



***Staphylococcus aureus* and *Streptococcus agalactiae*: prevalence, resistance to antimicrobials, and their relationship with the milk quality of dairy cattle herds in Minas Gerais state, Brazil¹**

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ABSTRACT- Mesquita A.A., Rocha C.M.B.M., Bruhn F.R.P., Custódio D.A.C., Braz M.S., Pinto S.M., Silva D.B. & Costa G.M. 2019. ***Staphylococcus aureus* and *Streptococcus agalactiae*: prevalence, resistance to antimicrobials, and their relationship with the milk quality of dairy cattle herds in Minas Gerais state, Brazil.** *Pesquisa Veterinária Brasileira* 39(5):308-316. Departamento de Medicina Veterinária, Universidade Federal de Lavras, Campus Universitário, Cx. Postal 3037, Lavras, MG 37200-000, Brazil. E-mail: marciocostavet@gmail.com

Bovine mastitis is the most frequent disease worldwide in dairy herds, causing high economic losses to producers and industry, as well as having implications for public health due to the zoonotic potential of some agents involved in its etiology and the increased risk of antimicrobial residues in milk and its derivatives. Considering the multifactorial aspect of this disease, knowledge of the agents involved in its etiology and their antimicrobial susceptibility profiles is very important. This study was conducted with 306 dairy herds from the Campo das Vertentes region, located in the south of Minas Gerais state, whose owners were milk suppliers to a dairy in the same region. The study involved approximately 34,000 dairy cows and covered an area of approximately 12,564 km². In these herds, prevalence rates of *Staphylococcus aureus* and *Streptococcus agalactiae* and their relationship with bulk milk somatic cell counts (BMSCC), total bacterial counts (TBC), and daily production were evaluated. In addition, analyses of resistance of these pathogens to the antimicrobials most commonly used in the treatment of mastitis in dairy herds were performed. Microbiological analyses of milk samples from collect from bulk milk tanks were performed aiming to evaluate the prevalence of *S. aureus* and *S. agalactiae*. For these proposes, the modified Baird-Parker Agar medium was used for detection of *S. aureus* and the modified Edwards Agar medium, enriched with 5% defibrinated sheep blood, was used for detection of *S. agalactiae*. The disc diffusion technique was applied to evaluate antimicrobial resistance. Results show high prevalence rates of *S. aureus* (70.3%) and *S. agalactiae* (67.0%) in the dairy farms studied, with 47.71% of the herds showing both pathogens. Associations between BMSCC and the presence of pathogens *S. aureus* and *S. agalactiae* and between TBC and the presence of *S. agalactiae* were observed, demonstrating the influence of these pathogens in milk quality. No variation was observed in the distribution of *S. aureus* and *S. agalactiae* in the different strata of daily production. High levels of resistance and multi-resistance were observed among the pathogens *S. aureus*

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and *S. agalactiae*. The results indicate the need for more effective control measures for mastitis caused by *S. aureus* and *S. agalactiae* in the dairy herds of the region studied and more judicious use of antimicrobials in order to reduce the problem of resistance to them.

INDEX TERMS: *Staphylococcus aureus*, *Streptococcus agalactiae*, mastitis, antimicrobial resistance, milk quality, dairy herds, Minas Gerais, Brazil, cattle, bacterial diseases, bacterioses, pathology.

RESUMO.- [Staphylococcus aureus e Streptococcus agalactiae: prevalência, resistência aos antimicrobianos e suas relações com a qualidade do leite em rebanhos leiteiros de Minas Gerais, Brasil.]

A mastite bovina é a doença de maior frequência em rebanhos leiteiros em nível mundial, acarretando grandes prejuízos econômicos aos produtores e à indústria. Além disso, esta enfermidade tem implicações na saúde pública, devido ao potencial zoonótico de alguns agentes envolvidos em sua etiologia e por aumentar os riscos de resíduos de antimicrobianos no leite e derivados. Considerando o aspecto multifatorial da mastite bovina, o conhecimento dos agentes envolvidos em sua etiologia e os perfis de suscetibilidade aos antibióticos é de suma importância. O estudo envolveu 306 fazendas de leite da região de Campo das Vertentes, localizada no sul de Minas Gerais, cujos proprietários eram fornecedores de leite para um laticínio da região, totalizando aproximadamente 34.000 animais e abrangendo uma área aproximada 12.564 km². Nestes rebanhos, avaliaram-se a prevalência de *Staphylococcus aureus* e *Streptococcus agalactiae* e a relação destes agentes com os índices de contagem de células somáticas do leite do tanque de expansão (CCSt), contagem bacteriana total (CBT) e produção diária. Analisou-se também a resistência destes patógenos aos antimicrobianos mais comumente utilizados no tratamento da mastite em rebanhos leiteiros. Análises microbiológicas de amostras de leite dos tanques de expansão foram realizadas para se determinar as prevalências dos patógenos *S. aureus* e *S. agalactiae*. 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 apontaram altas prevalências de *S. aureus* (70,3%) e de *S. agalactiae* (67,0%), com 47,71% dos rebanhos examinados apresentando ambos os agentes. Verificaram-se associações entre a CCSt e a presença dos patógenos *S. aureus* e *S. agalactiae*, e também entre a CBT e a presença de *S. agalactiae*, demonstrando a interferência negativa destes patógenos nestes quesitos de qualidade. Não se observaram variações nas distribuições dos patógenos *S. aureus* e nem *S. agalactiae* em função da produção diária das propriedades estudadas. Níveis elevados de resistência e de multirresistência foram observados para ambos os agentes. Os resultados apontam a necessidade de medidas mais efetivas de controle para *S. aureus* e *S. agalactiae* nos rebanhos da região estudada e do uso mais criterioso dos antimicrobianos, visando minimizar o problema da resistência aos mesmos.

TERMOS DE INDEXAÇÃO: *Staphylococcus aureus*, *Streptococcus agalactiae*, mastite, resistência a antimicrobianos, qualidade do leite, rebanhos leiteiros, Minas Gerais, Brasil, bovinos, bacterioses, patologia.

INTRODUCTION

Bovine mastitis is considered the main disease in dairy cattle farming worldwide (Lopes et al. 2012, Ruegg 2012, Costa et al. 2013). It is an infectious process usually caused by the presence of pathogenic microorganisms in the mammary gland, affecting the production and composition of quarter milk, with reflexes mainly in somatic cell counts (SCC), total bacterial counts (TBC), and contents of casein and fat (Freitas et al. 2005, Santos & Fonseca 2007, Langoni et al. 2017). Bovine mastitis can also cause economic losses owing to expenses with veterinary medicines and services and, in some cases, to death or early discard of affected animals (Oviedo-Boyso et al. 2007, Lopes et al. 2011, 2012). In addition, agents involved in its etiology, which in many cases are multi-resistant, may pose a risk to public health because of their involvement in food poisoning, and these microorganisms may carry resistance genes that can be horizontally transferred to bacteria in the human microbiota (De Vliegher et al. 2012, Forsythe 2013).

Bovine mastitis can be caused by different agents, including bacteria, viruses, fungi, and algae (Ranjan et al. 2006, Reyher et al. 2012). Among the bacteria, *Staphylococcus aureus* and *Streptococcus agalactiae* are the most frequently isolated pathogens in intramammary infections in Brazilian cattle. Previous studies have reported that *S. agalactiae* was found in 60% of the rural properties in the Zona da Mata region in Minas Gerais state (Brito et al. 1999) and in 39.7% of the farms in different municipalities of this state (Elias et al. 2012). Oliveira et al. (2013) studied the etiology of mastitis in approximately 6,000 lactating dairy cows from 112 herds of farms located in the states of Minas Gerais and Rio de Janeiro, and they found prevalence of *S. aureus* and *S. agalactiae* in 93.0% and 41.0% of the herds assessed, respectively. Cunha et al. (2015), in a study conducted in the municipality of Viçosa, in the Zona da Mata region of Minas Gerais state, observed prevalence of subclinical mastitis of 55.4% in the dairy herds evaluated, with *Corynebacterium* sp. (32.99%), *S. aureus* (28.35%), and *S. agalactiae* (13.66%) as the most frequent pathogens.

Combating bovine mastitis presents a high demand for the use of antibiotics, which are administered mainly for the treatment of clinical cases and in the dry cow therapy (Erskine et al. 2004); however, when antibiotic therapy is conducted indiscriminately, it becomes a determining factor for the selection of resistant strains. Antimicrobial resistance associated with indiscriminate use of antibiotics and management failures are the major obstacles to the success of antibiotic therapy in combating mastitis (Medeiros 2015); this combination can disseminate resistant strains in the environment and cause serious public health problems (Santos et al. 2006, OIE 2010). Therefore, it is important to emphasize that these medicines should be used more judiciously and antibiogram tests should be performed to choose the most appropriate drugs for the treatment and

prevention of bovine mastitis (Diniz et al. 2010, Jain et al. 2012, Costa et al. 2013).

Considering the economic relevance of bovine mastitis and its implications on human and animal health, it is essential to monitor the agents most commonly involved in its etiology, as well as the resistance indices of these pathogens to the antimicrobials used to combat them, thus enabling monitoring of their resistance dynamics and adoption of more effective therapeutic protocols for their control. In this context, the present study aimed to evaluate the prevalence rates of *Staphylococcus aureus* and *Streptococcus agalactiae* in dairy herds from Campo das Vertentes region, located in the south of Minas Gerais state, whose owners were milk suppliers to a dairy in the same region, and their relationship with bulk milk somatic cell counts (BMSCC), total bacterial counts (TBC), and daily production, as well as evaluate the resistance of these pathogens to the antimicrobials most commonly used in the treatment of mastitis in dairy cattle.

MATERIALS AND METHODS

This study was conducted with 306 dairy herds from the Campo das Vertentes region, located in the south of Minas Gerais state, involving approximately 34,000 dairy cows and covering an area of approximately 12,564 km² (FAEMG 2006). The owners of these herds were milk suppliers to a dairy in the region and expressed an interest in participating in the study. Thus, study sampling for choosing the participating properties was conducted for convenience, and the inclusion criteria comprised the previous relationship of the owners with the dairy and the research unit.

A non-probabilistic, descriptive study was conducted to determine the prevalence rates of pathogens *Staphylococcus aureus* and *Streptococcus agalactiae* in all properties linked to dairy (306). To this end, milk samples of approximately 40mL were aseptically collected from the expansion tanks after homogenization by trained technicians of the dairy and they were stored in sterile vials at -20°C until analysis. Milk samples were collected directly from the bulk milk tanks of the farms, with a small time variation (<30 days).

For detection of *S. aureus*, a 50µL aliquot of each milk sample was cultured on Petri plates containing modified Baird-Parker Agar selective medium and incubated at 37°C for 24-48h. The samples that, after incubation, showed 1-5mm diameter, black colonies with glossy appearance, convex shape, and formation of two halos in their contour, indicating lipolytic and proteolytic activity, were considered positive for *S. aureus* (Siqueira 1995). For isolation of *S. agalactiae*, a 50µL aliquot of each milk sample was cultured on Petri plates containing modified Edwards Agar selective medium enriched with 5% defibrinated sheep blood. Samples that showed growth of 1-2mm diameter, bluish colonies with bright appearance and convex shape, with or without formation of opaque zone at their contour were considered positive. All dishes were incubated at 37°C for 24-48h. All cultures were performed in duplicate. From the cultures that tested positive, five colonies suggestive of each of the agents of interest (*S. aureus* and *S. agalactiae*) were randomly selected and submitted to confirmatory tests according to the National Mastitis Council (NMC 2004). After phenotypic characterization, the isolates were stored in Brain-heart infusion (BHI) broth containing glycerol (15% v/v) at -20°C until performance of the other assays.

The disc diffusion technique was applied to evaluate *in vitro* antimicrobial resistance according to the specifications of the Clinical and Laboratory Standards Institute (CLSI 2013). To this end, we used three isolates of *S. aureus* from 99 different properties randomly

selected among those that tested positive for this bacterium and three isolates of *S. agalactiae* from 93 different farms randomly selected among those that tested positive to this bacterium. The three isolates of each agent from each farm were used to compose a pool that was used to perform the antimicrobial susceptibility tests. The following antimicrobials were tested: ampicillin, cephalothin, cefoperazone, ceftiofur, enrofloxacin, florfenicol, gentamicin, lincomycin, neomycin, nitrofurantoin, novobiocin, polymyxin B, penicillin G, oxacillin, sulfamethoxazole-trimethoprim, tetracycline, streptomycin and ciprofloxacin. Isolates of *S. aureus* (ATCC-25923) and *Escherichia coli* (ATCC 25992) were used as control in the antibiogram tests. Based on the standards previously defined by the CLSI (2013), isolates were classified as susceptible, moderately susceptible, or resistant. Isolates classified as moderately susceptible were considered resistant for statistical calculations. The multiple antibiotic resistance (MAR) index was calculated from these results. The index MAR was determined by the percentage ratio between the number of antimicrobials for which the isolate tested resistant and the total number of antimicrobials tested. MAR ≥0.2 characterizes multi-resistance according to Krumperman (1983).

Data on total bacterial counts (TBC) and bulk milk somatic cell counts (BMSCC) were obtained at the dairy from the biweekly evaluations conducted in laboratories of the Brazilian Milk Quality Network (RBQL). Results of the five most recent TBC and BMSCC analyses of each property were used to obtain the arithmetic means of these factors, from which the existence of association with the microbiological data (presence/absence of pathogens *S. aureus* and *S. agalactiae*) of the farms was assessed.

Aiming to identify whether the presence of pathogens *S. aureus* and *S. agalactiae* was associated with the daily production of the farms and the impact of these agents on the TBC and BMSCC indices of the properties, we assessed the existence of a correlation between presence of pathogens *S. aureus* and *S. agalactiae* in different strata of daily milk production (≤100 L/day; >100 and ≤500 L/day; >500 and ≤1000 L/day; ≥1000 L/day), as well as in different mean scores of BMSCC (≤200 cell/mL; >200 and ≤500 cell/mL; >500 and ≤1,000 cell/mL; >1,000 cell/mL) and TBC (≤10 CFU/mL; >10 and ≤50 CFU/mL; >50 and ≤100 CFU/mL; >100 CFU/mL).

Statistical analyses were processed using the Statistical Package for the Social Sciences (SPSS, Chicago, USA) software, version 20.0 for Windows®. Correlations between the presence/absence of agents *S. aureus* and *S. agalactiae* and the BMSCC and TBC indices and the daily milk production of the farms were assessed using the Pearson's chi-squared test (χ^2) at 5.0% significance level. Since application of the Kolmogorov-Smirnov test showed that data distribution was not normal, the non-parametric Mann-Whitney test was used to verify the existence of difference in the distribution of the quantitative variables (daily milk production, BMSCC, and TBC) and between the qualitative variables (presence of *S. aureus* and *S. agalactiae*).

RESULTS AND DISCUSSION

Results of this study showed high prevalence rates of pathogens *Staphylococcus aureus* (70.3%) and *Streptococcus agalactiae* (67.0%) among the assessed dairy herds (306), with 47.71% showing presence of both agents, 89.54% showing presence of at least one agent, and only 10.45% farms showing absence of both agents (Table 1). These results corroborate the findings of previous studies in the literature. Brito et al. (1999) verified prevalence rates of 77.80% for *S. aureus* and 60% for *S. agalactiae* when analyzing 6,315 milk samples from 48 dairy herds in the regions of Zona da Mata and Campo das

Vertentes in Minas Gerais state. Arcuri et al. (2006) evaluated the microbiological quality of milk in 24 cattle herds in the southeast of Minas Gerais state and north of Rio de Janeiro state and observed prevalence rates of 91.66% for *S. aureus* and 50% for *S. agalactiae*. In a study conducted with 112 dairy herds in the states of Minas Gerais and Rio de Janeiro involving approximately 6,000 dairy cows, Oliveira et al. (2013) found prevalence rates of 93.0% and 41.0% for *S. aureus* and *S. agalactiae*, respectively.

The high prevalence rates observed in the present survey for pathogens *S. aureus* and *S. agalactiae* (Table 1) suggest that the control measures for infectious mastitis are not being correctly applied, a finding that corroborates the results reported by Rodrigues et al. (2017). This fact emphasizes the need to adopt specific measures for the control of these contagious pathogens that affect the daily production of herds and the milk quality, especially the BMSCC index.

Analyses of the BMSCC and TBC indices as a function of the daily milk production of the properties are presented in Tables 2 and 3. The legislation actual in Brazil - Normative

Instruction no. 07 (Brasil 2016) - establishes a limit up to 400,000 cell/mL for BMSCC. However, BMSCC scores >200,000 cell/mL are indicative of losses in milk production and quality, indicating subclinical mastitis at high levels in the herd (Barbosa et al. 2002, Coldebella et al. 2004, Magalhães et al. 2006).

Findings of the present study showed that 50% of the herds presented BMSCC \geq 400,000 cell/mL, with mean of 483,000 cell/mL. These results are in agreement with those reported by Rodrigues et al. (2017), who verified a geometric annual BMSCC mean in herds in southeastern Brazil of 518,000 cell/mL, with a confidence interval from 491,000 to 546,000 cell/mL; these results are similar to those found in the present study - 483,000 cell/mL. High levels of BMSCC can cause numerous losses to the dairy industry, because they directly affect the composition of milk and, consequently, reduce its shelf life and that of its derivatives (Silva & Nogueira 2010).

The results presented in Table 4 show that, among the farms with lower BMSCC scores, the prevalence rates of

Table 1. Prevalence of pathogens *Staphylococcus aureus* and *Streptococcus agalactiae* in the bulk milk tanks of dairy herds in the region of Campo das Vertentes/MG, 2017

Factor	Unit	Values	%	CI
<i>S. aureus</i>	Present	215	70.3	
	Absent	91	29.7	(24.9-35.1)
<i>S. agalactiae</i>	Present	205	67.0	
	Absent	101	33.0	(28.0-38.5)
Presence of both agents	Present	146	47.71	
Absence of both agents	Absent	32	10.45	
Presence of one or both agents	Present	274	89.54	

Table 2. Bulk milk somatic cell counts (BMSCC) of dairy herds in the region of Campo das Vertentes/MG, 2017

Daily production	BMSCC x 1,000 (cell/mL)				Herd total
	\leq 200	201-500	501-1.000	\geq 1.000	
\leq 100	10 (16.67%)	33 (55.00%)	12 (20.00%)	5 (8.33%)	60
100-500	18 (11.11%)	81 (50.00%)	55 (33.95%)	8 (4.94%)	162
500-1000	3 (6.98%)	28 (65.12%)	8 (18.60%)	4 (9.30%)	43
\geq 1000	3 (7.32%)	22 (53.66%)	13 (31.71%)	3 (7.32%)	41
Total	34 (11.11%)	164 (53.59%)	88 (28.76%)	20 (6.54%)	306

Table 3. Total bacterial counts (TBC) in bulk milk tanks of dairy herds in the region of Campo das Vertentes/MG, 2017

Daily production	TBC x 1,000 (CFU/mL)				Herd total
	\leq 10	11-50	51-100	\geq 101	
\leq 100	18 (30.00%)	29 (48.33%)	7 (11.29%)	6 (9.68%)	60
100-500	45 (27.77%)	86 (53.08%)	17 (10.56%)	14 (8.70%)	162
500-1000	14 (32.56%)	24 (55.81%)	3 (6.98%)	2 (4.65%)	43
\geq 1000	11 (27.50%)	23 (56.09%)	5 (12.50%)	2 (5.00%)	41
Total	89 (29.08%)	161 (52.61%)	32 (10.46%)	24 (7.84%)	306

Table 4. Prevalence of pathogens *Staphylococcus aureus* and *Streptococcus agalactiae* as a function of bulk milk somatic cell counts (BMSCC) of dairy herds in the region of Campo das Vertentes/MG, 2017

Variable	BMSCC x 1,000 (cell/mL)				Total
	\leq 200	201-500	501-1000	>1000	
Presence of <i>S. aureus</i>	19 (55.88%)	114 (69.51%)	66 (75.00%)	17 (85.00%)	216 (70.30%)
Presence of <i>S. agalactiae</i>	15 (44.12%)	112 (68.29%)	65 (73.86%)	13 (65.00%)	205 (66.99%)
Presence of both agents	8 (23.53%)	76 (46.34%)	50 (56.82%)	12 (60.00%)	146 (47.71%)
Absence of both agents	8 (23.53%)	15 (9.15%)	7 (7.95%)	2 (10.00%)	32 (10.46%)

pathogens *S. aureus* and *S. agalactiae* are generally lower, and it can be observed that 23.53% of the properties with BMSCC <200,000 cell/mL showed absence of both agents, whereas only 10% of the farms with BMSCC >1,000,000 cell/mL had the same result. In the latter, the prevalence rates verified for pathogens *S. aureus* and *S. agalactiae* were 85% and 65%, respectively.

The BMSCC indices obtained in the present study suggest that improvements need to be made in the control of mastitis, especially regarding contagious pathogens, because the BMSCC scores observed in most properties are above those accepted in the international market, especially in the European Union, where the limit is <400,000 cell/mL, but with a decreasing trend in the coming years, since the national BMSCC scores in the Scandinavian countries ranged from 200,000 to 250,000 cell/mL (More 2009).

Current legislation (Brasil 2016) establishes a TBC limit of 100,000 CFU/mL for milk from bulk milk tanks. Among the properties assessed in the present study, 92.16% showed TBC ≤100,000 CFU/mL, with only 7.84% of them presenting scores >100,000 CFU/mL. Increased TBC indices can be caused by failures in the washing of the utensils that come in contact with the milk (milking machine and expansion tank), and faults in cleaning and antiseptics of udder prior to milking and in post-milking cooling, as well as by the presence of intramammary infection, especially when infected

with streptococci that cause subclinical mastitis (Santos & Fonseca 2007, Vargas et al. 2013). This fact may explain the increased TBC indices found in the herds affected by the *S. agalactiae* pathogen, as it can be verified in Table 5. Such finding corroborates the study by Zadoks et al. (2004), who reported increased TBC of milk from bulk milk tanks in dairy herds affected by *S. agalactiae*.

Prevalence of *S. aureus* was greater than that of *S. agalactiae* in all daily production strata, except for the 500-1,000 L/day stratum, in which a lower prevalence rate was observed for *S. aureus* (60.47%), with a prevalence rate of *S. agalactiae* of 67.44% (Table 6). The smallest prevalence rate for *S. agalactiae* was found in the daily production stratum of >1,000 L/day; however, no significant difference was verified with respect to any of the daily milk production strata ($p>0.05$). Also, no significant correlation was observed between the pathogens *S. aureus* and *S. agalactiae* ($p=0.601$), that is, they may be present in the herds regardless of each other.

No significant correlation was found between daily milk production and presence of pathogens (*S. aureus* and *S. agalactiae*) ($p>0.05$), which shows that they are equally distributed among the herds, regardless of the production stratum (Table 7). No association was verified between daily production and TBC ($p>0.05$) and between daily production and BMSCC ($p>0.05$), demonstrating that the daily production of the properties did not influence these variables (Table 7).

Table 5. Prevalence of pathogens *Staphylococcus aureus* and *Streptococcus agalactiae* in the different mean scores of total bacterial counts (TBC) in bulk milk tanks of dairy herds in the region of Campo das Vertentes/MG, 2017

Variable	TBC x 1,000 (CFU/mL)				Total
	≤10	11-50	51-100	≥101	
Presence of <i>S. aureus</i>	59 (66.29%)	120 (75.00%)	22 (40.00%)	15 (62.50%)	215 (70.59%)
Presence of <i>S. agalactiae</i>	51 (57.30%)	111 (69.38%)	26 (47.27%)	17 (70.83%)	205 (66.99%)
Presence of both agents	34 (38.20%)	85 (53.13%)	33 (60.00%)	21 (87.50%)	173 (56.54%)
Absence of both agents	13 (14.61%)	15 (9.38%)	2 (3.64%)	2 (8.33%)	32 (10.46%)

Table 6. Variation in the prevalence rates of *Staphylococcus aureus* and *Streptococcus agalactiae* and inadequacies in bulk milk somatic cell counts (BMSCC) and total bacterial counts (TBC), as a function of daily production, in bulk milk tanks of dairy herds in the region of Campo das Vertentes/MG, 2017

Daily production (L)	N(%)	Presence of <i>S. aureus</i> N(%)	Presence of <i>S. agalactiae</i> N(%)	BMSCC >500,000 cell/mL N(%)	TBC >100,000 CFU/mL N(%)
≤100	62 (20.26%)	46 (73.02%)	43 (68.25%)	19 (30.16%)	1 (1.59%)
100-500	163 (52.94%)	115 (70.55%)	111 (68.10%)	64 (53.27%)	5 (1.63%)
500-1000	42 (14.05%)	26 (60.47%)	29 (67.44%)	12 (27.91%)	1 (2.33%)
>1000	39 (12.75%)	28 (71.79%)	22 (56.41%)	14 (35.90%)	1 (2.56%)
All farms	306 (100%)	215 (70.30%)	205 (67.00%)	109 (35.62%)	8 (2.61%)

Table 7. Correlation between quantitative variables: daily production, bulk milk somatic cell counts (BMSCC), and total bacterial counts (TBC) of dairy herds in the region of Campo das Vertentes/MG that tested positive for *Staphylococcus aureus* and *Streptococcus agalactiae* in 2017

Variable	<i>S. aureus</i>		p value	<i>S. agalactiae</i>		p value
	Positive	Negative		Positive	Negative	
Prevalence N (%)	215 (70.3)	91 (29.7)		205 (67.0)	101 (33.0)	
Average daily production (L)	467	528	0.223	460	538	0.216
Mean BMSCC (cell/mL)	503,000	434,000	0.003*	503,000	442,000	0.002*
Mean TBC (CFU/mL)	40,000	38,000	0.867	46,000	38,000	0.009*

*Significant by the Mann-Whitney test at 5%.

Significant associations were observed between BMSCC and presence of *S. aureus* ($p=0.003$), BMSCC and presence of *S. agalactiae* ($p=0.002$), and TBC and presence of *S. agalactiae* ($p=0.009$) in the dairy herds investigated, which suggests that the presence of these agents in the herds negatively influence the BMSCC and that the presence of *S. agalactiae* is associated with increased TBC. In this study, herds affected by *S. agalactiae* showed mean TBC of 46,000 CFU/mL, whereas herds affected by *S. aureus* presented mean TBC of 40,000 CFU/mL (Table 7). Table 5 evidences that the properties with TBC >100,000 CFU/mL had higher levels of infection caused by *S. aureus* and *S. agalactiae* (87.50%), whereas presence of both agents was observed in only 38.20% of the farms with TBC <10,000 CFU/mL. These results corroborate the findings by Lopes Júnior et al. (2012), who verified correlation between BMSCC, TBC, and mastitis pathogens, and found correlation between BMSCC and TBC greater than 0.60 for the agents assessed, in which the coefficient of linear regressions for *S. agalactiae* (0.542) was higher compared with those for the other mastitis pathogens, followed by that of *S. aureus* (0.503), suggesting that the intensity of the inflammatory process measured by the BMSCC was associated with the bacterial burden present in the milk of animals affected by mastitis.

Tables 8 and 9 show the results of the antimicrobial susceptibility tests for pathogens *S. aureus* and *S. agalactiae*. It can be observed that the resistance indices of *S. aureus* varied from 2% to 72%, with the following most efficient drugs and percentages of susceptible isolates: sulfamethoxazole-trimethoprim (98%), florfenicol (97%), nitrofurantoin (94%), and ciprofloxacin (91%). The highest resistance levels were observed for polymyxin B (72%), penicillin G (69%), ampicillin (60%), streptomycin (58%), and tetracycline (49%) (Table 8). The Multiple Antibiotic Resistance (MAR) indices for *S. aureus* ranged from 0.00 to 0.84 - mean of 0.33, with 62,6% of the isolates presenting multi-resistance (Table 9).

The antimicrobial susceptibility profiles verified for *S. aureus* in the present study are in agreement with the findings of previous surveys. Cardoso et al. (2000) isolated and subjected to antibiogram tests 127 strains of *S. aureus* in 23 municipalities in Minas Gerais state from 1994 to 1997. The most effective *in vitro* antimicrobials were cefotaxime (100%), enrofloxacin (98.4%), gentamicin (98.4%), rifampicin (96.1%), chloramphenicol (90.4%), sulfamethoxazole-trimethoprim (86.6%), and novobiocin (85.8%). The following medicines showed the highest resistance levels: polymyxin B (8.7%), ampicillin (28.6%), and penicillin G (29.1%).

Table 8. Results of antimicrobial susceptibility tests on strains of *Staphylococcus aureus* and *Streptococcus agalactiae* isolated from milk bulk tanks of dairy herds in the region of Campo das Vertentes/MG, 2017

Antibiotic	<i>Staphylococcus aureus</i>			<i>Streptococcus agalactiae</i>		
	N of resistant strains	% of resistant strains	CI 95% (Lower limit - Upper limit)	N of resistant strains	% of resistant strains	CI 95% (lower limit - upper limit)
Ampicillin	59	60	(49.8-68.7)	34	37	(27.5-46.7)
Cephalothin	24	24	(16.9-33.5)	62	67	(56.6-75.4)
Cefoperazone	28	28	(20.4-37.8)	79	85	(76.3-90.8)
Cefotaxime	35	35	(26.6-45.2)	82	88	(80.1-93.3)
Ceftiofur	25	25	(17.7-34.6)	73	78	(69.1-85.6)
Ciprofloxacin	9	9	(4.9-16.4)	23	25	(17.1-34.4)
Chloramphenicol	5	5	(2.2-11.3)	41	44	(34.4-54.2)
Enrofloxacin	17	17	(11.0-25.8)	44	47	(37.5-57.4)
Streptomycin	57	58	(47.7-66.3)	89	96	(89.5-98.3)
Florfenicol	3	3	(1.0-8.5)	1	1	(0.0-4.0)
Gentamicin	21	21	(14.3-30.3)	47	51	(40.6-60.5)
Lincomycin	34	34	(25.7-44.1)	88	95	(88.0-97.3)
Neomycin	31	31	(23.0-41.0)	76	82	(72.6-88.3)
Nitrofurantoin	6	6	(2.8-12.6)	22	24	(16.2-33.2)
Novobiocin	45	45	(36.0-55.3)	90	97	(96.0-100)
Oxacillin	38	38	(29.4-48.2)	90	97	(90.9-98.9)
Penicillin G	68	69	(57.9-76.1)	80	86	(77.5-91.7)
Polymyxin B	71	72	(62.2-79.7)	89	96	(89.5-98.3)
sulfamethoxazole-trimethoprim	2	2	(0.6-7.1)	14	15	(9.2-23.7)
Tetracycline	49	49	(39.9-59.2)	72	77	(67.9-84.7)

Table 9. Cumulative frequency of resistance to antimicrobials of *Staphylococcus aureus* and *Streptococcus agalactiae* isolated from bulk milk tanks of dairy herds in the region of Campo das Vertentes/MG, 2017

Pathogen	Number of antimicrobials cumulatively tested				
	0	1	2	3	>4*
<i>S. aureus</i>	7	7	15	8	62
<i>S. agalactiae</i>	0	0	2	0	91

*Resistance to four or more antimicrobials characterizes multi-resistance in this study.

Donatele et al. (2002) analyzed antimicrobial susceptibility in 180 strains of *S. aureus* isolated from subclinical mastitis in herds in Rio de Janeiro state and found high resistance rates for β -lactam antibiotics (82.9%) and tetracyclines (24.4%), with the highest susceptibility indices obtained for sulfamethoxazole-trimethoprim and gentamicin. Coelho et al. (2007) reported high rates of resistance to penicillin (67.7%) and ampicillin (64.4%) for *S. aureus*; however, differently from the findings of the present research, gentamicin was more effective against the tested isolates, with only 12.5% resistance. Nunes et al. (2007) evaluated antimicrobial resistance in 234 pathogens isolated from cases of mastitis in dairy properties in Portugal and observed high levels of resistance to penicillin (78.7%) and ampicillin (65.6%) in *S. aureus* isolates; results similar to those found in the present study. Costa et al. (2013), assessed the antimicrobial resistance profile in 352 *S. aureus* isolates in 35 dairy herds in the south of Minas Gerais state and observed that the polymyxin B presented the highest resistance index (82%), followed by the β -lactam antibiotics ampicillin and penicillin, which showed resistance indices of 80.92 and 80.45%, respectively. The same authors also found multi-resistance with MAR index, varying from 0.2 to 0.5, differently from what was verified in the present survey, which showed MAR values ranging from 0.00 to 0.76.

Results in disagreement with those of our study were also reported by Freitas et al. (2005), who verified resistance indices >50% for gentamicin, lincomycin, tetracycline, and oxacillin in *S. aureus* isolates in dairy herds in Pernambuco state, and by Fontana et al. (2010), who evaluated the resistance of *S. aureus* isolates of 174 dairy cows in nine different farms in the municipality of Jataí, Goiás state, and observed 100% resistance to oxacillin, penicillin and ampicillin - indices higher than those found in the present study.

Antimicrobial susceptibility tests for the *S. agalactiae* demonstrated that the overall resistance of the isolates varied from 1% to 97%. The most efficient *in vitro* medicines with their respective percentages of susceptible isolates are shown ahead: florfenicol (99%), sulfamethoxazole-trimethoprim (85%), nitrofurantoin (76%), ciprofloxacin (75%), and ampicillin (63%). The antimicrobials that showed the highest resistance rates were novobiocin (97%), oxacillin (97%), polymyxin B (96%), streptomycin (96%), and lincomycin (95%) (Table 8). Among the isolates of *S. agalactiae*, 97.8% of strains showed multi-resistance, with MAR indices varying from 0.11 to 0.95, and mean of 0.65 (Table 9).

Silva et al. (2017) analyzed the antimicrobial susceptibility profiles of *S. agalactiae* isolates in cattle herds in different Brazilian regions. Their study showed the following drug resistance rates: erythromycin (26.23%), tetracycline (47.54%), gentamycin (3.28%), sulfonamide (98.36%), and clindamycin (29.51%). All isolates assessed were susceptible to penicillin, ceftiofur, and cephalothin. The results of the aforementioned survey differed from those of the present research especially with respect to penicillin and gentamicin resistance rates, which were lower than those observed herein, with values of 86% and 51%, respectively. Jain et al. (2012) assessed the antimicrobial resistance profiles in *S. agalactiae* isolates from cases of subclinical mastitis and observed resistance indices, in general, lower than those obtained in the present study. Our results show higher overall indices of resistance and multi-resistance for *S. agalactiae*

compared with *S. aureus* in the farms investigated. Table 9 shows that 48.48% of the *S. aureus* isolates were resistant to five or more different antimicrobials, whereas this index was 95.70% for *S. agalactiae* isolates. According to Nunes et al. (2007) and Fontana et al. (2010), poorly directed antibiotic therapy and indiscriminate use of antimicrobials, resulting in increased selective pressure on resistant strains, contribute to increased antimicrobial resistance rates. Different authors have referred to the difficulty in treating intramammary infections caused by *S. aureus*, with low cure rates (Costa et al. 2012, 2013, Silva et al. 2014). This fact is justified not only by the resistance phenomenon, but also by its escape mechanisms of the immune system and of the antimicrobials in the infected gland, such as intracellular invasion and biofilm formation (Novick et al. 2001, DeLeo et al. 2009).

As for intramammary infections caused by *S. agalactiae*, increasing difficulty has been observed with regards to treatment, including failure of the classic blitz therapy protocols, which in part may be associated with the high resistance rates observed in the present study. The differences between the results of the susceptibility tests verified in this research for pathogens *S. aureus* and *S. agalactiae* and those of previous studies in the literature can be justified by spatial and temporal variations in the origin of the isolates, as well as by the different methodologies used in the antibiotic susceptibility tests (minimal inhibitory concentration (MIC); disc diffusion). Differences in the origin of the isolates (whether from clinical or subclinical mastitis, if from individual mammary quarters or expansion tanks, or from the environment) and in selection pressure due to the selective and/or indiscriminate use of antimicrobials may also justify this variation in the resistance indices.

Results of the present study demonstrated high resistance levels among *S. aureus* and *S. agalactiae* isolates for the main medicines used in the treatment of mastitis, which highlights the need for constant monitoring of susceptibility profiles with a view to a more careful selection of antimicrobials in order to improve high cure rates and minimize the increase in drug resistance. In addition, it is fundamental to periodically and permanently monitor the pathogens involved in the etiology of bovine mastitis and their resistance indices, because these parameters are very dynamic and present spatial and temporal variations. According to Myllys et al. (1994), resistance to antimicrobials is an important factor in the establishment and dissemination of bacterial clones in a herd, and shows close association with changes in management, such as the use of systematic antibiotic treatment, cattle stabling, and introduction of mechanical milking machines - factors that impose selective pressure on the pathogens that cause mastitis.

CONCLUSIONS

Results show high prevalence rates of pathogens *Staphylococcus aureus* and *Streptococcus agalactiae* in dairy farms of the study region and association between presence of these agents in the herd with total bacterial counts (TBC) and bulk milk somatic cell counts (BMSCC), demonstrating that the presence of these pathogens negatively influence milk quality.

No difference in the presence of pathogens *S. aureus* and *S. agalactiae* were observed as a function of the daily production of the properties, demonstrating homogeneous distribution of these agents in the herds regardless of the daily production.

High resistance and multi-resistance levels were observed among the pathogens *S. aureus* and *S. agalactiae*, emphasizing the need for more judicious use of antimicrobials used in mastitis control.

Conflict of interest statement. - The authors declare that there are no conflicts of interest.

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