

Article

Prevalence and antibiotic resistance of *Staphylococcus aureus* and *Streptococcus agalactiae* in family-owned dairy herds in the state of Minas Gerais, Brazil

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RESUMO

A mastite bovina é a doença mais frequente em rebanhos leiteiros no mundo todo, acarretando grandes prejuízos econômicos para os produtores e a indústria. A doença também tem implicações na saúde pública, devido ao potencial zoonótico de alguns agentes envolvidos em sua etiologia e à presença de resíduos de antibióticos no leite. Considerando o aspecto multifatorial da doença e a importância da produção de leite na agricultura familiar, o conhecimento dos agentes envolvidos em sua etiologia e os perfis de suscetibilidade aos antibióticos é de suma importância. Neste estudo, avaliaram-se as prevalências de *Staphylococcus aureus* e *Streptococcus agalactiae* em rebanhos de propriedades de agricultura familiar, na região do Campo das Vertentes, Minas Gerais, e também se analisou a resistência destes patógenos aos antibióticos mais comumente utilizados no tratamento da mastite em rebanhos leiteiros. O estudo envolveu 200 propriedades, abrangendo uma área de aproximadamente 12.564 km². As prevalências dos patógenos *S. aureus* e *S. agalactiae* foram obtidas por meio de análises microbiológicas de amostras de leite do tanque de expansão de cada propriedade. Para a detecção de *S. aureus*, utilizou-se o meio seletivo Ágar Baird-Parker modificado e, para a detecção de *S. agalactiae*, o meio seletivo Ágar Edwards modificado, enriquecido com 5% de sangue ovino desfibrinado. Foi utilizada a técnica de difusão em discos para a avaliação de resistência aos antimicrobianos. Os resultados indicaram altas prevalências dos patógenos *S. aureus* (71,0%) e *S. agalactiae* (68,0%), com elevados níveis de

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resistência frente à polimixina B, penicilina G e ampicilina. O fenômeno da multirresistência foi observado a níveis elevados para ambos os patógenos. Os resultados demonstram que há falhas no controle da mastite contagiosa nos rebanhos estudados, apontando para a necessidade de medidas mais efetivas para o controle destes patógenos e do uso mais criterioso dos antibióticos, visando minimizar o problema da resistência ao medicamento. **Palavras-chave:** Mastite, Qualidade do leite, Contagem de células somáticas, Produção de leite.

Introduction

The Brazilian food production is intrinsically related to small farms, especially family farming, which represents approximately 84% of all properties and yields US\$ 55.2 billion in revenue annually. The Brazilian family farming segment ranks in the eighth position amongst the largest food producers in the world (MAPA, 2017).

Milk production is one of the main activities developed in family farming, accounting for 52% of the revenue of these properties; it represents an important complement to household income, playing an important role in local economy (GUANZIROLI; CARDIM, 2000; BEZERRA; SCHLINDWEIN, 2017). The State of Minas Gerais is the largest milk producer in Brazil. In 2016, approximately 6.5 billion liters of milk were produced in the state, which represented 26% of the milk purchased by the Brazilian industry that year (IBGE, 2017).

Despite the importance of this activity, the Brazilian milk production segment has large bottleneck. One of the main ones is still the low productivity of animals, with a national average of only 1,381 kg milk/cow/year, while the US produced on average 9,790 kg milk/cow/year in 2012 (USDA, 2012). The low productivity of cows and consequently of Brazilian dairy herds has multifactorial causes. Sanitary aspects plays a leading role, especially because of the endemic occurrence of mastitis. This is the most frequent disease and the greater cause of losses in dairy farms worldwide (KEEFE, 2012).

The economic losses caused by mastitis are associated mainly with reduction in milk production, increased labor costs, including veterinary services, drug spending, and milk discard of affected animals, early death or animal disposal (OVIEDO-BOYSO et al. al., 2007; LOPES et al., 2012). In addition to direct losses to producers, this disease promotes changes in milk composition associated with increased bulk milk somatic cell count (BMSCC) and low milk quality (LANGONI et al., 2011).

Among the mastitis-causing bacteria, the contagious pathogens *Staphylococcus aureus* and *Streptococcus agalactiae* are usually associated with more severe damage to the mammary gland as compared to other microorganisms (REYHER et al., 2012). These bacteria are the most common causes of mastitis in Brazilian dairy herds (OLIVEIRA et al., 2009; SAAB et al., 2014). Studies focusing on *S. agalactiae* showed that it was present in 60% of the properties in the Zona da Mata region, State of Minas Gerais (BRITO et al., 1999), and in 39.7% of farms in different municipalities in the state (ELIAS et al., 2012). In addition to their high prevalence, other studies have pointed to their increased resistance to antimicrobials (BRITO; BRITO, 2001; OLIVEIRA et al., 2012; ALENCAR et al., 2014).

Oliveira et al. (2013) studied approximately 6,000 lactating cows from 112 herds in the States of Minas Gerais and Rio de Janeiro, and found a prevalence of 93.0% for *S. aureus* and 41.0% for *S. agalactiae*. In another study, Cunha et al. (2015) found a prevalence of 55.4% for subclinical mastitis in dairy herds in the municipality of Viçosa, in the Zona da Mata region (State of Minas Gerais), with *Corynebacterium* spp. (32.99%), *S. aureus* (28.35%) and *S. agalactiae* (13.66%) as the most frequent isolated etiological agents. Besides the direct losses that *S. aureus* and *S. agalactiae* cause to producers, the simple presence of these pathogens in milk can be indicative of low quality, especially when it comes to milk quality indicators such as total bacterial count (TBC) and BMSCC (PETER et al., 2013).

Bovine mastitis is a disease that requires antibiotics, which is mostly administered to treat clinical cases, and dry cow therapy (ERSKINE et al., 2004). This, however, increases the risk of antimicrobial residues in milk and derivatives. The continuous, reckless antibiotic treatment is a determining factor for selecting resistant strains, potentially harmful to public health (OIE, 2010). The dry cow therapy, associated with high BMSCC and a history of mastitis, has potential for selecting antibiotic resistant strains (RAJALA-SCHULTZ et al, 2009).

The high prevalence of mastitis in Brazilian herds and its economic and health impacts require periodic monitoring of both the pathogens involved in its etiology and their antibiotic susceptibility. In addition, it is important to study the resistance of the bacteria involved in intramammary infections (IMI) to antibiotics that are commonly used against mastitis. This allows for monitoring the resistance dynamics and adopting more effective treatment protocols to combat the disease.

This study evaluates the prevalence of *S. aureus* and *S. agalactiae* in bulk milk tanks of small dairy herds in the Campo das Vertentes region, in the south of Minas Gerais. It also analyzes their resistance to the antibiotics that have been commonly used to treat bovine mastitis.

Material and methods

This study analyzed the bulk milk tank samples from 200 dairy herds located in the Campo das Vertentes region (approximated of 12,564 km²). Since the properties were family farms, i.e., had less than 4 fiscal modules (less than 120 hectares), they produced less than 350 liters of milk/day and predominately employed family labor (ZOCCAL; SOUZA; GOMES, 2004). Probabilistic criteria were not used to choose the properties. The inclusion of farms was based on the previous relationship of the milk industry with the research unit.

A descriptive study was carried out in the properties to evaluate the prevalence of the pathogens *S. aureus* and *S. agalactiae*. To this end, samples of approximately 40 mL of bulk milk were collected in expansion tanks after homogenization. The samples were stored at -20°C for up to two weeks before the microbiological analyses. Freezing milk samples at -20°C for up to six weeks does not appreciably affect the recovery rate of *Staphylococcus aureus* and *Streptococcus agalactiae* in milk samples submitted to microbiological analysis (OLIVER et al., 2004).

To detect *S. aureus*, an aliquot of 50µL of each milk sample was spread in a Petri dish containing modified Baird-Parker Agar selective medium, which was incubated at 37°C for 24-48 hours. After incubation, colonies in dark gray to black color, glossy, convex shape, with 1-5mm of diameter and a clear 2-5-mm-wide halo at their periphery were characterized as positive for *S. aureus* (SCHOELLERS; INGHAM, 2001).

To detect *S. agalactiae*, an aliquot of 50µL of each milk sample was spread in modified Edwards Agar Selective Medium Petri dishes enriched with 5% defibrinated sheep blood. The plates were incubated for 24-48 hours at 37°C. Colonies that had a bluish color, bright appearance and convex shape, 1-5 mm in diameter, with or without an opaque zone on the periphery, were considered positive (SAWANT et al., 2002).

After evaluating the growth in the selective media, five suggestive colonies of each agent of interest were selected at random and submitted to confirmatory tests for precise species identification, including sugar fermentation tests and CAMP test for characterization of *S. agalactiae*, and acetoin production (VP test), hemolysis, coagulase and mannitol fermentation for identification of *S. aureus* (NMC, 2004). After characterization, the microorganisms were stored in BHI containing glycerol (15% v/v) at -20°C until susceptibility tests were performed.

For antibiotic susceptibility testing, three strains of *S. aureus* randomly selected from each of the *S. aureus* positive properties were pooled

and used to evaluate antimicrobial susceptibility. The same procedure was done to evaluate the susceptibility of *S. agalactiae* isolates. In total, 74 antibiograms were performed for *S. aureus* and 65 for *S. agalactiae*.

In-vitro antibiotic resistance tests were performed by the disk diffusion method (CLSI, 2013). To this end, the strains were thawed, and an aliquot of 10 µl was cultivated in tubes containing 2 ml of Mueller Hinton broth, incubated at 37°C for 18-24 hours after evaluation of growth and purity, and then diluted in sterile saline until 0.5 turbidity equivalent to McFarland standard scale to produce the inoculum.

The standardized inoculums were spread on a culture media plate surface containing Mueller-Hinton Agar, which is supplemented with 5% sheep blood for *S. agalactiae* susceptibility tests. The antibiotics tested were: ampicillin (10mcg), cephalothin (30mcg), cefotaxime (10mcg), cefoperazone (15mcg), ceftiofur (25mcg), chloramphenicol (10mcg), enrofloxacin (5mcg), florfenicol (30mcg), gentamicin (10mcg), lincomycin (15mcg), nitrofurantoin (20mcg), novobiocin (10mcg), polymyxin B (30mcg), penicillin G (10mcg), oxacillin (15mcg), sulfazotrim (25mcg), tetracycline (30mcg), streptomycin (25mcg), and ciprofloxacin (5mcg). Reference strains of *S. aureus* (ATCC-25923) and *Escherichia coli* (ATCC 25992) were used as control for the antibiogram tests (ANON, 2008).

The strains were classified as sensitive and resistant according to the standards defined by the CLSI (2013). Strains that had intermediate susceptibility were considered resistant in the statistical analysis. The Multiple Resistance Index (MAR) was calculated according to Krumperman (1983). All statistical analyses were descriptive.

Results and discussion

The results showed a high prevalence of *S. aureus* (71.0%) and *S. agalactiae* (68.0%) pathogens in the farms. In the herds, 49.5% had both agents, 91.0% had one single agent and only 9.0% of the studied properties were negative for both pathogens (see Table 1).

Several studies have pointed to the high prevalence of *S. aureus* and *S. agalactiae* in Brazilian dairy herds. Brito et al. (1999) found a prevalence of 77.8% for *S. aureus* and 60% for *S. agalactiae* when they analyzed 6,315 milk samples from 48 dairy herds in the regions of Zona da Mata and Campo das Vertentes, both in the State of Minas Gerais, which is consistent with the results obtained in this study. Oliveira et al. (2013) studied approximately 6,000 lactating cows from 112 herds located in the States of Minas Gerais and Rio de Janeiro, and found a prevalence of 93.0% and 41.0% for *S. aureus* and *S. agalactiae*, respectively. Arcuri et al (2006) studied herds in the Southeast of Minas Gerais and North of Rio de Janeiro and found a prevalence of 91.66% and 50% for *S. aureus* and *S. agalactiae*, respectively, which points to a high prevalence of these pathogens in dairy herds in these states. In contrast, Zimmermann and Araújo (2017) studied the main causative agents of bovine mastitis in the city of Campo Mourão, State of Paraná, and found *Staphylococcus* spp. in 58.97% of the properties and *Streptococcus* spp. isolates in 33.33% of the farms. Such finding shows that the prevalence of these pathogens can be variable among herds of different regions.

Table 1 - Prevalence of *S. aureus* and *S. agalactiae* in family-owned dairy herds in the Campo das Vertentes region, State of Minas Gerais.

Pathogen	Presence or absence	Number of positive farms	%
S. aureus	Present	142	71.0
	Absent	58	29.0
S. agalactiae	Present	136	68.0
	Absent	64	32.0
Both pathogens	Present	99	49.5
Both pathogens	Absent	18	9.0

The high prevalence found in the present study for *S. aureus* and *S. agalactiae* (see Table 1) indicates that control measures for contagious mastitis have not been applied correctly. This points to a need for specific measures to control these contagious pathogens that impact the productivity of herds and the quality of milk, especially when it comes to the BMSCC (RODRIGUES et al., 2017).

The results of the antibiogram tests (see Table 2) indicate that the resistance rates ranged from 1.45% to 73.91% for *S. aureus*. The highest resistance indices were observed for polymyxin B (73.91%), penicillin G (72.46%), ampicillin (58.11%), streptomycin (56.52%), and tetracycline (46.38%). The MARs for the isolates ranged from 0.00 to 0.76, with a mean of 0.31. Over 50% of the isolates showed multidrug resistance, with resistance to at least five different antibiotics (see Table 3). The most efficient antibiotics to combat *S. aureus* were sulfazotrim (with 98.55% of susceptible strains), florfenicol (95.65%), chloramphenicol (92.75%), nitrofurantoin (92.75%), and ciprofloxacin (91.30%). According to Santos, Leal and Rossi (2006), staphylococci are generally resistant to various antibiotics, including beta-lactams, aminoglycosides, chloramphenicol, quinolones, macrolides, and oxacillin.

Consistent with the present findings, Donatele, Motta, and Folly (2002) found high resistance rates for β -lactam antibiotics (82.9%) and tetracyclines (24.4%), and the highest sensitivity indices for sulfazotrim and gentamicin in 180 strains of *S. aureus* isolated from subclinical mastitis from herds in the State of Rio de Janeiro. Coelho et al. (2007) found high indices of resistance to penicillin (67.7%) and ampicillin (64.4%) in 29 clinical strains of *S. aureus* isolated from bovine mastitis.

Fontana et al (2010) studied the resistance of *S. aureus* strains isolated from 174 dairy cows from nine different properties in Jataí, State of Goiás. They found 100% resistance to oxacillin, penicillin, and ampicillin.

They claimed that indiscriminate use of antimicrobial drugs had contributed to the high rate of antibiotic resistance.

The present findings are consistent with the results of Costa et al. (2013). They evaluated the resistance of 352 *S. aureus* isolates from 35 dairy herds in the South of Minas Gerais. They found high resistance for polymyxin B (82%), followed by β -lactams: ampicillin (80.92) and penicillin (80.45%). They also observed multi-resistance in the strains, with a MAR index ranging from 0.2 to 0.5, unlike the present study that pointed to a MAR index ranging from 0.00 to 0.76.

Also consistent with the present findings, Nunes, Cavaco, and Vilela (2007) evaluated the resistance of 234 mastitis pathogens isolates in dairy herds in Portugal and found high levels of resistance to penicillin (78.7%) and ampicillin (65.6%) in *S. aureus* isolates. Unlike the present findings, Freitas et al. (2005) found resistance rates higher than 50% for gentamicin, lincomycin, tetracycline, and oxacillin in *S. aureus* isolates from dairy herds in the State of Pernambuco.

The antimicrobial susceptibility tests for *S. agalactiae* (Table 2) showed rates of resistance ranging from 19% to 90%. The most resisted antibiotics were novobiocin (100%), oxacillin (98.46%), polymyxin B (96.92%), streptomycin (96.92%), and lincomycin (95.38%). The most effective antibiotics were florfenicol (with 100% of susceptible isolates), followed by sulfazotrim (84.62%), nitrofurantoin (72.31%), ciprofloxacin (69.23%), and ampicillin (63.08%). The MARs scores ranged from 0.19 to 0.90, with an average of 0.62. Besides, 98% of the strains showed multidrug resistance to the antibiotics (Table 3).

Silva et al. (2017) investigated the resistance to antibiotics used to control mastitis against strains of *S. agalactiae* isolates from dairy herds in different Brazilian regions. They found high rates of resistance to several antimicrobials, with 26.23% of resistance to erythromycin, 47.54% to tetracycline, 3.28% to gentamicin, 98.36% to sulfonamide, and 29.51% to

clindamycin. All isolates were susceptible to penicillin, ceftiofur, and cephalothin. Such finding differs from those in the present study, especially regarding the resistance index for gentamicin, which reached 44.62% in this study. The high rate of resistance to *S. aureus* and *S. agalactiae* found in the present study might reflect the selective pressure of resistant strains due to misuse of antibiotics.

Table 2 - Results of antibiotic susceptibility tests in strains of *Staphylococcus aureus* and *Streptococcus agalactiae* isolates from bulk milk tanks from in family-owned dairy herds in Campo das Vertentes, State of Minas Gerais.

Antimicrobials	Staphylococcus aureus		Streptococcus agalactiae	
	Number of resistant strains	% of resistant strains	Number of resistant strains	% of resistant strains
Ampicillin	43	58.11	24	36.92
Cephalotin	20	28.99	42	64.62
Cefoperazone	22	31.88	55	84.62
Cefotaxime	25	36.23	58	89.23
Ceftiofur	19	27.54	52	80.00
Ciprofloxacin	6	8.70	20	30.77
Chloranphenicol	5	7.25	29	44.62
Enrofloxacin	14	20.29	33	50.77
Streptomycin	39	56.52	63	96.92
Florfenicol	3	4.35	0	0
Gentamicin	14	20.29	29	44.62
Lincomycin	24	34.78	62	95.38
Neomicin	23	33.33	55	84.62
Nitrofurantoin	5	7.25	18	27.69
Novobiocin	32	46.38	65	100
Oxacillin	28	40.58	64	98.46
Penicillin G	50	72.46	58	89.23
Polymyxin B	51	73.91	63	96.92
Sulfazotrim	1	1.45	10	15.38
Tetracycline	32	46.38	48	73.85

Table 3 shows that 38 (51.35%) in the 74 *S. aureus* strains were resistant to more than five out of the 20 antibiotics tested in this study. The antibiotic susceptibility tests for *S. agalactiae* indicated that 64 stains (98.46%) were resistant to more than five antibiotics. These results point to the high frequency of multi-resistance strains for both pathogens.

The present in-vitro results point to higher resistance rates for *S. agalactiae* as compared to *S. aureus*. Several studies have pointed to the greater difficulty in treating intramammary infections (IMI) caused by *S. aureus* as compared to IMI caused by *S. agalactiae*. This can be explained due to both increased antibiotic resistance rates and the mechanisms of escape to the immune system of the mammary glands, such as intracellular invasion, biofilm formation, leukotoxin production, IgA proteases, and other virulence factors in *S. aureus* strains adapted to bovine mammary glands (NOVICK; SCHLIEVERT; RUZIN, 2001; DELEO; CHAMBERS; CHAMBERS, 2009).

Variations in the antibiotic susceptibility found for the agents tested in the present study as compared to previous studies in the literature may be justified by spatial and temporal variations. They may also be due to the different methodologies employed in susceptibility testing (MIC or diffusion in discs), as to the origin of the isolates (clinical or subclinical mastitis, isolates of bulk milk tanks), and the selection pressure, due to the selective and indiscriminate use of antibiotics.

Table 3 - Multiple resistance in strains of *Staphylococcus aureus* and *Streptococcus agalactiae* isolates from bulk milk tanks in family-owned dairy herds in Campos das Vertentes, Minas Gerais.

Pathogens	Number of antimicrobials against which the strains were resistant					
	0	1	2	3	4	>5
<i>S. aureus</i>	4	6	8	6	12	38
<i>S. agalactiae</i>	0	0	0	0	1	64

According to Myllys et al. (1994), antimicrobial resistance is an important factor in the establishment and spread of bacterial clones in a herd. It is associated with changes in management, such as the use of systematic antibiotic treatment, confinement and introduction of mechanical milking. These factors act as selective forces on mastitis-causing pathogens.

The present results point to high rates of resistance to the main antibiotics used in the treatment of mastitis in herds. This requires constant monitoring of susceptibility of the main pathogens causing mastitis, with a view to making a more careful choice of antibiotics in order to obtain higher cure rates and minimize drug resistance. Thus, permanent monitoring of pathogens and their antibiotic resistance indexes are indispensable to both animal health and human health.

Conclusions

The findings point to a high prevalence of *S. aureus* and *S. agalactiae* pathogens in family-owned dairy herds in Campo das Vertentes, State of Minas Gerais, Brazil. High levels of resistance and multidrug resistance of *S. aureus* and *S. agalactiae* were also found. Multi-resistance was found for both agents, with higher levels for *S. agalactiae*.

Prevalence and antibiotic resistance of *Staphylococcus aureus* and *Streptococcus agalactiae* in family-owned dairy herds in the state of Minas Gerais, Brazil

ABSTRACT

Bovine mastitis is the most frequent disease in dairy herds worldwide, causing great economic losses to both producers and the industry. This disease has direct implications in public health due to the zoonotic potential of some agents involved in its etiology and the presence of antimicrobial residues in milk. Knowledge of the agents involved in its etiology and the susceptibility profiles to antimicrobials is of paramount

importance, give the multifactorial aspect of the disease and the importance of milk production in family farming. This study evaluated the prevalence of *Staphylococcus aureus* and *Streptococcus agalactiae* in family-owned herds in the Campo das Vertentes region, State of Minas Gerais, and the resistance of these pathogens to the most commonly used antibiotics in the treatment of mastitis in dairy herds. The study involved 200 properties, covering an area of approximately 12,564 km². The prevalence of pathogens was carried out through microbiological analyses of milk samples from each farm's milk tank. Modified Baird-Parker Agar medium was used to detect *S. aureus*, and modified Edwards Agar medium, enriched with 5% defibrinated sheep blood, was used to detect *S. agalactiae*. Disc diffusion method was used to evaluate resistance to antibiotics. The results showed high prevalence of *S. aureus* pathogens (71.0%) and *S. agalactiae* (68.0%) as well as high levels of multidrug resistance. The results show that the control of contagious mastitis in the herds is flawed, which indicates the need for more effective control of these pathogens and more judicious use of antibiotics to minimize resistance.

Keywords: Mastitis, Milk quality, Somatic cell count, BMSCC, Milk production.

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