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

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# 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 mecA (gene encoding penicillin binding protein 2a) mecALGA251 (mecA homologue), blaZ (gene encoding penicillin resistance), femA and femB (genes encoding essential factors - A and B - for the expression of methicillin resistance) and aacA-aphD (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 femA and femB genes were the most frequent. Regarding the variables’ detection (N = number of strains) and expression (% of strains), the following results were obtained: blaZ (N = 40 – 82.5%), femA (N = 147 – 47.6%), aacAaphD (N = 30 – 43.3%), femB (N = 138 – 29.7%), mecA (N = 33 – 27.3%), mecALGA251 (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 blaZ 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 ineffectiveness 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 blaZ 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 mecA gene [6]. Penicillin binding proteins
(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, *mecC*, which is not detected by conventional PCR for the *mecA* gene. Both studies detected isolates positive for resistance to methicillin and absence of *mecA*, and verified the presence of homologue *mecC*, emphasizing its importance as a gene associated with resistance to beta-lactams.

The *femA* and *femB* (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: *femA* is involved in the addition of glycines 2 and 3 and *femB* 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 *femA* and *femB* is related to glycin reduction in the peptidoglycan, thus making the cell wall more susceptible to beta-lactams [10]. Thus, the *femA* and *femB* genes, present in the *Staphylococcus aureus* 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 antibiotic 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 *aacA-aphD* 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 mecA, mecALGA251, blaZ, femA, and femB genes, while the resistance to gentamicin was evaluated by the occurrence and expression of the aacA-aphD 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 NASpin 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.

<table>
<thead>
<tr>
<th>Function</th>
<th>Genes</th>
<th>Genetic Sequence (5’-3’)</th>
<th>Amplified Product Size</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endogenous control</td>
<td>16sRNA - F</td>
<td>GTAGGTGGCAAGCGTTATCC</td>
<td>228bp</td>
<td>[19]</td>
</tr>
<tr>
<td></td>
<td>16sRNA - R</td>
<td>CGCACATCAGCGTCAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resistance to Beta</td>
<td>blaZ - F</td>
<td>AAGAGATTTCCTATGCTTC</td>
<td>517bp</td>
<td>[20]</td>
</tr>
<tr>
<td>lactams</td>
<td>blaZ - R</td>
<td>GCTTAGACACTTATATCAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>mecA - R</td>
<td>CCTGAATC[W]GCTAATAATATTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resistance to MRSA</td>
<td>mecALGA251 - F</td>
<td>GCTCCTAATGTCAATGCA</td>
<td>304bp</td>
<td>[9]</td>
</tr>
<tr>
<td></td>
<td>mecALGA251 - R</td>
<td>TAAGCAATAATGACTACC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resistance to MRSA</td>
<td>femA - F</td>
<td>AGACAAATAGAGAATGAT</td>
<td>509bp</td>
<td>[6]</td>
</tr>
<tr>
<td></td>
<td>femA - R</td>
<td>AAACTCAGACTGAGTGATA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resistance to MRSA</td>
<td>femB - F</td>
<td>TTACAGAGTTAATGTTACC</td>
<td>651bp</td>
<td>[6]</td>
</tr>
<tr>
<td></td>
<td>femB - R</td>
<td>ATACAAATCCAGACGGCTCT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resistance to Aminoglycosides</td>
<td>aacA-aphD - F</td>
<td>TAATCAGAGGACAATAAGGGC</td>
<td>227bp</td>
<td>[13]</td>
</tr>
<tr>
<td></td>
<td>aacA-aphD - R</td>
<td>GCCACACTATCAATCACC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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); MIC50 was ≤ 1; MIC90 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); MIC50 was ≤ 1; MIC90 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); MIC50 for this antimicrobial was ≤ 4 and MIC90 ≤ 32. Isolates resistant
to gentamicin (N = 15) presented MICs in the range of 16 to 256 μg/mL; MIC50 was ≤ 128 and MIC90 was ≤ 256.

**Detection and expression of genes related to bacterial resistance to antimicrobials**

One or more genes encoding resistance to different antimicrobials (mecA, mecALGA251, blaZ, femA, femB, and aacA-aphD) 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 femA (60.5%) and femB (56.8%); their frequencies of occurrence were higher than those of the other genes (p < 0.0001). The mecA gene was found in a *S. hyicus* strain, but the mecA gene was not found in this isolate. Data concerning cephalothin were not shown in the table as 100% of sensitivity was not found in this isolate. Data concerning cephalothin was found in a *S. hyicus* strain, but the mecA 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 femA-femB (N = 99; 40.74%) (p < 0.0001). Regarding *S. aureus*, the most frequent gene combination was also femA-femB (N = 95; 58%) (p < 0.0001), followed by blaZ-femA-femB (N = 22; 13.4%) (p = 0.02).

In relation to the occurrence of femA 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 femA 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: *blaZ* (80%, N = 32/40), mecA (27.3%, N = 9/33), mecALGA251 (0%, N = 0/1), femA (25.2%, N = 37/147), femB (15.9%, N = 22/138) and aacA-aphD (40%, N = 12/30). The results for penicillin were similar except for femA (23.8%, N = 35/147) and femB (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: *blaZ* (15%, N = 6/40), mecA (18.2%, N = 6/33), mecALGA251 (0%, N = 0/1), and aacA-aphD (26.6%, N = 8/30), femA (0%, N = 0/147), femB (0%, N = 0/138) (Table 3).

The femA and femB gene expression occurred in *S. aureus* (51.88% and 33.06%, respectively). FemA 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 femA and femB, 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

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**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.

<table>
<thead>
<tr>
<th>Coagulase species</th>
<th>Staphylococcus species</th>
<th>femA</th>
<th>femB</th>
<th>mecA</th>
<th>aacA-aphD</th>
<th>mecALGA251</th>
<th>blaZ</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>N %</strong></td>
<td><strong>N %</strong></td>
<td><strong>N %</strong></td>
<td><strong>N %</strong></td>
<td><strong>N %</strong></td>
<td><strong>N %</strong></td>
<td><strong>N %</strong></td>
<td><strong>N %</strong></td>
</tr>
<tr>
<td>CPS*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>164</td>
<td>133</td>
<td>81.1</td>
<td>69</td>
<td>51.88</td>
<td>124</td>
<td>75.6</td>
</tr>
<tr>
<td>Others CPS</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>S. hyicus</em></td>
<td>40</td>
<td>7</td>
<td>17.5</td>
<td>100</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>S. agnetis</em></td>
<td>2</td>
<td>50.0</td>
<td>1</td>
<td>100</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>S. epidermidis</em></td>
<td>18</td>
<td>4</td>
<td>22.2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>13</td>
</tr>
<tr>
<td><em>S. sciuri</em></td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td><strong>CNS</strong>*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. chromogenes</em></td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>S. saprophyticus</em></td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>S. simulans</em></td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>CNS total</strong></td>
<td>37</td>
<td>6</td>
<td>16.2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>18</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>243</td>
<td>147</td>
<td>60.5</td>
<td>70</td>
<td>47.62</td>
<td>138</td>
<td>56.8</td>
</tr>
</tbody>
</table>

*a*1 coagulase-positive *Staphylococcus* (CPS); *a*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 mecA 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 *blaZ* gene expression was higher (82.5%) than that of other genes (p < 0.0001). In the presence of *blaZ* 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 *aacAaphD* 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.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene detection</th>
<th>N</th>
<th>%</th>
<th>Gene expression</th>
<th>N</th>
<th>%</th>
<th>Ampicillin / Amoxicillin</th>
<th>Oxacillin</th>
<th>Penicillin</th>
<th>Gentamicin</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>N</td>
<td></td>
<td></td>
<td>N</td>
<td>%</td>
<td>N</td>
<td>%</td>
</tr>
<tr>
<td>femA</td>
<td>present</td>
<td>147</td>
<td>60.5</td>
<td>present</td>
<td>70</td>
<td>47.6</td>
<td>37</td>
<td>52.9</td>
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<td>1.4</td>
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<tr>
<td></td>
<td>absent</td>
<td>96</td>
<td>39.5</td>
<td>absent</td>
<td>77</td>
<td>52.4</td>
<td>51</td>
<td>66.2</td>
<td>7</td>
<td>9.1</td>
</tr>
<tr>
<td>femB</td>
<td>present</td>
<td>138</td>
<td>56.8</td>
<td>present</td>
<td>41</td>
<td>29.7</td>
<td>57</td>
<td>59.4</td>
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<td>27.1</td>
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<tr>
<td></td>
<td>absent</td>
<td>105</td>
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<td>70.3</td>
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<tr>
<td>mecA</td>
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<td>present</td>
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<td>66</td>
<td>62.9</td>
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<td></td>
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<td>72.7</td>
<td>124</td>
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<td>8.6</td>
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<td>present</td>
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<td>100.0</td>
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</tr>
<tr>
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<td>145</td>
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<td>34</td>
<td>14.0</td>
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<tr>
<td>blaz</td>
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<td>40</td>
<td>16.5</td>
<td>present</td>
<td>33</td>
<td>82.5</td>
<td>32</td>
<td>97.0</td>
<td>6</td>
<td>18.2</td>
</tr>
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<td></td>
<td>absent</td>
<td>203</td>
<td>83.5</td>
<td>absent</td>
<td>7</td>
<td>17.5</td>
<td>1</td>
<td>14.3</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>aacA-aphD</td>
<td>present</td>
<td>30</td>
<td>12.3</td>
<td>present</td>
<td>13</td>
<td>43.3</td>
<td>12</td>
<td>92.3</td>
<td>12</td>
<td>92.3</td>
</tr>
<tr>
<td></td>
<td>absent</td>
<td>213</td>
<td>87.7</td>
<td>absent</td>
<td>17</td>
<td>56.7</td>
<td>8</td>
<td>47.1</td>
<td>2</td>
<td>11.8</td>
</tr>
</tbody>
</table>

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penicillin (MIC<sub>50</sub> ≤ 4; MIC<sub>90</sub> ≤ 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 femA and femB 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 femA 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 femA 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 femA 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 femA 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 femA gene are *S. aureus*.

The detection frequency of the femB gene was similar (p = 0.46) to that of the femA gene. Kobayashi et al. [6] detected the femB 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 femA-femB (40.74%).

In relation to the presence of the femA and femB 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 femB 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 mecA gene can also be used to confirm the diagnosis of MRSA. In the present study the presence of the mecA 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 mecA gene was verified in 48.3% of *S. aureus* isolates, out of which 23.3% were MRSA. The mecA gene was also detected in CNS (N = 18 - 48.6%). The presence of the mecA 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 mecA gene, it was observed that, out of the 33 *Staphylococcus* isolates in which mecA 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 mecA 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 mecA-positive *S. aureus* (OS-MRSA) [29].

According to Kobayashi et al. [6], between the concomitant and the separate detection of mecA, femA, and femB 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 femA-femB (58%).

García-Alvarez et al. [8] reported that the mecA homologous gene, mec<sub>ALGA251</sub> has 70% similarity to mecA, 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<sub>ALGA251</sub> gene was identified in only one sample (*S. hyicus*), susceptible to all beta-lactams evaluated in the antibiograms. The mec<sub>ALGA251</sub> gene was detected, along with the mecA gene, in a *S. hyicus* strain. García-Alvarez et al. [8] also detected the presence of the mec<sub>ALGA251</sub> gene at a low frequency of occurrence (1.4% of *Staphylococcus* isolates from bovine mastitis), and the strains were also negative for mecA and methicillin resistant (in the phenotypic analysis).

The blaz 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 blaZ gene in 70% of the S. aureus isolates. In relation to the occurrence of samples of S. aureus 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 phenotypic profile of antimicrobial resistance of 100 CNS isolates obtained from bovine mastitis and found the blaZ 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 aacA-aphD 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 aacA-aphD 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 aacA-aphD 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 aacA-aphD 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 mecA, icaADBC, 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 mecA. The percentages of gene expression were as follows: blaZ 82.5%, femA 47.6%, aacAaphD 43.3%, femB 29.7%, and mecA 27.27%. The occurrence of expression of the blaZ 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 femA and femB genes was observed only in S. aureus (with the exception of the femA gene observed in S. agnetis isolate). None of the 7 S. aureus isolates in which the mecA gene was detected showed expression of this gene, although 9 (50%) CNS samples were positive for detection and expression of this gene. The mecA_ALGA251 gene was detected in an isolate (S. hyicus), but no expression was observed. Regarding the expression of the aacAaphD 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 femA, femB, and mecA_ALGA251 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 mecA gene also showed phenotypic resistance to the beta-lactams which were tested; in the presence of blaZ gene expression, there was a higher occurrence of resistant strains for amoxicillin (97%), ampicillin (97%), and penicillin (97%); for aacAaphD 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 Zootecny, University of São Paulo (USP).

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References

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