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Pulsed-field gel electrophoresis genotyping and antimicrobial resistance profiles of *Staphylococcus aureus* isolated from subclinical mastitis in dairy cattle in Brazil

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ABSTRACT. *Staphylococcus aureus* is a contagious pathogen frequently associated with bovine subclinical mastitis (SCM) cases in Brazil. Molecular characterization of *S. aureus* allows monitoring of specific features at the strain level, such as transmission routes and antimicrobial resistance, and it can be a helpful tool for implementation of prevention measures among and within herds. We evaluated molecular typing and antimicrobial resistance profiles of *S. aureus* isolates from lactating cows with SCM. A total of 79 *S. aureus* isolates recovered from bovine SCM were submitted to pulsed-field gel electrophoresis (PFGE) analysis and tested for antimicrobial susceptibility against 13 antimicrobials, based on minimum inhibitory concentrations. Based on the band patterns generated by PFGE, dendrograms were constructed to compare *S. aureus* pulsotypes (n = 17). Resistance was observed for amoxicillin (100% of the isolates), erythromycin (96%) and for ampicillin and

penicillin (77%). All *S. aureus* isolates were susceptible to gentamicin, enrofloxacin, ciprofloxacin and tetracycline. One methicillin-resistant *S. aureus* strain was identified based on resistance to cefoxitin. We found a wide genotypic diversity of *S. aureus* causing SCM among the isolates. In general, *S. aureus* was sensitive to quinolones and aminoglycosides, while we observed β-lactams resistance in most of the isolates. Our findings are similar to those of previous results that reported high resistance of *S. aureus* mainly to β-lactams. Consequently, control measures for this bacterium need to be implemented in order to control the spread of the disease and establish more assertive treatment protocols.

Key words: *Staphylococcus aureus*; PFGE; MIC; Bovine mastitis

INTRODUCTION

Staphylococcus aureus is frequently isolated from raw milk (Boufaida et al., 2012; Saidi et al., 2013); its prevalence in Brazilian dairy farms is variable and can reach up to approximately 70% (Mesquita et al., 2019). Cows with *S. aureus* intramammary infection (IMI) may present high somatic cell counts (SCC), but with a cyclic pattern of SCC and bacterial shedding. This cyclic pattern of *S. aureus* shedding makes it difficult to identify the infected cows in dairy herds, which facilitates the contagious transmission of *S. aureus* (Zecconi et al., 2005). Intramammary infections caused by *S. aureus* usually become persistent, in part because of expression of virulence (e.g. biofilm formation) and antimicrobial (ATM) resistance factors (Haveri et al., 2007). The mechanisms by which *S. aureus* induces chronic IMI involve modulation and evasion of the immune system, such as adherence and internalization in mammary epithelial cells, as well as high levels of ATM resistance (Haveri et al., 2007; Zecconi and Scali, 2013).

There is a major public and animal health concern about infections caused by antimicrobial resistant *S. aureus*. Recent studies evaluating *S. aureus* isolated from bovine mastitis reported resistance of this pathogen to antimicrobials widely used in veterinary and human medicine, especially to β-lactam antibiotics (Oliveira et al., 2012; Xavier et al., 2017; Benites et al., 2021; Freu et al., 2022). Understanding the epidemiology of antimicrobial resistance of *S. aureus* causing mastitis may allow the development of preventive strategies to decrease existing resistance and prevent the emergence of new strains of resistant *S. aureus*.

Molecular characterization of *S. aureus* can be a helpful tool for understanding the epidemiology of this pathogen and for implementation of prevention measures, among and within herds. Previous studies suggested that within herds few strains cause bovine mastitis, whereas other studies suggest that IMIs are caused by a diverse genetic variability of strains (André et al., 2008; Lee et al., 2012). Several molecular typing methods based on DNA banding patterns are available for *S. aureus* characterization. Pulsed-field gel electrophoresis (PFGE) is often used because it has a high discriminatory power (Dingwell et al., 2006; Ikawaty et al., 2009; Adkins et al., 2016). *S. aureus* resistance characterization in Brazilian dairy herds allows constant monitoring of the strains and their association with the main antimicrobials used in these herds.

The objective of the present study was to a) assess the molecular typing diversity of *S. aureus* isolated from subclinical mastitis (SCM) in Brazilian dairy herds using PFGE; and b) identify the ATM resistance profiles of *S. aureus* by the minimum inhibitory concentration (MIC) test.

MATERIAL AND METHODS

Ethics statement

All the experimental procedures were performed according to the National Council of Control of Animal Experimentation (CONCEA) directives and approved by the Animal Use Ethics Committee of the Faculty of Veterinary Medicine and Animal Science of the University of São Paulo (CEUA/FMVZ), protocol no. 8178270617.

Characterization of isolates

A total of 79 isolates of *Staphylococcus* spp. tentatively classified as *S. aureus* by conventional microbiological culture were obtained from a bacteria collection of Milk Quality Laboratory (Qualileite, FMVZ, University of São Paulo) that had been cryopreserved in brain heart infusion (BHI) solution and 20% glycerin at -80°C. The criteria for selecting the isolates was based on the suspected isolation of *S. aureus* from subclinical cases during lactation from composite milk samples or from individual quarters. The selected isolates were obtained between 2015 and 2016 from four Brazilian dairy farms in southeastern regions of the country (Minas Gerais and São Paulo states), markedly known to have a history of *S. aureus* SCM cases. An interval of 14 days was considered for categorization of new cases of SCM and all possible *S. aureus* isolates per cow (e.g. more than one-quarter SCM infected) were considered.

To certify the correct identification of *S. aureus* isolates after thawing according to National Mastitis Council recommendations (NMC, 2017), an aliquot of all cryopreserved isolates was first inoculated on blood agar plates (35°C/24 h) and then a single colony was streaked onto the surface of tryptic soy agar (TSA) plates, followed by incubation at 35°C/24 h. Afterward, one colony was applied to the steel plate spot with a disposable loop and submitted to Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) identification, according to (Barcelos et al., 2019). For our study, all 79 *S. aureus* isolates grew at re-cultivation (i.e., no contamination) and were identified to the species level by MALDI-TOF MS (score > 2).

PFGE genotyping

PFGE analyses were performed according to a protocol defined by the Centers for Disease Control and Prevention. After a 24 h incubation in the TSA plate, some colonies were inoculated in BHI broth and incubated at 37°C for approximately 18 h. After this period, the plugs were prepared by adding 2.5 µL of lysostaphin and 150 µL of 1.8% low melting agarose (BioRad, France) previously diluted in 0.5x TBE buffer.

This mixture was quickly dispensed in a mold and the plugs were dried at room temperature. After drying, the plugs were soaked in EC solution (Tris HCl 1M; NaCl 2M; EDTA tetrasodium 1M; sodium deoxycholate 2%; sarcosyl 5%) and incubated overnight at 37 °C. Subsequently, the plugs were washed and digested with 30 U of *SmaI* restriction enzyme (FastDigest, Thermo Scientific), according to the manufacturer's recommendations.

The gel was made in a 15-well mold (12 samples + 3 standards) and PFGE was performed in a CHEF-DR III system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) according to the following parameters: 6 V / cm, temperature 14°C, initial pulse 5 s, final pulse 40 s, angle included 120°C and operating time 22 h. The gel was then stained in ethidium bromide solution (final concentration 1 mg / ml) for 30-40 min in a covered container. The images were captured by an UV transilluminator and the DNA fragments of ATCC BAA-664 (*Salmonella* serotype Braenderup strain H9812) were used as molecular weight markers for *XbaI* digestion standards.

Antimicrobial susceptibility test

The MIC analyses were performed for *S. aureus* isolates using a microdilution broth method according to Clinical and Laboratory Standards Institute (CLSI, 2015). Seventy-nine isolates and the *S. aureus* ATCC 29213 strain were used to determine the MIC of 13 ATMs (amoxicillin, ampicillin, ceftiofur, gentamicin, penicillin, enrofloxacin, oxytetracycline, tetracycline, ciprofloxacin, cephalexin, lincomycin, erythromycin and cefoxitin). Twelve serial dilutions were performed (concentrations ranging from 0.03 to 64 µg / mL) using stock solutions at a concentration of 1 mg / mL prepared using specific solvents for each ATM.

Briefly, selected colonies were inoculated on blood agar and after 24 h at 37 ºC, they were transferred to BHI broth and reincubated under the same conditions. Upon BHI growth, the colonies were suspended in 2 mL of sterile 0.9% saline, and turbidity was adjusted to 0.5 McFarland scale containing approximately 1.5×10^8 cfu / mL, using a nephelometer (Uniscience, São Paulo, Brazil).

For microdilution assay, sterile microplates with 96 wells were used, filled with Mueller Hinton broth (MH) adjusted to pH 7.2 ± 0.2. For each isolate, 2 microplate wells (A and B) were used, and the control rows were distributed along with the ATMs. A total of two-fold serial dilutions were made to obtain concentrations of the ATMs ranging from 64 to 0.03 µg / mL. After dilutions, 12.5 µL of the inoculum of *S. aureus* isolates were added to each well (one well per inoculum, final volume = 137.5 µL) and incubated aerobically for 24 h at 37°C. The inoculum dilution methods resulted in an appropriate inoculum concentration of approximately 5×10^5 cfu/ml. Following the 24-h incubation, 20 µL of the MTT reagent (Thiazolyl Blue Tetrazolium Bromide, Sigma) was pipetted into each well, and were visually read after 3 h. The purple staining generated by MTT indicated the presence of viable cells. The dilution ranges and the interpretation criteria for determining the susceptible, intermediate and resistant were done according to CLSI (2015) for amoxicillin, ampicillin, ceftiofur, gentamicin, penicillin, oxytetracycline, tetracycline, lincomycin, erythromycin and cefoxitin;

European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2020) for ciprofloxacin and cephalexin; and Rubin et al., (2011) for enrofloxacin. Isolates with intermediate susceptibility were considered resistant. Strains of *S. aureus* resistant to cefoxitin were screened as MRSA (Fernandes et al., 2005).

Data analysis

The DICE coefficient and average association method (UPGMA) were used to compare the fingerprints obtained by PFGE, using a 2% tolerance according the BioNumerics software version 7.6 (Applied Maths, Sint-Martens-Latem, Belgium). Comparisons were made following the 80% similarity criteria for the clusters, considered the gold standard (McDougall et al., 2003; Van Belkum et al., 2007).

Survival analysis was performed by PROC LIFETEST (SAS ver. 9.4, SAS Institute Inc., Cary, NC, USA) to determine MIC and concentrations tested as a time variable (Cortinhas et al., 2013). The isolates that grew at the highest concentration tested (64 μg / mL) were considered resistant. The MIC value was given by the lowest concentration of each ATM that visibly inhibited the growth of *S. aureus*. MIC_{50} and MIC_{90} were determined based on inhibition of 50 and 90% of the tested isolates, respectively. The ATM concentration was defined as variable over time, while the non-difference between the survival curves was tested using the log-rank and Wilcoxon tests, on the Kaplan-Meier curves. Values of $P < 0.05$ were considered significant.

RESULTS

PFGE analysis

Figure 1 shows the dendrogram of all *S. aureus* isolates (n = 79). This analysis indicated that the 79 isolates were grouped into 17 distinct pulsotypes, numbered from 1 to 17 and named from A to Q, with pulsotypes K and M being the most prevalent (n = 21 and 13). The isolates designated as pulsotypes K and M originated from herds 1, 2 and 4. Of the total evaluated, eight isolates had unique PGFE patterns.

MIC analysis

The ATM susceptibility testing of *S. aureus* isolates showed 100% susceptibility to gentamicin, enrofloxacin, ciprofloxacin and tetracycline hydrochloride, followed by oxytetracycline hydrochloride (98.7%) and ceftiofur (94.9%; Table 1). On the other hand, lower ATM susceptibilities were observed for ampicillin and penicillin, both with 22.7%. For amoxicillin, resistance was observed in 100% of the isolates, followed by erythromycin with 96.2% of resistance for the evaluated isolates. Only one isolate showed cefoxitin resistance.

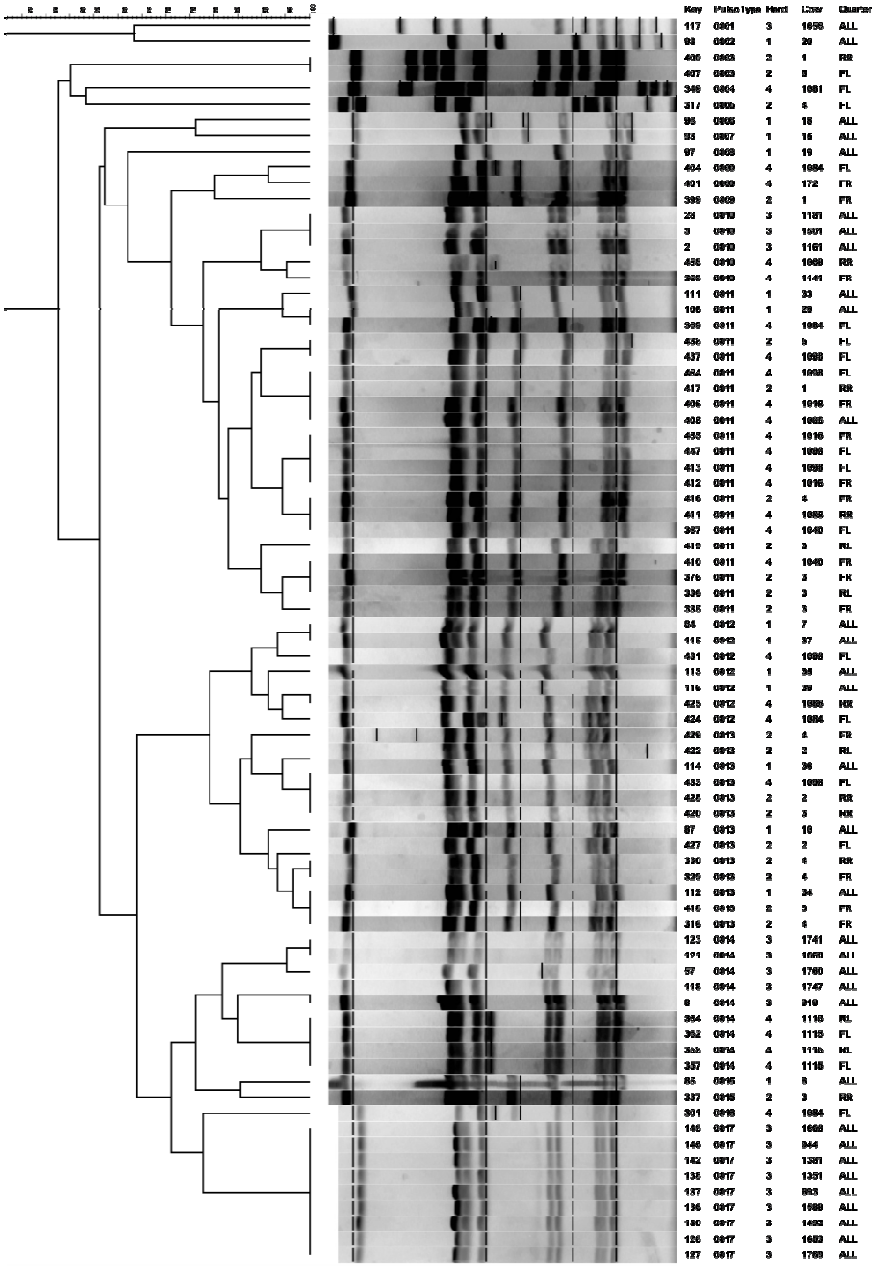

Figure 1. Pulsed Field Gel Electrophoresis (PFGE) dendrogram of 79 isolates of *Staphylococcus aureus* obtained from cows with subclinical mastitis during lactation. The dotted vertical line indicates the cutoff point (80% similarity). Key = isolates. Mammary quarters are left rear (LR), right rear (RR), left front (LF), right front (RF), and all quarters (ALL).

Table 1. Antimicrobial susceptibility of 79 strains of *Staphylococcus aureus* isolated from subclinical mastitis in four dairy herds in Brazil.

Antimicrobial[2]	Res.[3] (%)	Frequency (%) of isolates at each indicated MIC[1] (µg / mL)												MIC_{50} [4]	MIC_{90} [5]
		0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32	64		
Amoxicillin	92.4	-	-	-	-	-	2.53	1.27	0.0	0.0	1.27	1.27	1.27	≥64	≥64
Ampicillin	46.8	17.7	3.8	0.0	1.27	3.8	0.0	0.0	1.27	3.8	5.0	7.6	10.12	≥64	≥64
Cefalexin	38.0	-	-	-	7.6	14.0	19.0	7.6	1.27	2.53	0.0	3.8	6.4	≥8	≥64
Cefoxitin	0	-	-	-	-	-	11.4	74.7	12.6	1.27	-	-	-	≥2	≥4
Ceftiofur	0	-	-	-	-	1.27	51.9	41.8	5.0	-	-	-	-	≥1	≥2
Ciprofloxacin	0	-	20.3	73.4	5.0	1.27	-	-	-	-	-	-	-	≥0.125	≥0.125
Enrofloxacin	0	14.0	64.5	20.2	1.27	-	-	-	-	-	-	-	-	≥0.06	≥0.125
Erythromycin	0	-	-	-	-	3.8	78.4	17.8	-	-	-	-	-	≥1	≥2
Gentamicin	0	-	-	8.9	63.3	25.3	2.53	-	-	-	-	-	-	≥0.25	≥0.5
Lincomycin	0	-	-	-	5.0	46.8	36.7	6.4	2.53	1.27	1.27	-	-	≥0.5	≥2
Oxytetracycline	0	-	-	-	-	-	1.27	56.9	40.5	1.27	-	-	-	≥2	≥4
Penicillin	60.7	20.2	1.27	1.27	0.0	0.0	0.0	1.27	0.0	0.0	1.27	2.53	11.4	≥64	≥64
Tetracycline	0	1.27	1.27	0.0	7.6	54.4	34.1	1.27	-	-	-	-	-	≥0.5	≥1

[1] MIC: Minimum inhibitory concentration; [2] The results were interpreted according to Rubin et al. (2011), CLSI (2015) and EUCAST (2020). The resistant category included those isolates classified as intermediate or resistant. Light shading represents the sensitivity zone and dark shading represents the resistant zone; [3] Isolates resistant to the highest concentration of antimicrobial tested; [4] MIC_{50} = MIC (µg / mL) that inhibited 50% of the isolates; [5] MIC_{90} = MIC (µg / mL) that inhibited 90% of the isolates.

The lowest MIC_{90} values for *S. aureus* were observed for enrofloxacin and ciprofloxacin, both ≥0.0125 µg/mL; while amoxicillin, ampicillin, cephalexin and penicillin had the highest MIC_{90} values (≥64 µg/mL; Table 1). According to the MIC_{50}, enrofloxacin was the ATM evaluated that had the lowest value (≥0.06 µg/mL), while amoxicillin and penicillin had the highest values (≥64 µg/mL).

The Kaplan-Meier survival curves showed homogeneous curves between ceftiofur, ciprofloxacin, enrofloxacin, erythromycin, gentamicin, oxytetracycline and tetracycline. For amoxicillin, ampicillin, cefalexin, cefoxitin, lincomycin and penicillin heterogeneous curves were observed against *S. aureus* ($P < 0.0001$ for log-rank test and $P = 0.0015$ for Wilcoxon test).

DISCUSSION

In this study, *S. aureus* isolates collected from SCM were submitted for PFGE to characterize the genotypic diversity, followed by susceptibility antimicrobial analyses. A great diversity of strains was observed in the isolates evaluated which corroborates the hypothesis of the genetic variability of *S. aureus* that causes IMI although frequent pulsotypes (K and M) were identified. In addition, high levels of ATM resistance were observed, especially for β-lactam antibiotics. Ours is another study that confirms the high diversity of *S. aureus* and the need for specific control strategies against the bacteria spread in the herd. In addition, we emphasize the importance of correct antimicrobial choice for a better biological cure.

The predominance of some pulsotypes within specific herds indicates that the transmission between cows occurred from a common source in these herds. However, the diversity of *S. aureus* pulsotypes observed in our study suggests the presence of different sources of strains causing SCM. Like our study, Rabello et al. (2005), Vitale et al. (2018) and Srednik et al. (2018) also reported a wide genotypic diversity of *S. aureus* when PFGE was used. Dorneles et al. (2019) also observed a wide genotypic variability in 79 *S. aureus* isolates where 34 different pulsotypes were found (using 100% of similarity). Rabello et al.

(2005) found among nine dairy herds 16 distinct pulsotypes (15%) and 24 subtypes considering 107 *S. aureus* isolates. A greater genetic variability of *S. aureus* (29%) was reported in a study using 80 *S. aureus* isolates from clinical and SCM, in which 23 distinct pulsotypes were observed, using 80% of similarity, like the present study (Srednik et al., 2018), showing the high capacity of transmission of specific pulsotypes within dairy herds.

In the present study, it was possible to observe 17 pulsotypes among all isolates (n = 79), with pulsotypes K and M being more prevalent, distributed in more than one herd. However, the pulsotype Q was observed only in the herd 3, suggesting a common source of infected cows in this herd, which could spread from cow to cow during milking. This prevalence of the same *S. aureus* genotype (persistent one) within a specific herd could be partially explained by the ability of the pathogen to adapt to a specific condition of the herd (Goerke and Wolz, 2004; Tuchscherr et al., 2010), such as hygiene conditions or milking routine.

The high percentage of strains resistant to β-lactams in the present study was similar to that observed in other previous studies (Daka et al., 2012; Xavier et al., 2017; Benites et al., 2021). In Brazil, resistance to β-lactams has also been observed from 18 to 95% in *S. aureus* isolated from bovine milk (Medeiros et al., 2009; Silva et al., 2012; Ferreira et al., 2016; Girardini et al., 2016; Carvalho et al., 2018). Xavier et al. (2017) reported high resistance (>76.0%) to amoxicillin, ampicillin and penicillin of *S. aureus* recovered from SCM cases; and more recently, Freu et al. (2022) showed resistance of 60.7% to penicillin from *S. aureus* recovered from cows with clinical mastitis. This high β-lactams resistance values in Brazil may result from the high use of this antimicrobial class, either by intramammary or systemic route. Tomazi and Santos (2020) carried out a survey to quantify antimicrobial consumption for treatment of clinical mastitis in Brazil and observed that among intramammary drugs, aminoglycosides had the highest ATI (11.7 DDD per 1,000 lactating cow-days), followed by tetracycline, aminoglycoside and polypeptide.

In the present study, the resistance against cefoxitin was used as a predictor of methicillin-resistant (MRSA) strains, as it was used in susceptibility tests to identify strains resistant to methicillin (Fernandes et al., 2005), since methicillin is no longer manufactured (Swenson et al., 2009; Zurita et al., 2010; Ho et al., 2016;). We observed only 1 (1.2%) isolate resistant to cefoxitin, classified as MRSA, which indicated a low MRSA diffusion among bovine mastitis isolates as previously reported (Monistero et al., 2018). Although high rates of MRSA are not generally found in mastitis samples, some factors may be the cause of variations in responses to MRSA, both in prevalence and in the expression of *mecC* or *mecA* genes. In humans, the geographical variation in MRSA can be explain by differences in diseases control practices and pathogen-specific characteristics of the circulating clones (Lee et al., 2018). In dairy cows, the prevalence of MRSA has been associated with lack of some milking hygienic measures, such pre- and post-milking teat dip that can result to high MRSA prevalence in milk (Guimarães et al., 2017). This lack of analyses for the detection of *mecC* or *mecA* genes was a limitation of our study, although the cefoxitin test has been considered fully accurate as a marker for the detection of MRSA, both with disc diffusion and agar dilution methods (Fernandes et al., 2005).

Finally, the survival curve suggested that all *S. aureus* isolates were susceptible to gentamicin, enrofloxacin and tetracycline hydrochloride, as observed in previous studies (Teixeira et al., 2014; Yang et al., 2016; Dorneles et al., 2019). In Brazil, Teixeira et al. (2014) evaluated the MIC in 278 *S. aureus* isolates from bulk tank milk and observed 94%

and 92.54% of sensitivity to gentamicin and enrofloxacin, respectively. Similarly, a high frequency of susceptible isolates of *S. aureus* isolated from bovine mastitis (n = 44) was observed for gentamicin (90.91%) and tetracycline (81.82%) in an ATM resistance study in China (Yang et al., 2016). More recently, Dorneles et al. (2019) observed high frequency of susceptible isolates for gentamicin (97.2%) and enrofloxacin (98.6%) against *S. aureus* (n = 71) causing mastitis.

In the present study, 100% of the isolates showed sensitivity to ciprofloxacin, and this high frequency of susceptible isolates was also found by Scapin et al. (2010) and Sun et al. (2018), 97.4% and 86.5%, respectively. Although the use of ciprofloxacin has been restricted in some countries and has not been allowed to use in food producing animals (Jacoby, 2005), in Brazil ciprofloxacin is allowed to use as an intramammary ATM option for treatment of mastitis due to its effectiveness (Joshi and Gokale, 2006), alone or combined with anti-inflammatory drugs. A possible explanation for these rates of sensitivity to ciprofloxacin is due to its ability, as well as other antibiotics in the quinolone group, to accumulate in the phagocytic cell, possibly leading to the death of the mastitis-causing pathogen (Qin and Sun, 2009).

Additional epidemiological studies are needed to search for the expression of resistance genes among *S. aureus* isolates distributed in dairy herds, particularly focused on the detection of those that are methicillin-resistant, which constitutes a risk factor for public health.

CONCLUSION

A wide genotypic diversity of *S. aureus* was observed among the isolates evaluated. In general, *S. aureus* was sensitive to quinolones and aminoglycosides, but we observed β-lactams resistance in most *S. aureus* isolates evaluated.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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