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Genotypic evaluation of antimicrobial resistance in *Staphylococcus* spp. isolated from bovine clinical mastitis

[*Avaliação genotípica da resistência antimicrobiana em* Staphylococcus *spp. isolados de mastite clínica bovina*]

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ABSTRACT

Bovine clinical mastitis caused by *Staphylococcus* spp. is a serious and widespread disease in the world of dairy farming. Antimicrobial therapy is of fundamental importance in the prevention and treatment of infectious mastitis, but the indiscriminate use of antimicrobials acts as a determining factor for the spread of the disease. The present study evaluated the resistance profiles of 57 *Staphylococcus* spp. isolated from bovine clinical mastitis to beta-lactams and gentamicin, relating characteristics of phenotype (in vitro susceptibility tests) and genotype (detection and expression of genes encoding resistance - *mecA, mecA$_{LGA251}$, blaZ, femA, femB, and aacA-aphD* – using PCR and RT-PCR, respectively). One or more genes coding for resistance to different antimicrobials were detected in 50 Staphylococcus spp. isolates. The *femA* and *femB* genes were the most frequent (75.4% for both). The observed expression of the genes was as follows: *blaZ (60%), femA (39.5%), aacA-aphD (50%), femB (32.6%), mecA (8.3%), and mecA$_{LGA251}$* (0%). Considering the relevance of the genus Staphylococcus to bovine mastitis, this study aimed to elucidate aspects regarding the genotypic and phenotypic profiles of these microorganisms so as to contribute to the development of effective strategies for mastitis control.

Keywords: staphylococcal mastitis, antimicrobial resistance genes, gene expression

RESUMO

A mastite clínica bovina causada por Staphylococcus *spp. é uma doença grave e generalizada no mundo da pecuária leiteira. A terapia antimicrobiana é de fundamental importância na prevenção e no tratamento da mastite infecciosa, mas o uso indiscriminado de antimicrobianos atua como fator determinante para a disseminação da doença. O presente estudo avaliou os perfis de resistência de 57* Staphylococcus *spp. isolados de mastite clínica bovina em relação ao uso de betalactâmicos e gentamicina, relacionando características do fenótipo (testes de suscetibilidade* in vitro*) e genótipo (detecção e expressão de genes que codificam resistência* - mecA, mecA$_{LGA251}$, blaZ, femA, femB, e aacA-aphD – *usando PCR e RT-PCR, respectivamente). Um ou mais genes que codificam resistência a diferentes antimicrobianos foram detectados em 50* Staphylococcus *spp. isolados. Os genes* femA e femB *foram os mais frequentes (75,4% para ambos). A expressão observada dos genes foi a seguinte:* blaZ *(60%),* femA *(39,5%),* aacA-aphD *(50%),* femB *(32,6%),* mecA *(8,3%) e* mecA$_{LGA251}$ *(0%). Considerando-se a relevância do gênero* Staphylococcus *para a mastite bovina, este estudo teve como objetivo elucidar aspectos referentes aos perfis genotípico e fenotípico desses microrganismos, a fim de contribuir para o desenvolvimento de estratégias eficazes para o controle da mastite.*

Palavras-chave: mastite estafilocócica, genes de resistência a antimicrobianos, expressão gênica

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INTRODUCTION

Mastitis is an inflammatory process of the mammary gland that interferes directly in milk production and is extremely important for dairy farming. The bacteria most commonly isolated from infectious mastitis are those of the genus *Staphylococcus* (Côté-Gravel and Malouin, 2018). The control of mastitis has great importance and should be based on therapeutic and preventive measures. Drug therapy assists the animal's defenses in eliminating the invading agent. In turn, microorganisms often seek to nullify the defense response of the host using the most diverse resistance mechanisms, which have been the targets of extensive studies (Ceniti *et al.*, 2017).

Bacterial resistance is related to the existence of different biochemical mechanisms that act to prevent the action of the drugs. Phenotypic and genotypic diagnostic methods for the evaluation of the *Staphylococcus* spp. resistance profile have been widely used, especially when considering herds of cattle and the possibility of multi-resistant pathogens transfer to other animals and humans (Cuny *et al.*, 2011; García-Alvarez *et al.* 2011; Boireau *et al*. 2018).

The indiscriminate use of antimicrobials is a risk factor for infection by strains resistant to several antibiotic classes. The increasing rate of bacterial resistance is associated with an exponential increase in the frequency of multi-resistant strains and treatment failures, resulting in a high percentage of morbidity and mortality of animals (Cohn and Middleton, 2010; Ceniti *et a*l. 2017). Considering that the genus *Staphylococcus* is of great relevance as a mastitis-causing agent, further studies are required regarding their resistance to antimicrobials, particularly those commonly used for the treatment of bovine clinical mastitis.

The use of phenotypic (*in vitro* antimicrobial susceptibility) and genotypic (molecular techniques for gene detection and gene expression verification) methods to identify the profile of resistant *Staphylococcus* may help in the development of strategies to control the spread of resistant strains. The objectives of the present study were the evaluation of characteristics of *Staphylococcus* spp. isolated from bovine clinical mastitis regarding phenotypic and genotypic resistance to the beta-lactams and aminoglycosides most commonly used for the treatment and prevention of this condition.

MATERIAL AND METHODS

A total of 57 *Staphylococcus* spp. strains isolated from bovine milk samples presenting clinical mastitis were evaluated. These isolates were previously obtained from the cultivation of milk samples of cows from different dairy farms in the state of Minas Gerais (Brazil). The project was previously approved by the Ethical Committee in the Use of Animals of the Faculty of Veterinary Medicine and Animal Science, University of São Paulo (CEUA/FMVZ) under protocol number 8515270415. All samples were reactive to the Tamis Test (Radostitis *et al.* 2000) and were submitted to Polimerase Chain Reaction (PCR) for *rpob* gene detection for genus confirmation, with subsequent DNA sequencing of the amplified region for determining the species (Mellmann *et al.* 2006).

In vitro susceptibility tests were conducted using the diffusion technique described by Bauer *et al.* (1966). The concentrations and interpretation criteria used were those standardized by the Clinical and Laboratory Standards Institute (Performance…, 2008, 2013). The antimicrobials evaluated were amoxicillin (10µg), ampicillin (10µg), oxacillin (10µg), penicillin (10 I.U.), cephalothin (30µg), and gentamicin (10µg). The isolates that presented resistance in the diffusion tests were submitted to the minimum inhibitory concentration (MIC) evaluation using the M.I.C.Evaluator™ (M.I.C.E.™) (Thermo Fisher Scientific, Basingstoke, UK) according to the manufacturer's instructions and the criteria used to interpret the results were those described by the Clinical and Laboratory Standards Institute (Performance…, 2008, 2013).

Regarding genotypic analysis, the bacterial resistance to beta-lactams was evaluated by the occurrence and expression of the genes *mec*A, $mec_{ALGA251}$, *bla*Z, *fem*A, and *fem*B, whereas the resistance to gentamicin was evaluated by the occurrence and expression of the *aac*A-*aph*D gene. The DNA and RNA extraction were performed using Illustra™ Bacteria Genomic Prep Mini Spin Kit (GE Healthcare®) and Illustra™ RNASpin Mini Isolation Kit (GE Healthcare®), respectively, according to the

manufacturer's instructions. The extracts were stored at -20°C for further PCRs to detect antimicrobial resistance encoding genes. After RNA extraction, the samples were quantified using NanoDrop™ 2000 Spectrophotometers (ThermoFisher Scientific®).

Subsequently, the RNA was subjected to cDNA production using SuperScript™ III (Invitrogen®) according to the manufacturer's instructions. For gene detection by PCR and evaluation of gene expression by Reverse transcription-polymerase chain reaction (RT-PCR), a pair of primers was used as the endogenous control 16sRNA – F (GTAGGTGGCAAGCGTTATCC) and 16sRNA – R (CGCACATCAGCGTCAG), 228 bp (Monday and Bohac, 1999). To investigate beta-lactams resistance, five pairs of primers were used: *bla*Z – F (AAGAGATTTGCCTATGCTTC) and *bla*Z – R (GCTTGACCACTTTTATCAGC), 517 bp (Sawant *et al.,* 2009); *mec*A – F (TCACCAGGTTCAAC[Y]CAAAA) and *mec*A – R (CCTGAATC[W]GCTAATAATATTTC), 356 bp (García-Álvarez *et al*., 2011); $mec_{ALGA251}$ – F (GCTCCTAATGCTAATGCA) and $mec_{ALGA251}$ – R (TAAGCAATAATGACTACC), 304 bp (Cuny *et al.* 2011); *fem*A- F (AGACAAATAGGAGTAATGAT) and *fem*A – R (AAATCTAACACTGAGTGATA), 509 bp, and *fem*B – F (TTACAGAGTTAACTGTTACC) and *fem*B – R (ATACAAATCCAGCACGCTCT), 651 bp (Kobayashi *et al.,* 1994); for resistance to gentamicin the primer *aac*A-*aph*D was used – F (TAATCCAAGAGCAATAAGGGC) and *aac*A-*aph*D – R (GCCACACTATCATAACCACTA), 227 bp (Strommenger *et al.,* 2003).

Standard ATCC strains containing the genes under study were used as positive controls: ATCC 700699 for *fem*A and *fem*B; ATCC 700698 for *mec*A and *aac*A-*aph*D; ATCC 35984 for *bla*Z; ATCC BAA-2312 for $mec_{ALGA251}$. PCRs were performed using the PlatinumTM Taq DNA Polymerase kit (Invitrogen®) according to the manufacturer's recommendations. Statistical analysis was carried out using the the software GraphPad Instat (Statistical Analysis Systems for Personal Computers 1990-1993) using Fisher's test.

RESULTS

The antimicrobial susceptibility tests were conducted using 57 *Staphylococcus* spp. isolates. All samples were sensitive to cephalothin (100%). High levels of sensitivity were observed in relation to oxacillin and gentamicin, 93% and 91.2%, respectively. The highest resistance indices (42.1%) were found for amoxicillin, ampicillin, and penicillin when compared to the other antimicrobials tested ($P<0.0001$). The ranges of minimum inhibitory concentrations (MICs) including the MIC_{50} and MIC_{90} for the isolates that showed resistance in the diffusion tests are presented in Table 1.

Table 1. Ranges of minimum inhibitory concentrations (MICs), MIC_{50} and MIC_{90} regarding the isolates that presented resistance in the diffusion tests

Antimicrobial	MIC (Range for most strains - µg/mL)	MIC_{50}	MIC_{90}
Oxacillin (N = 4)	0.5–2	≤ 0.5	≤ 1
Penicillin (N=24)	0.25–16	≤ 2	≤ 16
Amoxicillin (N=24)	0.5–2	≤ 1	≤ 2
Ampicillin (N=24)	0.5–2	≤ 1	≤ 2
Gentamicin (N=5)	16–128	≤ 64	≤ 128

In relation to the detection of genes related to bacterial resistance it was observed that one or more genes that were investigated (*mec*A, $mec_{ALGA251}$, *bla*Z, *fem*A, *fem*B, and *aac*A-*aph*D) were detected in 50 (87.7%) *Staphylococcus* spp. isolates. Table 2 presents the occurrence of the genes associated with antimicrobial resistance as well as the occurrence of the expression of these genes according to the *Staphylococcus* species. The *fem*A and *fem*B genes were the most frequent (N = 43, 75.4%) ($P<0.0001$). Different genes combinations were detected: *fem*A/*fem*B (N = 28; 49.1%), *fem*A/*fem*B/*aac*A-*aph*D (N = 6; 10.5%), and *fem*A/*fem*B/*mec*A/*aac*A-*aph*D (N = 5; 8.8%) among others that occurred to a lesser extent. For *S. aureus*, the most frequent gene combination was *fem*A/*fem*B (65.9%) ($P<0.007$).

Regarding the presence of the *fem*A gene in *Staphyococcus* species, it was possible to verify its occurrence in species other than *S. aureus* (two samples of *S. agnetis* and one of *S. xylosus*). The

*fem*A gene was detected in 97.6% of *S. aureus*. The expression of the genes *fem*A and *fem*B occurred only in *S. aureus* (42.5% for *fem*A and 35% for *fem*B) (Table 2). Regarding the *mec*A gene, none of the isolates of *S. aureus* or the other coagulase-positive *Staphylococcus* isolates showed expression of this gene, whereas a sample of *S. haemolyticus* was positive. The *mec*A$_{LGA251}$ gene was detected in an *S. equorum* isolate but expression of the gene was not observed. The *blaZ* gene was detected in five samples, and the expression of the gene was verified in one *S. aureus* and two *S. haemolyticus* isolates. Regarding the *aac*A-*aph*D gene, its expression was observed in 100% of coagulase negative *Staphylococcus* and in 55.6% of *S. aureus* (Table 2).

Table 2. Classification of 57 *Staphylococcus* strains (*S. aureus, S. agnetis, S. xylosus, S. epidermidis, S. equorum, S. haemolyticus*) isolated from bovine clinical mastitis according to the presence and expression of genes coding for resistance to different antimicrobials

Coagulase	*Staphylococcus Species*	N	fem*A*				*fem*B				*mec*A				aacA-aph*D*				*mecA*$_{LGA251}$				*blaZ*			
			detection		expression		detection		expression		detection		expression		detection		expression		detection		expression		detection		expression	
			N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%
Positive	*S. aureus*	41	40	97.6	17	42.5	40	97.6	14	35	7	17.1	0	0	9	22.0	5	55.6	0	0.0	0	0	1	2.4	1	100
other CPS	*S. hyicus*	6	0	0.0	0	0	0	0.0	0	0	3	50.0	0	0	2	33.3	0	0	0	0.0	0	0	1	16.7	0	0
	S. agnetis	2	2	100.0	0	0	2	100.0	0	0	1	50.0	0	0	2	100.0	0	0	0	0.0	0	0	0	0.0	0	0
	CPS total	8	2	25.0	0	0	2	25.0	0	0	4	50.0	0	0	4	50.0	0	0	0	0.0	0	0	1	12.5	0	0
Negative CNS	*S. xylosus*	1	1	100.0	0	0	1	100.0	0	0	0	0.0	0	0	0	0.0	0	0	0	0.0	0	0	0	0.0	0	0
	S.epidermidis	2	0	0.0	0	0	0	0.0	0	0	0	0.0	0	0	0	0.0	0	0	0	0.0	0	0	0	0.0	0	0
	S. equorum	2	0	0.0	0	0	0	0.0	0	0	0	0.0	0	0	0	0.0	0	0	1	50.0	0	0	1	50.0	0	0
	S.haemolyticus	3	0	0.0	0	0	0	0.0	0	0	1	33.3	1	100	3	100.0	3	100	0	0.0	0	0	2	66.7	2	100
	CNS total	8	1	12.5	0	0	1	12.5	0	0	1	12.5	1	100	3	37.5	3	100	1	12.5	0	0	3	37.5	2	66.7
	Total	57	43	75.4	17	39.5	43	75.4	14	32.6	12	21.1	1	8.33	16	28.1	8	50	1	1.8	0	0	5	8.8	3	60

Table 3 shows the results regarding the detection and expression of genes associated with antimicrobial resistance, and the association of these results with those of resistance obtained in the phenotypic tests. Regarding the *mec*A gene, the only sample positive for expression also presented phenotypic resistance to ampicillin, amoxicillin, oxacillin, and penicillin. It should be noted that of the 12 samples in which the gene was detected, in 11 (91.7%) of these there was no gene expression (Table 3). Regarding the *bla*Z gene, three isolates showed expression and phenotypic resistance to the beta-lactams which were tested, except for oxacillin.

When considering the *fem*A and *fem*B genes, it was verified that the expression of these genes was not associated with the occurrence of resistance in phenotypic tests with amoxicillin, ampicillin, and penicillin ($P<0.05$). The same was observed for the *aac*A-*aph*D gene and the resistance to gentamicin (Table 3). Concerning the *mec*A gene the concordance between expression of the genes and phenotypic resistance was very good for oxacillin and in the case of the *bla*Z gene, very good agreement was observed for ampicillin, amoxicillin, and penicillin.

Table 3. Classification of 57 *Staphylococcus* strains (*S. aureus, S. agnetis, S. xylosus, S. epidermidis, S. equorum, S. haemolyticus*) isolated from bovine clinical mastitis, according to the detection and expression of genes associated with antimicrobial resistance, and to the occurrence of phenotypic resistance to amoxicillin, ampicillin, oxacillin, penicillin, and gentamicin

gene	Gene Detection	N	%	Gene Expression	N	%	Ampicillin/ Amoxicillin		Oxacillin		Penicillin		Gentamicin	
							N	%	N	%	N	%	N	%
*fem*A	present	43	75.4	present	17	39.5	6	35.3	0	0.0	6	35.3	3	17.6
				absent	26	60.5	11	42.3	1	3.8	11	42.3	0	0.0
	absent	14	24.6	--	--	--	7	50.0	3	21.4	7	50.0	2	14.3
*fem*B	present	43	75.4	present	14	32.6	4	28.6	0	0.0	4	28.6	3	21.4
				absent	29	67.4	13	44.8	1	3.4	13	44.8	0	0.0
	absent	14	24.6	--	--	--	7	50.0	3	21.4	7	50.0	2	14.3
*mec*A	present	12	21.1	present	1	8.3	1	100.0	1	100.0	1	100.0	1	100.0
				absent	11	91.7	3	27.3	0	0.0	3	27.3	0	0.0
	absent	45	78.9	--	--	--	20	44.4	3	6.7	20	44.4	4	8.9
$mecA_{LGA251}$	present	1	1.8	present	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
				absent	1	100.0	0	0.0	0	0.0	0	0.0	0	0.0
	absent	56	98.2	--	--	--	24	42.9	4	7.1	24	42.9	5	8.9
*bla*Z	present	5	8.8	present	3	60.0	3	100.0	1	33.3	3	100.0	1	33.3
				absent	2	40.0	0	0.0	0	0.0	0	0.0	0	0.0
	absent	52	91.2	--	--	--	21	40.4	3	5.8	21	40.4	4	7.7
*aac*A-*aph*D	present	16	28.1	present	8	50.0	3	37.5	1	12.5	3	37.5	5	62.5
				absent	8	50.0	0	0.0	0	0.0	0	0.0	0	0.0
	absent	41	71.9	--	--	--	21	51.2	3	7.3	21	51.2	0	0.0

DISCUSSION

Antimicrobial therapy is a very important tool in mastitis control and treatment, although the increasing resistance to antimicrobials presented by microorganisms represents an important cause of therapeutic failure (Boireau *et al.* 2018). Different types of antimicrobials are used to treat and control bovine mastitis for lactating or drying cows. The use of beta-lactams and aminoglycosides as important antimicrobials used in bovine mastitis therapies has been reported (Srednik *et al*. 2017a; Martins *et al.* 2016).

Krewer *et al*. (2015) emphasized that intramammary treatment is not always effective, and resistance to the beta-lactams group may occur as they are routinely used to treat intramammary infections. Other researchers also report the occurrence of penicillin resistance (Rabello *et al*. 2005; Schmidt *et al*., 2015). In the present study, the resistance rate found for penicillin was 42.1%, and the same was found for amoxicillin and ampicillin. Costa *et al.* (2013) conducted a study with 35 dairy herds located in Minas Gerais, Brazil, that had cases of mastitis caused by *Staphylococcus aureus* and found higher resistance rates of 80.9% and 80.5% to ampicillin and penicillin, respectively. Sensitivity to oxacillin was also observed in over 90% of samples. Mistry *et al*. (2016) obtained 100% of *S. aureus* isolated from bovine intramammary infections sensitive to oxacillin.

The presence of genes associated with antimicrobial resistance was investigated in all *Staphylococcus* strains. The most frequently detected genes were *fem*A (75.4%) and *fem*B (75.4%), and according to Hussain *et al.* (2013) these are genes associated with the expression of methicillin resistance (MRSA - methicillin resistant *Staphylococcus aureus*) and are specific genes related to *S. aureus*. In the present study, it was found that 97.6% (40/41) of *S. aureus* isolates presented the *fem*A gene, which was also found in two samples of *S. agnetis* and one of *S. xylosus*. Therefore, the *fem*A gene can also be found in *Staphylococcus* species other than coagulase positive *Staphylococcus* (CPS). Lange *et al*. (2011) evaluated 100 *S. aureus* isolates from milk of cows with clinical or subclinical mastitis and detected *fem*A in 83 of them, and the gene was not detected in *S. chromogenes* (N=13) and *S. hyicus* (N=4).

The presence of the *fem*B gene was detected in 75.4% (43/57) of the isolates. When the presence

of the gene was evaluated in *S. aureus*, 97.6% (40/41) isolates were detected as positives, similar to the detection of the *fem*B gene in 97% (192/198) of *S. aureus* samples by Kobayashi *et al.* (1994). The presence of the gene was also observed in other species, such as *S. agnetis* and *S. xylosus*, contrary to the results of Kobayashi *et al.* (1994) who did not verify the presence of the gene in SCN. López-Vazques *et al.* (2015) obtained different results than those verified by the present study, as they isolated 28.1% (N=85/302) strains of *S. aureus* positive for the *fem*B gene.

The most frequent gene combination that was detected was *fem*A/*fem*B. The presence or absence of these genes was not a determinant for the occurrence of phenotypic resistance to the beta-lactams which were evaluated, indicating that other factors besides the presence of the genes probably influence the occurrence of resistance. Until the mid-1990s, MRSA exhibited phenotypic multidrug resistance and over time, the presence of the *mec*A gene became an important marker for indicating MRSA infections in view of their increased occurrence (Cuny *et al.* 2011). In the present study, the presence of the *mec*A gene was detected in 12 samples (21.1%): seven *S. aureus*, one *S. agnetis*, one *S. haemolyticus*, and three *S. hyicus*.

Regarding *S. aureus*, the frequency was 17.1% (N = 7/41). The presence of the *mec*A gene was also detected in CNS (one *S. haemolyticus*), similar to the results obtained by other researchers such as Soares *et al*. (2012) who detected the *mec*A gene in 13.8% (4/29) of oxacillin resistant CNS (*Staphylococcus xylosus*) isolated from bovine mastites and Frey *et al*. (2013) who isolated CNS (n = 370) from milk obtained from cows with clinical (n = 115) and subclinical (n = 255) mastitis and detected 19 strains containing *mec*A gene - *S. epidermidis*, *S. fleurettii*, *S. haemolyticus* and *S. xylosus*.

Fessler *et al.* (2010a) investigated the occurrence of resistance to oxacillin and the presence of the *mec*A gene in 121 CNS isolates from bovine mastitis on properties in Germany; 16 of these isolates showed resistance to oxacillin and the gene was detected in 15 of them. Frey *et al*. (2013) identified the *mec*A gene in 9.7% (n = 19) of the oxacillin-resistant CNS isolates (n = 196) and the gene was not detected in the other 177 oxacillin-resistant isolates (90.3%). Soares *et al*. (2012) isolated 100 CNS strains from milk from dairy cows in six different towns comprising an important milk production region of the State of Rio de Janeiro, Brazil; four isolates were positive to *mec*A gene (4%), all *S. xylosus* and the gene was detected in 13.8% (4/29) of the oxacillin resistant CNS.

In the present study, considering the 12 *Staphylococcus* isolates in which *mec*A was detected, it was observed that one (8.3%) was oxacillin-resistant and 11 (91.7%) oxacillin-sensitive. Regarding the *S. aureus* samples, all seven samples in which the gene was detected were oxacillin sensitive. It was found that in the presence of the gene there was a higher frequency of phenotypic resistance than in its absence (P<0.0001). In turn, Silva *et al*. (2014) isolated twenty-six methicillin-resistant CNS (*S. epidermidis*, *S. chromogenes*, *S. warneri*, *S. hyicus* and *S. simulans*) from milk of mastitic cows in Brazil, and all isolates carried *mec*A and were oxacillin resistant.

The *mec*A_{LGA251} (*mec*C), a *mec*A homolog gene, was found in a sample of *S. equorum* which was sensitive for all beta-lactams which were evaluated and was negative for the evaluation of gene expression. Srednik *et al*. (2017b) reported for the first time, one CNS (*Staphylococcus saprophyticus*) isolated from bovine mastitis in South America that was resistant to beta-lactams and positive for the *mec*C gene. Dhaouadi *et al*. (2020) also reported the occurrence of the *mec*C gene in MR-CNS (three *Staphylococcus sciuri* isolates) from cows' milk and manure in Tunisia and Africa. Unnerstad *et al.* (2013) investigated 730 *S. aureus* samples isolated from bovine mastitis and confirmed MRSA, having detected the *mec*A_{LGA251} gene in 4 (0.5%) of these.

The *bla*Z gene, that can be found on both the chromosome and plasmids, was detected at reduced frequency when the totality of the isolates was considered (8.8%) as well as in the *S. aureus* samples (2.4%). In turn Ruegg *et al.* (2015) evaluated 35 *S. aureus* isolates and 51 CNS isolates from bovine mastitis samples and detected the presence of the *bla*Z gene in 46 (53.5%) isolates. Regarding the 41 *S. aureus* isolates, the concomitant presence of the *mec*A, *fem*A, and *fem*B genes - important for a safer identification of MRSA (Kobayashi *et al.* 1994) -

had a low frequency of occurrence (7.3%). The most frequent gene combination was *fem*A/*fem*B for both *Staphylococcus* spp. (49.1%) and *S. aureus* (65.9%).

The presence of the *aac*A-*aph*D gene, which codes for enzymes that act on aminoglycosides, was detected in 28.1% (16/57) of the *Staphylococcus* spp. isolates and 22% (9/41) of the *S. aureus* isolates. The *aac*A-*aph*D gene was detected in 62.5% (5/16) of the isolates phenotypically resistant to gentamicin. Fessler *et al.* (2010b) evaluated 27 MRSA isolates (25 isolates from clinical bovine mastitis and two isolates from dairy farm workers) and 100% of the samples that were phenotypically resistant to gentamicin (N = 6) also presented the *aac*A-*aph*D gene.

One of the objectives of the present study, considering the lack of information on the subject, was to relate data on the detection and expression of genes encoding resistance to antimicrobials most commonly used in clinical bovine practice in *Staphylococcus* isolated from cases of clinical bovine mastitis. The occurrence of expression was observed for all genes that were evaluated except for *mec*A$_{LGA251;}$ *bla*Z (60%), *fem*A (39.5%), *aac*A-*aph*D (50%), *fem*B (32.6%), and *mec*A (8.3%). It was observed that the presence of the gene was not necessarily associated with its expression when all the cases in which they were detected were considered.

It was also verified that, for the *fem*A and *fem*B genes, the expression of these genes was observed only in *S. aureus* and that none of the 7 *S. aureus* isolates in which the *mec*A gene was detected showed gene expression. Only one of eight CNS samples presented the *mec*A gene positive for gene expression. Regarding the *aac*A-*aph*D, the expression of the gene was observed in all CNSs (N = 3) in which the gene was detected, and in 55.6% of the *S. aureus* samples positive for the occurrence of the gene. The evaluation of the variables genes expression *versus* phenotypic resistance showed very good concordance regarding *bla*Z gene *versus* ampicillin, amoxicillin, and penicillin, as well as *mec*A *versus* oxacillin. From this perspective, it is possible that the *bla*Z gene may have a more relevant participation in the occurrence of phenotypic resistance to ampicillin, amoxicillin, and penicillin as well as *mec*A against oxacillin.

CONCLUSIONS

The results show that the presence of a specific gene is not necessarily associated with its genotypic and/or phenotypic expression, as well as the occurrence of gene expression may not be necessarily associated with its phenotypic expression. The results of the present study reinforce the importance of conducting further research in order to clarify aspects involved in the occurrence of gene expression associated with antimicrobial resistance, whether genetic or not. Research on this subject will shed more light on the behavior of microorganisms towards antimicrobials, and consequently, should help in the development of new strategies for the control and treatment of mastitis in dairy herds around the world.

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