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Original Article

Expression of genes encoding resistance in *Staphylococcus* spp. isolated from bovine subclinical mastitis in Brazil

Eveline Zuniga[1#], Nilson Roberti Benites[1#], Aline Santana da Hora[2#], Priscila Luiza Mello[3#], Marco Antonio Laes[1#], Lilian Abgail Ribeiro de Oliveira[1#], Paulo Eduardo Brandão[1#], Sheila Oliveira de Souza Silva[1#], Sueli Akemi Taniwaki[1#], Priscilla Anne Melville[1#]

[1] Department of Preventive Veterinary Medicine and Animal Health, Faculty of Veterinary Medicine and Zootechnics, University of São Paulo (USP), São Paulo, Brazil
[2] Department of Preventive Veterinary Medicine, School of Veterinary Medicine, Federal University of Uberlandia (UFU), Minas Gerais, Brazil
[3] University of Guarulhos, Guarulhos, SP, Brazil

# Authors contributed equally to this work.

Abstract

Introduction: *Staphylococci* are the most important agents associated with bovine mastitis. This study aimed at characterizing resistance factors to antimicrobials in *Staphylococcus* spp. isolated from the milk of cows with subclinical mastitis.

Methodology: *In vitro* resistance of 243 *Staphylococcus* spp. isolates to antimicrobials commonly used in clinical practice was evaluated. The detection and expression of genes encoding resistance *mec*A (gene encoding penicillin binding protein 2a) $mec_{ALGA251}$ (*mec*A homologue), *bla*Z (gene encoding penicillin resistance), *fem*A and *fem*B (genes encoding essential factors - A and B - for the expression of methicillin resistance) and *aac*A-*aph*D (gene encoding for a bifunctional enzyme that confers resistance to gentamicin) using PCR and RT-PCR was investigated.

Results: One or more genes encoding resistance to different antimicrobials were detected in 184 *Staphylococcus* spp. samples. The *fem*A and *fem*B genes were the most frequent. Regarding the variables' detection (N = number of strains) and expression (% of strains), the following results were obtained: *bla*Z (N = 40 – 82.5%), *fem*A (N = 147 – 47.6%), *aac*A*aph*D (N = 30 – 43.3%), *fem*B (N = 138 – 29.7%), *mec*A (N = 33 – 27.3%), $mec_{ALGA251}$ (N = 01 – 0.0%). There was a higher occurrence of phenotypic resistant strains for amoxicillin, ampicillin and penicillin in isolates positive for detection and/or expression of *bla*Z gene when compared with the other genes.

Conclusions: The present study provides new information on genotypic traits of *Staphylococcus* isolates from bovine subclinical mastitis especially regarding the evaluation of expression of genes associated with antimicrobial resistance in *Staphylococcus* spp. using molecular tools.

Key words: Antimicrobial resistance; bovine subclinical mastitis; gene expression; *Staphylococcus* spp..

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Introduction

Bovine mastitis is one of the most prevalent livestock diseases; it is challenging for field veterinarians and dairy farmers, due to the economic losses it incurs in the global dairy industry. *Staphylococcus* spp. is one of the most common pathogens that cause bovine mastitis [1]. Treatment and prevention are fundamental measures for the control of this condition. The indiscriminate use of antimicrobials, however, is commonly viewed as a factor contributing to the increase of bacterial resistance and inefficiency of drug therapies [2].

Although penicillins do not distribute adequately in the inflamed mammary gland, these antimicrobials are still constantly used to treat mastitis caused by *Staphylococcus* spp. [3]. Cephalosporins, therefore, have been widely used, with satisfactory results [3]. Bacterial resistance to beta-lactam antimicrobials that has been described in isolates obtained from milk produced by mastitis infected cows [4], is the result of different mechanisms. One of them is related to the production of the extracellular enzyme beta-lactamase (encoded by the *bla*Z gene), which inactivates the antibiotic through the hydrolysis of the beta-lactam ring [5]. A second mechanism of resistance is associated with the production of penicillin binding protein 2a (PBP2a), a modified penicillin binding protein encoded by the *mec*A gene [6]. Penicillin binding proteins

(PBPs) are enzymes found in the membrane that catalyzes the cross-linking reactions of cell wall peptidoglycan polymers. Beta-lactams bind covalently to these proteins, changing their conformation and resulting in bacterial destruction. PBP2a proteins replace PBPs and inhibit the drug's action; this mechanism is present in methicillin resistant strains [7].

García-Alvarez *et al.* [8] and Cuny *et al.* [9] report that methicillin-resistant *Staphylococcus aureus* (MRSA) may contain a homologous gene, $mec_{ALGA251}$, also known as *mec*C, which is not detected by conventional PCR for the *mec*A gene. Both studies detected isolates positive for resistance to methicillin and absence of *mec*A, and verified the presence of homologue $mec_{ALGA251}$, emphasizing its importance as a gene associated with resistance to beta-lactams.

The *fem*A and *fem*B (fem - factor essential for methicillin resistance) genes encode proteins that influence the level of methicillin resistance. Both are involved in the formation of the cell wall: *fem*A is involved in the addition of glycines 2 and 3 and *fem*B in the addition of glycines 4 and 5, thus forming pentaglycine, which is essential for the formation of the wall's peptidoglycan [10]. The absence of *fem*A and *fem*B is related to glycine reduction in the peptidoglycan, thus making the cell wall more susceptible to beta-lactams [10]. Thus, the *fem*A and *fem*B genes, present in the *Staphylococcus* chromosome, are also considered important indicators for MRSA identification [6].

Aminoglycosides are also used in the treatment of mastitis, often in combination with beta-lactams [11,12]. The main mechanism of resistance to aminoglycosides is the enzymatic modification of the antimicrobial agent with consequent inactivation. The enzymes responsible for the inactivation of these antibiotics are phosphotransferases (APH), adenyltransferases (ANT), or acetyltransferases (AAC). These enzymes act by modifying the amino and hydroxyl groups of the antibiotics, thus preventing them from binding to the ribosomes; the genes encoding these enzymes are usually located on plasmids. Concurrent resistance to gentamicin, tobramycin, and kanamycin, is mediated by a bifunctional enzyme that exhibits AAC and APH activity and is encoded by the *aac*A-*aph*D gene [13].

Considering the importance of the *Staphylococcus* genus in mastitis etiology, we evaluated the resistance of *Staphylococcus* spp. isolated from bovine subclinical mastitis to the most commonly used antimicrobial agents in clinical practice (beta-lactams and gentamicin), evaluating phenotype (*in vitro* antimicrobial susceptibility tests) and genotype (detection and expression of genes coding for resistance) characteristics. The results could be useful in the development of new approaches regarding therapies and measures for the prevention of bovine mastitis. Studies on mastitis-causing *Staphylococcus* are important for a better understanding of the characteristics of these microorganisms and their pathogenic potential.

Methodology

The area chosen for the study is part of an important dairy region located in the south of the state of Minas Gerais - Brazil. The predominant dairy breed in this area is the black and white Holstein Friesians. The cattle are raised on pasture, but receive feed based on corn silage and concentrate with 20% crude protein offered in quantities according to individual milk production. A total of 2,509 mammary glands from 633 cows were screened using California Mastitis Test [14]. A total of 910 mammary glands from 431 cows tested positive in the CMT and were collected for microbiological examination. Those animals belonged to eight different dairy farms.

A total of 243 *Staphylococcus* spp. strains isolated from bovine milk samples presenting subclinical mastitis were evaluated. After bacterial cultivation, the isolates were submitted to PCR for *rpob* gene detection for genus confirmation, with subsequent DNA sequencing of the amplified region, for determining the species [15].

In vitro susceptibility testing of Staphylococcus spp.

To evaluate the susceptibility profile of strains under study, the diffusion technique described by Bauer was applied [16]. The concentrations, as well as the interpretation criteria used, were those standardized by the Clinical and Laboratory Standards Institute [17,18]. The susceptibility to the following antimicrobials was evaluated: amoxicillin (10 μg), ampicillin (10 μg), oxacillin (10 μg), penicillin (10 I.U.), cephalothin (30 μg), and gentamicin (10 μg).

The isolates that presented resistance in the diffusion tests were submitted to the minimum inhibitory concentration (MIC) evaluation regarding the antimicrobials to which they presented resistance. The E-test commercial kit was used to perform the tests, according to the manufacturer's instructions; the criteria used to interpret the results were those described by the Clinical and Laboratory Standards Institute [17,18].

Detection and expression of genes related to bacterial resistance to antimicrobials

The bacterial resistance to beta-lactams was evaluated by the occurrence and expression of *mec*A, *mec*$_{ALGA251}$, *bla*Z, *fem*A, and *fem*B genes, while the resistance to gentamicin was evaluated by the occurrence and expression of the *aac*A-*aph*D gene. DNA and RNA were extracted from *Staphylococcus* isolates previously cultured in blood agar and incubated at 37 °C for 24 hours. DNA extraction was performed using the Illustra Bacteria Genomic Prep Mini Spin Kit (GE Healthcare, Chalfont, St. Giles, UK), according to the manufacturer's instructions. RNA was extracted using the IllustraTM RNASpin Mini Isolation Kit, GE Healthcare (Buckinghamshire, UK), according to the manufacturer's recommendations. Immediately after extraction, samples were quantified using NanoDrop™ 2000/2000c Spectrophotometers, ThermoFisher Scientific (Wilmington – Delaware, USA). Subsequently, RNA was used for cDNA production using SuperScript III (Invitrogen, Carisbad, California, USA), according to the manufacturer's instructions.

Six pairs of primers were used for gene detection and evaluation of gene expression by using PCR and RT-PCR (Reverse-Transcription PCR), respectively. A pair of primers was used as endogenous control when the sample was negative for all PCR and RT-PCR reactions (Table 1). As positive controls, standard ATCC strains containing the genes under study and strains from the Laboratory of Bacteriology and Mycology (FMVZ-VPS-USP) were used. PCRs were performed using a Platinum Taq DNA Polymerase kit (Invitrogen, Carisbad, California, USA), according to the manufacturer's recommendations.

Statistical analysis

Statistical analysis was carried out in the software GraphPad Instat (Statistical Analysis Systems for Personal Computers, version 2.01/ 1990-1993) using Fisher's test.

Results

Evaluation of in vitro susceptibility of Staphylococcus spp*. against different antimicrobials*

Sensitivity and resistance profiles of 243 isolates of *Staphylococcus* spp. were evaluated. All samples showed sensitivity to cephalothin. According to CLSI [18] standardization, the interpretation of the results obtained for ampicillin susceptibility tests can be applied to amoxicillin. High sensitivity to gentamicin (N = 233; 95.9%) and oxacillin (N = 209; 86%) were observed. The highest resistance was found for amoxicillin (N = 145; 59.7%), ampicillin (N = 145; 59.7%), and penicillin (N = 142; 58.4%) when compared to the other antimicrobials that were evaluated ($p < 0.0001$).

All isolates that showed resistance in the diffusion tests were submitted to the MIC evaluation against the antimicrobials to which they were resistant. Regarding amoxicillin and ampicillin, it was found that a higher frequency of isolates (N = 100; 76.3%) presented MICs in the range of 0.5 – 2 µg/mL ($p < 0.05$); MIC_{50} was ≤ 1; MIC_{90} was ≤ 8. Regarding oxacillin, it was observed that a higher frequency of isolates (N = 31; 91.2%) presented MICs in the range of 0.5 – 8 µg/mL ($p < 0.05$); MIC_{50} was ≤ 1 and MIC_{90} was ≤ 8. For 166 penicillin-resistant samples, there was a higher frequency of isolates (N = 58; 40.8%) distributed in the range of MICs 2 – 4 µg/mL ($p < 0.05$); MIC_{50} for this antimicrobial was ≤ 4 and MIC_{90} ≤ 32. Isolates resistant

Table 1. Primers used to amplify endogenous control and genes related to antimicrobial resistance, extended product size and references.

Function	Genes	Genetic Sequence (5´- 3´)	Amplified Product Size	Reference
Endogenous control	16sRNA - F 16sRNA - R	GTAGGTGGCAAGCGTTATCC CGCACATCAGCGTCAG	228bp	[19]
Resistance to Beta lactams	*bla*Z - F *bla*Z - R	AAGAGATTTGCCTATGCTTC GCTTGACCACTTTTATCAGC	517bp	[20]
Resistance to MRSA	*mec*A - F *mec*A - R	TCACCAGGTTCAAC[Y]CAAAA CCTGAATC[W]GCTAATAATATTTC	356bp	[8]
Resistance to MRSA	*mec*$_{ALGA251}$ - F *mec*$_{ALGA251}$ - R	GCTCCTAATGCTAATGCA TAAGCAATAATGACTACC	304bp	[9]
Resistance to MRSA	*fem*A- F *fem*A - R	AGACAAATAGGAGTAATGAT AAATCTAACACTGAGTGATA	509bp	[6]
Resistance to MRSA	*fem*B - F *fem*B - R	TTACAGAGTTAACTGTTACC ATACAAATCCAGCACGCTCT	651bp	[6]
Resistance to Aminoglycosides	*aac*A-*aph*D - F *aac*A-*aph*D - R	TAATCCAAGAGCAATAAGGGC GCCACACTATCATAACCACTA	227bp	[13]

to gentamicin (N = 15) presented MICs in the range of 16 to - 256 μg/mL; MIC_{50} was ≤ 128 and MIC_{90} was ≤ 256.

Detection and expression of genes related to bacterial resistance to antimicrobials

One or more genes encoding resistance to different antimicrobials (*mec*A, $mec_{ALGA251}$, *bla*Z, *fem*A, *fem*B, and *aac*A-*aph*D) were detected in 184 *Staphylococcus* spp. samples. It should be noted that in three strains (*S. chromogenes, S. saprophyticus, S. simulans*) the evaluated genes were not detected. The most frequently appearing genes were *fem*A (60.5%) and *fem*B (56.8%); their frequencies of occurrence were higher than those of the other genes ($p < 0.0001$). The $mec_{ALGA251}$ gene was found in a *S. hyicus* strain, but the *mec*A gene was not found in this isolate. Data concerning cephalothin are not shown in the table as 100% of sensitivity was detected for this antimicrobial (Table 2).

The most frequently detected combination of genes was *fem*A-*fem*B (N = 99; 40.74%) ($p < 0.0001$). Regarding *S. aureus*, the most frequent gene combination was also *fem*A-*fem*B (N = 95; 58%) ($p < 0.0001$), followed by *bla*Z-*fem*A-*fem*B (N = 22; 13.4%) ($p = 0.02$).

In relation to the occurrence of *fem*A in coagulase-positive *Staphylococcus* (CPS) and coagulase-negative *Staphylococcus* (CNS) species, the presence of the gene was verified in species other than *S. aureus*: *S. hyicus* (N = 7; 17.5% of isolates), *S. agnetis* (N = 1; 50%), *S. epidermidis* (N = 3; 16.7%), and *S. haemolyticus* (N = 3; 21.4%). It should be emphasized that the *fem*A gene was not present in all *S. aureus* isolates (detected in 133 isolates or 81.1%).

When the occurrence of phenotypic resistance for amoxicillin and ampicillin was considered regarding the isolates which were positive for the genes, the following results were observed: *bla*Z (80%, N = 32/40), *mec*A (27.3%, N = 9/33), $mec_{ALGA251}$ (0%, N = 0/1), *fem*A (25.2%, N = 37/147), *fem*B (15.9%, N = 22/138) and *aac*A-*aph*D (40%, N = 12/30). The results for penicillin were similar except for *fem*A (23.8%, N = 35/147) and *fem*B (15.2%, N = 21/138) (Table 3).

In relation to the occurrence of phenotypic resistance for oxacillin regarding the isolates which were positive for the genes, the following results were observed: *bla*Z (15%, N = 6/40), *mec*A (27.3%, N = 9/33), $mec_{ALGA251}$ (0%, N = 0/1), *fem*A (0.7%, N = 1/147), *fem*B (0%, N = 0/138) and *aac*A-*aph*D (40%, N = 12/30) (Table 3).

When the occurrence of phenotypic resistance for gentamicin was considered regarding the isolates which were positive for the genes, the following results were observed: *bla*Z (5%, N = 2/40), *mec*A (18.2%, N = 6/33), $mec_{ALGA251}$ (0%, N = 0/1), and *aac*A-*aph*D (26.6%, N = 8/30). *fem*A (0%, N = 0/147), *fem*B (0%, N = 0/138) (Table 3).

The *fem*A and *fem*B gene expression occurred in *S. aureus* (51.88% and 33.06%, respectively). *fem*A gene expression occurred in a *S. agnetis* isolate (12.5% of a total of 42 non-*S. aureus* SCP). It should be noted that for *fem*A and *fem*B, expression of these genes was not observed in any of the 37 SCN samples in which they were detected (Table 2). When considering these two

Table 2. Classification of 243 *Staphylococcus* strains isolated from subclinical mastitis according to the bacterial species, the presence of genes that confer resistance, as well as the occurrence of expression of the genes that were detected.

Coagulase	*Staphylococcus species*	N	fem*A*				*fem*B				*mec*A				aacA-aph*D*				*mec*A_{LGA251}				*bla*Z			
			detection		expression		detection		expression		detection		expression		detection		expression		detection		expression		detection		expression	
			N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%
CPS*1	*S. aureus*	164	133	81.1	69	51.88	124	75.6	41	33.06	7	4.3	0	0	6	3.7	0	0	0	0.0	0	0	28	17.1	26	92.86
others CPS	*S. hyicus*	40	7	17.5	0	0	8	20.0	0	0	8	20.0	0	0	8	20.0	1	12.5	1	2.5	0	0	7	17.5	2	28.57
	S. agnetis	2	1	50.0	1	100	1	50.0	0	0	0	0.0	0	0	0	0.0	0	0	0	0.0	0	0	0	0.0	0	0
	CPS total	42	8	19.0	1	12.5	9	21.4	0	0	8	19.0	0	0	8	19.0	1	12.5	1	2.4	0	0	7	16.7	2	28.57
CNS*2	*S. haemolyticus*	14	3	21.4	0	0	1	7.1	0	0	3	21.4	0	0	3	21.4	0	0	0	0.0	0	0	2	14.3	2	100
	S. epidermidis	18	3	16.7	0	0	4	22.2	0	0	13	72.2	9	69.23	13	72.2	12	92.31	0	0.0	0	0	3	16.7	3	100
	S. sciuri	2	0	0.0	0	0	0	0.0	0	0	2	100.0	0	0	0	0.0	0	0	0	0.0	0	0	0	0.0	0	0
	S. chromogenes	1	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. saprophyticus	1	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. simulans	1	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
	CNS total	37	6	16.2	0	0	5	13.5	0	0	18	48.6	9	50	16	43.2	12	75	0	0.0	0	0	5	13.5	5	100
	Total	**243**	**147**	**60.5**	**70**	**47.62**	**138**	**56.8**	**41**	**29.71**	**33**	**13.6**	**9**	**27.27**	**30**	**12.3**	**13**	**43.33**	**1**	**0.4**	**0**	**0**	**40**	**16.5**	**33**	**82.5**

*1 coagulase-positive *Staphylococcus* (CPS); *2 coagulase-negative *Staphylococcus* (CNS).

genes, it was possible to verify that the presence or absence of their expression was not associated with the occurrence of phenotypic resistance to amoxicillin, ampicillin, oxacillin, penicillin, and gentamicin ($p < 0.05$).

In relation to the *mec*A gene, neither *S. aureus* (N = 7) nor other CPS isolates (N = 8) in which the gene was detected, showed expression of the gene, whereas nine (50% of the 18 strains of CNS in which the gene was detected) samples of *S. epidermidis* were positive for expression (Table 2). All samples that tested positive for expression also showed phenotypic resistance to ampicillin ($p < 0.01$), amoxicillin ($p < 0.01$), oxacillin ($p < 0.0003$), and penicillin ($p < 0.01$). It should be noted that, of the 33 strains in which the gene was detected, gene expression was not verified by molecular tests in 24 (72.7%) (Table 3). Similarly, the presence of gene expression was related to a higher frequency of resistance to gentamicin ($p < 0.005$).

The *mec*A_{LGA251} gene, in turn, was detected in one isolate (*S. hyicus*) in which no gene expression was observed (Table 2).

The *bla*Z gene was detected in 40 strains, and gene expression was observed in 82.5% isolates. The expression was verified in a higher percentage in *S. aureus* (92.86%) ($p < 0.0001$) when compared to the other CPS and CNS isolates (Table 2). The occurrence of *bla*Z gene expression was higher (82.5%) than that of other genes ($p < 0.0001$). In the presence of *bla*Z gene expression, there was a higher occurrence of resistant strains for amoxicillin (97%), ampicillin (97%), and penicillin (97%) ($p < 0.0001$) (Table 3).

Regarding the *aac*A*aph*D gene, it was observed in a higher percentage ($p < 0.0001$) in CNS (43.33%) than in *S. aureus* and other CPS isolates, with the majority (92.3%) of *S. epidermidis* isolates ($p < 0.0001$) showing expression of this gene (Table 2). For this gene, most strains in which detection and expression were present, also showed antibiotic resistance to gentamicin (8/13 or 61.5%) ($p < 0.001$) (Table 3). When the expression of this gene was observed, a higher occurrence of strains resistant to other antimicrobials was verified ($p < 0.05$).

Discussion

Bovine mastitis is a multifactorial disease and has a significant impact on the agricultural sector around the world [19]. Antimicrobials are commonly used in the treatment and control of bovine mastitis for dry and lactating cows. Researchers report the occurrence of bacterial resistance to the most commonly used drugs, such as beta-lactams and aminoglycosides [4,20,12].

Beta-lactam resistance results from the widespread use of this class of antibiotics for the treatment and control of mastitis in dairy herds, as well as from the inadequate use of these drugs (such as low dosage and short treatment time) [21]. For these reasons, bacterial resistance is often associated with a low cure rate [22]. The results of the present study reported high MICs for

Table 3. Classification of 243 *Staphylococcus* strains isolated from subclinical mastitis according to the presence of genes that confer antimicrobial resistance, as well as the occurrence of expression of the genes that were detected and occurrence of phenotypic resistance to amoxicillin, ampicillin, oxacillin, penicillin, and gentamicin.

gene	Gene detection	N	%	Gene expression	N	%	Ampicillin / Amoxacillin		Oxacillin		Penicillin		Gentamicin	
							N	%	N	%	N	%	N	%
*fem*A	present	147	60.5	present	70	47.6	37	52.9	1	1.4	35	50.0	0	0.0
				absent	77	52.4	51	66.2	7	9.1	50	64.9	2	2.6
	absent	96	39.5	--	--	--	57	59.4	26	27.1	57	59.4	8	8.3
*fem*B	present	138	56.8	present	41	29.7	22	53.7	0	0.0	21	51.2	0	0.0
				absent	97	70.3	57	58.8	7	7.2	55	56.7	3	3.1
	absent	105	43.2	--	--	--	66	62.9	27	25.7	66	62.9	7	6.7
*mec*A	present	33	13.6	present	9	27.3	9	100.0	9	100.0	9	100.0	6	66.7
				absent	24	72.7	212	883.3	7	29.2	12	50.0	3	12.5
	absent	210	86.4	--	--	--	124	59.0	18	8.6	121	57.6	1	0.5
mecA_{LGA251}	present	1	0.4	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	242	99.6	--	--	--	145	59.9	34	14.0	142	58.7	10	4.1
*bla*Z	present	40	16.5	present	33	82.5	32	97.0	6	18.2	32	97.0	2	6.1
				absent	7	17.5	1	14.3	0	0.0	1	14.3	0	0.0
	absent	203	83.5	--	--	--	112	55.2	28	13.8	109	53.7	8	3.9
*aac*A-*aph*D	present	30	12.3	present	13	43.3	12	92.3	12	92.3	12	92.3	8	61.5
				absent	17	56.7	8	47.1	2	11.8	8	47.1	1	5.9
	absent	213	87.7	--	--	--	125	58.7	20	9.4	122	57.3	1	0.5

penicillin ($MIC_{50} \leq 4$; $MIC_{90} \leq 32$), possibly related to a higher resistance of the evaluated pathogens.

The present study investigated the presence of genes encoding antimicrobial resistance in *Staphylococcus*, isolated from bovine subclinical mastitis. The *fem*A and *fem*B genes were the most frequently detected genes (60.5% and 56.8%, respectively). Lange *et al.* [23] evaluated 100 *Staphylococcus* spp. isolated from bovine mastitis and detected the *fem*A gene in 83% of the samples, which were confirmed to be *S. aureus*. In the present study, 81.1% (133/164) of *S. aureus* isolates carried the *fem*A gene; the same was true for 17.5% (7/40) of *S. hyicus*, 16.7% (3/18) of *S. epidermidis*, 21.4% (3/14) of *S. haemolyticus*, and 50% (1/2) of *S. agnetis* isolates. All species were confirmed by sequencing, which indicated that the *fem*A gene can also be found in species of *Staphylococcus* other than *S. aureus*, including CNS. It should be noted that other researchers have also found that *S. aureus* isolates carry the *fem*A gene [23,24], however the gene was not verified in all samples (81.1% of samples), inferring a possible error of 18.9% considering the statement that all *Staphylococcus* species that carry the *fem*A gene are *S. aureus.*

The detection frequency of the *fem*B gene was similar ($p = 0.46$) to that of the *fem*A gene. Kobayashi *et al.* [6] detected the *fem*B gene in 192 (97%) of 198 *S. aureus* isolates, but not in CNS isolates. In the present study the gene was detected in 124 (75.6%) *S. aureus*, 04 (22.2%) *S. epidermidis* and 01 (7.1%) of *S. haemolyticus* isolates. It should be noted that the most frequently ($p < 0.0001$) detected gene combination in the present study was *fem*A-*fem*B (40.74%).

In relation to the presence of the *fem*A and *fem*B genes and the resistance presented by the antibiogram, it was observed that the presence or absence of these genes was not a determinant of the occurrence of phenotypic resistance to antimicrobials that were evaluated; in other words, other factors could be influencing the occurrence of resistance. Similarly, other researchers did not observe differences regarding the presence or absence of the *fem*B gene in *Staphylococcus* oxacillin-resistant strains [6].

The first case of MRSA in mastitis was reported in 1972; since then, new reports have appeared in relation to dairy cattle [9]. Phenotypic resistance is determined by the *in vitro* susceptibility test, but the detection of the *mec*A gene can also be used to confirm the diagnosis of MRSA. In the present study the presence of the *mec*A gene was detected in 33 samples (13.6%); in *S. aureus*, the frequency was 4.3% (N = 7/164). Guimarães *et al.* [25] isolated *Staphylococcus* (98.4% *S. aureus* and 1.6% *S. epidermidis*) from 61 bovine mastitis samples; the presence of the *mec*A gene was verified in 48.3% of *S. aureus* isolates, out of which 23.3% were MRSA. The *mec*A gene was also detected in CNS (N = 18 - 48.6%). The presence of the *mec*A gene has already been detected in other *Staphylococcus* species including *S. epidermidis, S. xylosus, S. sciuri,* and *S. haemolyticus*, among others [26-28]. In this study, the presence of the gene in CPS and CNS species was verified.

When considering the variables' susceptibility to oxacillin and the presence of the *mec*A gene, it was observed that, out of the 33 *Staphylococcus* isolates in which *mec*A was detected, 16 (48.48%) were oxacillin resistant. Considering only *S. aureus*, it was observed that all seven strains in which the gene was detected were oxacillin sensitive. There was a greater frequency of the occurrence of phenotypic resistance in the presence than in the absence of the gene ($p < 0.0001$). Researchers investigated MRSA occurrence in 103 *S. aureus* isolates from bovine mastitis samples; the *mec*A gene was detected in 49 (47.6%) of the strains, while 37 (75.5%) of them were sensitive to oxacillin in antimicrobial susceptibility tests and were classified as oxacillin-susceptible *mec*A-positive *S. aureus* (OS-MRSA) [29].

According to Kobayashi *et al.* [6], between the concomitant and the separate detection of *mec*A, *fem*A, and *fem*B genes, the former proved to be a more reliable tool for MRSA identification and this combination of genes was verified in 135 (68.2%) out of a total of 198 *S. aureus* isolates from different sources. In relation to the 164 *S. aureus* studied, this combination of genes was observed with low frequency (1.3%), and the most frequent combination was *fem*A-*fem*B (58%).

García-Alvarez *et al.* [8] reported that the *mec*A homologous gene, $mec_{ALGA251}$ has 70% similarity to *mec*A, and described its occurrence in MRSA, isolated from bovine mastitis in the UK and from human samples in Denmark. In the present study, the $mec_{ALGA251}$ gene was identified in only one sample (*S. hyicus*), susceptible to all beta-lactams evaluated in the antibiograms. The $mec_{ALGA251}$ gene was detected, along with the *mec*A gene, in a *S. hyicus* strain. García-Alvarez *et al.* [8] also detected the presence of the $mec_{ALGA251}$ gene at a low frequency of occurrence (1.4% of *Staphylococcus* isolates from bovine mastitis), and the strains were also negative for *mec*A and methicillin resistant (in the phenotypic analysis).

The *bla*Z gene was detected in *S. aureus* at a low frequency (17.1%). The presence of the gene was verified in 40 (16.5%) out of the 243 *Staphylococcus*

isolates studied. Marques *et al.* [30] isolated 59 *Staphylococcus* spp. strains (20 were *S. aureus*) from bovine subclinical mastitis, and detected the *bla*Z gene in 70% of the *S. aureus* isolates. In relation to the occurrence of samples *bla*Z positive it was verified that 80% and 15% of the isolates showed phenotypic resistance against amoxicillin/ampicillin and oxacillin, respectively. Soares *et al.* [31] evaluated the phenogenotypic profile of antimicrobial resistance of 100 CNS isolates obtained from bovine mastitis and found the *bla*Z gene in 16% of the cases; in all of these cases the strains were resistant to the beta-lactams which were tested (penicillin, ampicillin, cephalotin, and oxacillin).

The main mechanism of resistance regarding aminoglycosides in *Staphylococcus* is the enzymatic inactivation of these antimicrobials [32]. The *aac*A-*aph*D gene encodes acetyltransferase and phosphotransferase enzymes that act leading to the loss of the antimicrobials' ability to bind to ribosomes, no longer impeding the protein synthesis of bacterial cells, which develop resistance. In the present study, the presence of the *aac*A-*aph*D gene was detected in 30 (12.3%) of the 243 isolates. Phenotypically resistance to gentamicin was observed for 26.6% (8/30) of the isolates *aac*A-*aph*D positive. A group of researchers evaluated 128 *S. aureus* isolates (83 from animals with subclinical mastitis and 45 from animals with clinical mastitis) and detected the *aac*A-*aph*D gene in 26.6% of the cases; phenotypic resistance to gentamicin was detected in only one of the cases [33].

In the literature examined, no reports were found on the evaluation of gene expression in *Staphylococcus* spp. relative to expression of genes associated with antimicrobial resistance. As in the present study, some researchers also used RT-PCR to evaluate gene expression [12]. They performed the characterization of virulence factors and resistance to antimicrobials in different CNS species isolated from sheep's milk. After the identification of the isolates, DNA was extracted to detect (by PCR) the presence of *mec*A, *ica*ADBC, *bap*, *bhp,* and toxin genes (*sea, seb, sec, sed, tst*, and *luk*-PV). Expression of toxins was evaluated by the RT-PCR technique. Seventy samples (62.5%) had some toxin encoding gene. However, none of the samples expressed the toxin genes studied.

In the present study, the occurrence of expression was observed for all genes except for *mec*A$_{LGA251}$. The percentages of gene expression were as follows: *bla*Z 82.5%, *fem*A 47.6%, *aac*A*aph*D 43.3%, *fem*B 29.7%, and *mec*A 27.27%. The occurrence of expression of the *bla*Z gene was higher than that of the other genes ($p < 0.0001$). It was observed that the presence of the gene is not associated with its expression when the totality of the cases in which they were detected was considered. The expression of the *fem*A and *fem*B genes was observed only in *S. aureus* (with the exception of the *fem*A gene observed in a *S. agnetis* isolate). None of the 7 *S. aureus* isolates in which the *mec*A gene was detected showed expression of this gene, although 9 (50%) CNS samples were positive for detection and expression of this gene. The *mec*A$_{LGA251}$ gene was detected in an isolate (*S. hyicus*), but no expression was observed. Regarding the expression of the *aac*A*aph*D gene, it was observed in a higher percentage in CNS (75%) compared to *S. aureus* and other CPS ($p < 0.0004$) isolates.

The evaluations of the occurrence of gene expression and phenotypic resistance, showed that both characteristics do not always occur together, and produced variable results; for example, when considering *fem*A, *fem*B, and *mec*A$_{LGA251}$ genes, it was possible to verify that the presence or absence of expression was not associated with the occurrence of phenotypic resistance to amoxicillin, ampicillin, oxacillin, penicillin, and gentamicin; all samples that tested positive for expression of *mec*A gene also showed phenotypic resistance to the beta-lactams which were tested; in the presence of *bla*Z gene expression, there was a higher occurrence of resistant strains for amoxicillin (97%), ampicillin (97%), and penicillin (97%); for *aac*A*aph*D gene, most strains in which detection and expression were present, also showed antibiotic resistance to gentamicin (61.5%).

Conclusions

The present study provides new information on genotypic traits of *Staphylococcus* isolates from bovine subclinical mastitis especially regarding the evaluation of expression of genes associated with antimicrobial resistance in *Staphylococcus* spp. using molecular tools. The results helped to clarify genetic factors that may be involved in the occurrence of antimicrobial resistance.

Given the diversity of the results obtained this study, it is important to conduct more studies in order to elucidate certain results. Further studies should be performed to better clarify which factors may be interfering with the expression of these genes. Future researches could further evaluate genetic and non-genetic factors that may be involved in the occurrence of gene expression associated with phenotypic antimicrobial resistance; they could also examine other characteristics of the microorganisms that may be involved in virulence mechanisms.

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Authors' contribution
EZ, PAM, NRB, ASH, PLM, conceptualized the study; participated in its design, coordination, NRB, MAL performed statistical analysis EZ, NRB, MAL, PAM drafted the manuscript and approved the final version. EZ, PAM, NRB, ASH, PLM, LARO, PEB, SOS, SAT performed laboratorial analysis and EZ, PAM, NRB, MAL evaluated the results.

Ethical considerations
The study received ethical clearance from the Institutional Animal Care and Use Committee (protocol number 8515270415) from Faculty of Veterinary Medicine and Zoothecny, University of São Paulo (USP).

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Corresponding author

Eveline Zuniga PhD in Animal Science
Department of Preventive Veterinary Medicine and Animal Health,
Faculty of Veterinary Medicine and Zoothecnics,
University of São Paulo (USP),
Av. Prof. Dr. Orlando Marques de Paiva, 87, CEP 05508-270, São Paulo, Brazil.
Tel: +55 11 3091-7651
Fax: +55 11 3091-7651
Email: eveline.zuniga@usp.br

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