muzzles.

Material and Methods

All bacterial strains were collected from two dairy herds with approximately 125 lactating dairy cows per herd between January 2013 and January 2014. Both herds had a high bulk tank milk somatic cell count ($\geq$ 5.0 x 10^5 cells mL^{-1}) and a history of a high level of mastitis caused by *S. aureus* (as determined by veterinarian and herd statistics). The dairy farms located in Minas Gerais state, Brazil, are geographically distant (approximately 450 km).

S. aureus isolates from milk samples

Here, we used 182 *S. aureus* isolates previously identified by biochemical tests (Souza et al., 2016; Cunha et al. 2020). All *S. aureus* isolates were further speciated by matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI TOF MS), as previously described by Nonnemann et al. (2019). Furthermore, *S. aureus* identification was confirmed by polymerase chain reaction (PCR) targeting a portion of the termonuclease gene conserved in *S. aureus* (*nuc*; Sasaki et al. 2010). All *S. aureus* isolates from milk samples were analysed by an antimicrobial susceptibility test.

The *S. aureus* strains isolated from milk samples were further categorized into those derived from persistent and transient IMIs. A quarter was defined as having an IMI caused by *S. aureus* if $\geq$ 100 colony forming unit colonies mL^{-1} were detected in the milk microbiological culture. An IMI was regarded as transient if *S. aureus* was isolated at only one sampling of the consecutive samplings, in the midpoint between the first and the last samplings. A persistent IMI was assumed if a quarter had an IMI at $\geq$ 3 consecutive samplings (at least one-month interval) caused by the same *S. aureus* strain. From those isolates, we selected 100 *S. aureus* isolates from milk samples according to their epidemiological behaviour (transient vs. persistent IMIs) for *spa* typing.

S. aureus isolates from muzzle/nare samples

From the same herds and period, the nares/muzzles of dairy cows were sampled by swabbing the muzzle and the inner nares with a single moistened sterile cotton swab, as previously described (Roberson et al.

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1994). The swabs were spread inoculated onto the surface of Baird-Parker agar (Oxoid) with 5% egg yolk tellurite emulsion and incubated at 37°C for 24-48 h under aerobic conditions. From each plate, if existing, three grey to black colonies with clear zones and three grey to black colonies without clear zones were selected, transferred separately to microtubes containing brain heart infusion (BHI) broth, and incubated overnight at 37°C; 10% glycerol (final concentration) was added, and the samples were stored at -80°C until identification. Afterwards, the bacterial isolates were spread inoculated onto BHI agar for 24-48 h at 37°C. The bacterial colonies (n = 159) were first subjected to Gram staining and catalase and coagulase tests and further speciated by MALDI-TOF MS (Nonnemann et al. 2019). Furthermore, *S. aureus* identification was confirmed by PCR targeting a portion of the *S. aureus nuc* gene (Sasaki et al. 2010). Among the 159 bacterial isolates, all *S. aureus isolates* identified by both MALDI-TOF MS and PCR were used for *spa* typing and antimicrobial susceptibility tests.

DNA sequencing of the spa gene

First, DNA was extracted from the bacterial culture in BHI broth by a method adapted from the boiling methodology described by Fan et al. (1995), where phosphate-buffered saline (PBS PO_4 0.01 M, NaCl 0.15 M, pH 7.2) was replaced with tris-EDTA buffer (TE tris-HCl 10 mM, EDTA 1 mM, pH 8.0). The repeated region of *S. aureus* protein A was amplified with the primers previously described by Harmsen et al. (2003), and the DNA sequences were obtained with an ABI-3500 automatic sequencer (Applied Bisystems®, Foster, USA). *Spa* types were determined with the protocol recommended by the Ridom spa Server (http://www.spaserver.ridom.de). The obtained *spa* type sequences were analysed using the *spa* plugin included in Bionumerics 7.6 (Applied Maths NV, Sint-Martens-Latem, Belgium).

Antimicrobial susceptibility tests

Here, a broad antimicrobial susceptibility profile was performed by the automated Vitek 2® compact system (BioMériux, Inc., Durham, NC, USA) by determining minimum inhibitory concentration (MIC) using veterinary susceptibility AST-GP69 card (BioMériux, Inc., Durham, NC, USA) panels for gram-positive bacteria. The following antimicrobials were tested by the Vitek 2® kit: ampicillin/sulbactam, benzylpenicillin, cefoxitin screen, chloramphenicol, clindamycin, inducible resistance to clindamycin, enrofloxacin, erythromycin, fusidic acid, gentamicin, imipenem, kanamycin, marbofloxacin, mupirocin, nitrofurantoin, oxacillin, rifamycin, tetracycline, trimethoprim/sulfamethoxazole, and vancomycin. *S.*

aureus isolates were regarded as multidrug resistance if they were not susceptible to three or more classes of distinct antimicrobials. Furthermore, *S. aureus* resistance to penicillin was excluded for the definition of multidrug resistance because of the widespread resistance of *S. aureus* to this antimicrobial agent, as proposed by Magiorakos et al. (2012). All isolates were also tested by the Kirby Bauer's disk diffusion technique using disks for oxacillin (1 µg) and cefoxitin (10 UI/30 µg) for the prediction of methicillin-resistant *S. aureus* (MRSA). All antimicrobial susceptibility criteria were interpreted according to the Clinical and Laboratory Standards Institute (CLSI 2018a; CLSI 2018b) and European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2019).

In addition, all *S. aureus* isolates phenotypically regarded as oxacillin and/or cefoxitin resistant by MIC using the Vitek 2® compact system or Kirby Bauer's disk diffusion technique were further investigated by Etest® (bioMérieux, Basingstoke, UK) and the presence of the methicillin resistance gene (*mecA* and *mecC*). PCR analysis for the detection of the *mecA* and *mecC* genes was performed as previously described by Mehrotra et al. (2000) and Paterson et al. (2014), respectively.

Results and discussion

Apart from few available studies that have focused on the ecology of *S. aureus* (Roberson et al. 1994; Capurro et al. 2010) and those that have investigated non-*aureus* staphylococci isolated from body sites (Adkins et al. 2018), information on the frequency of bacterial isolates from nares/muzzles in dairy cows is limited. In the present study, the speciation of 159 bacteria isolates from nasal/muzzles swabs using MALDI-TOF MS resulted in the identification of *Staphylococcus chromogenes* (n = 87, 54.72%), *Staphylococcus haemolyticus* (n = 21; 13.21%), *Bacillus pumilus* (n = 12; 7.55%), *Staphylococcus hyicus* (n = 10; 6.29%), *S. aureus* (n = 9; 5.66%), *Staphylococcus xylosus* (n = 3; 1.89%), *Corynebacterium efficiens* (n = 3; 1.89%), *Enterococcus casseli* (n = 2; 1.26%), *Enterococcus faecium* (n = 2; 1.26%), *Staphylococcus saprophyticus* (n = 1; 0.63%), *Staphylococcus warneri* (n = 1; 0.63%), *Staphylococcus nepalensis* (n = 1; 0.63%), *Macrococcus caseolyticus* (n = 1; 0.63%), *Enterococcus mundtii* (n = 1; 0.63%), *Arthrobacter gandavensis* (n = 1; 0.63%), *Arthrobacter koreensis* (n = 1; 0.63%), *Arthrobacter protophormiae* (n = 1; 0.63%), *Bacillus subtilis* (n = 1; 0.63%), and *Cellulosimicrobium cellulans* (n = 1; 0.63%). All bacteria identified as *S. aureus* using MALDI-TOF MS were confirmed by the presence of the *nuc* gene using PCR. While nares represent a primary reservoir of *S. aureus* humans, nares/muzzles

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did not appear to be a major reservoir for *S. aureus* in dairy cows, corroborating the findings of Capurro et al. (2010), although nares/muzzles appeared to be mainly colonized by non-*aureus* staphylococci.

Among the 107 *S. aureus* isolates obtained, t605 (93.46%; 99.00% of them from milk), t189 (1.87%; one from milk and one from nares/muzzles), t098 (3.74%; from nares/muzzles swabs) and t127 (0.93%; from nares/muzzles swabs) were molecularly identified (Figure 1). Thus, our study did not strengthen the idea that extramammary niches (i.e., nares/muzzles) are an important source for *S. aureus* strains that cause persistent IMIs in dairy cows, which also substantiates their contagious behaviour. The only *S. aureus* strain ST89 originating from transient IMI was also isolated in the nose of another cow, indicating that this *S. aureus* strain can cause IMI in this herd, but it is not associated with persistent IMIs. However, one *S. aureus* strain isolated from herd A was the same strain that caused persistent IMI, suggesting that this strain might adapt to extramammary niches and colonize them, as previously suggested for teat skin (Paduch and Krömker 2011). Thus, even though *S. aureus* can colonize extramammary niches of dairy cows (i.e., nares/muzzles) (Roberson et al. 1994; Capurro et al. 2010), our study showed that the udder is the most important reservoir of these bacteria.

We also showed that one *S. aureus* strain was widespread in the two herds investigated, although the herds are geographically distant; this *S. aureus* strain was responsible for almost all IMIs, probably because it is well adapted to the udder, leading to the persistence of IMIs. Thus, our results are in agreement with other reports that indicated that few specialized *S. aureus* types with broad geographic distribution are responsible for most of the IMIs in dairy herds (Srednik et al. 2018).

Another important finding was that the herd that only had one *S. aureus* strain was a closed herd, where no cows were purchased from other herds in recent decades, in contrast to the other herd, an open herd, in which new *S. aureus* strains can be introduced by animals from foreign herds; we identified an additional *S. aureus* strain in the open herd. Although the owner of the open herd acquired several dairy cows from distinct dairy herds (personal communication), the most udder-adapted *S. aureus* strain might spread more efficiently, preventing the spread of other less well-udder adapted (i.e., opportunistic) *S. aureus* strains. In agreement with our outcomes, the *S. aureus* ST605 clone was the most important clone related to bovine IMIs in another Brazilian study (Silva et al. 2013). To the best of our knowledge, this study is the first report of the isolation of the *S. aureus* ST098 clone from bovine samples; in this study, the *S. aureus* ST098 clone was the most common *S. aureus* strain isolated from nasal samples. We also

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178 demonstrated that the persistence of bovine IMIs was probably mainly determined by cow factors, as the
179 same *S. aureus* strain (ST605 clone) caused persistent and transient IMIs.

180 The antimicrobial susceptibility results by the Vitek 2® Compact system are summarized in
181 Table 1. Our results showed that 46.56% (n = 88) of *S. aureus* isolates were not susceptible to at least
182 three distinct classes of antimicrobials. Beyond that, even if we excluded resistance to penicillin, as
183 proposed by Magiorakos et al. (2012), 30 (15.87%) isolates were regarded as multidrug resistant *S.*
184 *aureus*. Overall, our study showed that the *S. aureus* isolates from IMIs had considerable resistance to
185 antimicrobials, including resistance to critically important antimicrobials, such as macrolides (e.g.,
186 erythromycin) and glycopeptides (e.g., vancomycin). Moreover, some intermediate resistance to critically
187 important antimicrobials such as the group quinolones (e.g., marbofloxacin and enrofloxacin) was also
188 found. Altogether, our data emphasize that *S. aureus* IMIs are concerning to animal and public health. We
189 also found eight and four oxacillin-resistant and cefoxitin-resistant *S. aureus* isolates from milk samples,
190 respectively. Therefore, although some of them were confirmed by Kirby Bauer's disk diffusion
191 technique, none of the strains were confirmed by the E-test® or the presence of the *mecA* and *mecC* genes,
192 suggesting that none of them could be regarded as MRSA.

193 Although a limited number of *S. aureus* strains were isolated from nares/muzzles samples and
194 characterized in this study, a discrepancy in antimicrobial resistance between *S. aureus* isolated from
195 nasal/muzzles and milk samples was observed. For instance, while a great proportion of the *S. aureus*
196 isolates from milk samples were resistant to benzylpenicillin (94.50%), gentamycin (80%) and
197 tetracycline (43.96%), none of the *S. aureus* isolates from noses/muzzles were resistant to these
198 antimicrobials. In contrast, the *S. aureus* isolates from noses/muzzles were more prone to be resistant to
199 clindamycin and erythromycin. We hypothesized that this divergence in antimicrobial resistance could be
200 attributed to, at least in part, the distinct history of exposure to antimicrobials between *S. aureus* isolated
201 from noses/muzzles compared to those isolated from milk samples. For instance, *S. aureus* isolates from
202 noses/muzzles may have been exposed to antimicrobials used systemically since the early stages of life
203 for a longer time (since colonization), and these antimicrobials are totally divergent from the
204 antimicrobials used mainly locally for mastitis treatment after first parturition.
205
206 **Declarations**
207 **Funding**

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Conflict of interest

The authors declared that they have no potential conflicts of interest with respect to the research, authorship, publication of this article and/or financial and personal relationships that could inappropriately influence this study.

Ethics approval

This study was approved by the Animal Research Ethics Committee of the Federal University of Minas Gerais - Brazil under protocol number 201/2011.

Consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials.

Code availability

Not applicable.

Authors' contributions

RPS designed the experiments, performed all analyses, and drafted and edited the manuscript. FNS designed the experiments, collected samples, performed microbiological analysis, supervised the studies and drafted and edited the manuscript. ACDO provided technical help, performed microbiological analysis and edited the manuscript. AFC designed the experiments, collected samples, performed microbiological analysis and edited the manuscript. AFSF, JA, and LZM performed the molecular characterization of the *S. aureus* isolates, analysed the data and edited the manuscript. AMMPDL, MBH and MMOPC designed the experiments, supervised the studies and edited the manuscript. RPS and FNS should be regarded as co-first authors.

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310 **Figure 1.** Dendrogram of *S. aureus* strains isolated from milk (n = 100) and nares/muzzles samples (n =

311 7) discriminated by *spa* typing.