ORIGINAL ARTICLE

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Detection of β-lactamase, *blaZ* and *mecA* in penicillin-resistant *Staphylococcus aureus* isolated from bovine mastitis in Garanhuns, Brazil

Detecção de β-lactamase, blaZ e mecA em amostras de Staphylococcus aureus resistentes à penicilina isoladas de mastite bovina em Garanhuns, Brasil

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ABSTRACT: There are few reports in the literature about genetic determinants of resistance to β-lactams in *Staphylococcus aureus* isolated from dairy cattle located in the municipality of Garanhuns, state of Pernambuco, Brazil. Thus, this study aimed to investigate the production of β-lactamase and the presence of the *blaZ* and *mecA* genes in penicillin-resistant *S. aureus* isolated from cases of subclinical bovine mastitis in the city of Garanhuns. Forty-six strains of penicillin-resistant *S. aureus* were evaluated using the nitrocefin disc test and duplex PCR. The results revealed that 45 strains (97.8%) were positive for β-lactamase production and 44 (95.7%) carried the *blaZ* gene. Among the latter, 43 (97.7%) were β-lactamase producers and only one (2.3%) was not. The *mecA* gene was not detected in any of the isolates investigated. The results suggest that enzymatic inactivation is the main β-lactam resistance mechanism expressed by *S. aureus* in the herds analyzed.

KEYWORDS: staphylococcal mastitis; antimicrobial resistance; β-lactam antibiotic.

RESUMO: Existem poucos relatos na literatura sobre determinantes genéticos da resistência aos β-lactâmicos em *Staphylococcus aureus* isolados em rebanhos de bovinos leiteiros localizados no município de Garanhuns, estado de Pernambuco, Brasil. Dessa forma, este estudo teve como objetivo investigar a produção de β-lactamase e a presença dos genes *blaZ* e *mecA* em *S. aureus* resistentes à penicilina isolados de casos de mastite bovina subclínica na cidade de Garanhuns. Quarenta e seis amostras de *S. aureus* resistentes à penicilina foram avaliadas usando o teste do disco de nitrocefina e PCR duplex. Os resultados demonstraram que 45 amostras (97,8%) foram positivas para a produção de β-lactamase e 44 (95,7%) portavam o gene *blaZ*. Destes últimos, 43 (97,7%) eram produtores de β-lactamase e apenas um (2,3%) não produziu essa enzima. O gene *mecA* não foi detectado em nenhum dos isolados investigados. Os resultados sugerem que nos rebanhos avaliados a inativação enzimática é o principal mecanismo de resistência aos β-lactâmicos expresso por *S. aureus*.

PALAVRAS-CHAVE: mastite estafilocócica; resistência antimicrobiana; β-lactâmicos.

INTRODUCTION

Staphylococcus aureus (*S. aureus*) is one of the most important Gram-positive pathogens causing infectious diseases in several animal species. Diseases in humans range from mild infections located on the skin and soft tissues to severe diseases, such as endocarditis, pneumonia, septicemia, and toxic shock syndrome (AGUAYO-REYES et al., 2018; GARCÍA-ALVAREZ et al., 2011; OTTO, 2014). In worldwide, mastitis by that bacteria is one of the most important diseases, particularly in dairy herds (ABEBE et al., 2016; FELIPE et al., 2017; FREITAS et al., 2018; LAVOR et al., 2019).

From an economic point of view, mastitis is the most important illness in dairy herds because losses due to costs related to treatment, prevention, and technical assistance as well as discarded milk and a reduction in milk production

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(HOGEVEEN; VAN DER VOORT, 2017). Mastitis is also of considerable public health importance, as causal agents and antimicrobial residues can be transmitted through milk and other dairy products to consumers (ARAÚJO et al., 2014; BORELLI et al., 2006; MIRANDA; ARANGO, 2016).

The frequency of *S. aureus* mastitis in dairy cattle varies depending on the breed, management system and type of milking (ABEBE et al., 2016). In Brazilian herds, this pathogen is isolated at high frequencies (CUNHA et al., 2015; FREITAS et al., 2018; GIRARDINI et al., 2016; MESQUITA et al., 2019; SILVA et al., 2012) as well as in other countries (ABEBE et al., 2016; FELIPE et al., 2017; LIU et al., 2017).

Mastitis caused by *S. aureus* is usually treated with intramammary antimicrobials agents and β-lactams are the most extensively used class (Krewer et al., 2014). However, such use may contribute to the selection and dissemination of multidrug-resistant strains at a farm level (KLIMIENE et al., 2016).

In the genus *Staphylococcus*, resistance to β-lactam antimicrobials is encoded by chromosomes or plasmids genes. Two different resistance mechanisms are known: the production of β-lactamase and modification of the drug target. β-lactamase comprises a group of enzymes that hydrolyze the β-lactam ring of the β-lactams antimicrobials. The synthesis of different types of enzyme is encoded by the *bla*Z gene harbored in bacterial plasmids or chromosomes (FERREIRA et al., 2017; OLSEN; CHRISTESEN; AARESTRUP, 2006).

The modification of the drug target is represented by an altered penicillin-binding protein (PBP2a or PBP2') that has a poor affinity to the β-lactams antimicrobials (HARRISON et al., 2014; KLIMIENE et al., 2016). The *mec*A gene and its homologues (*mec*B and *mec*C) are the responsible for the genetic code of the altered PBP and are acquired as part of a mobile genetic element known as staphylococcal cassette chromosome mec (SCCmec) located on the bacterial chromosome. The *mec* genes, together with inducer/repressor and recombinase genes, comprise the well-studied mec complex (AGUAYO-REYES et al., 2018; ARÍZA-MIGUEL et al., 2014; LIU et al., 2016; LONCARIC et al., 2019; MacFADYEN et al., 2019).

In recent years, studies have reported a high incidence of resistance to penicillin and other β-lactams in *S. aureus* isolated from dairy cattle (FREITAS et al., 2018; GIRARDINI et al., 2016; KREWER et al., 2014; LIU et al., 2017; SILVA et al., 2012; YANG et al., 2015). However, investigations of the genetic determinants of β-lactam resistance are scarce, particularly in terms of *S. aureus* isolated from dairy cattle in the municipality of Garanhuns, state of Pernambuco, Brazil.

Thus, this study aimed to detect β-lactamase production and the presence of *bla*Z and *mec*A genes in penicillin-resistant *S. aureus* isolated from subclinical bovine mastitis in Garanhuns, PE, Brazil.

MATERIALS AND METHODS

Forty-six strains of *S. aureus* were isolated from the milk of cows with subclinical mastitis in commercial dairy herds located in the municipality of Garanhuns in the state of Pernambuco, Brazil. The collection of milk samples and bacteriological analyses were carried out in a previous study (Silva et al., 2012). The strains were kept frozen at -70°C in a skim milk based medium enriched with 15% glycerol. To carry out the studies, the samples were reactivated on 5% sheep blood agar.

The penicillin-resistance profile was previously investigated (Silva et al., 2012). All 46 *S. aureus* strains exhibited an inhibition zone of ≤ 28 mm to penicillin (penicillin G 10U, CECON, São Paulo, Brazil) in the agar diffusion test, according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2008).

The production of the β-lactamase was detected by nitrocefin disc test (CEFINASE DISCS®, Becton, Dickinson and Company, Franklin Lakes, NJ, USA), according to manufacturer's recommendations. Briefly, discs were moistened with 30 µL of sterile purified water and bacterial samples were spread over the surface. A positive reaction in the first five minutes was defined by a red color, whereas a lack of color change denoted a negative reaction. In this case samples were left at room temperature for 1 h, when the final reading was performed. *Staphylococcus aureus* ATCC 29213 was the positive control.

The genomic staphylococcal DNA extraction was performed using the heating method described by Hassanzadeh et al. (2016), with few modifications. Briefly, the isolates were grown in tryptic soy broth (TSB) and left approximately 16h at 37°C. After, 1 mL of each cultured bacteria was retrieved by centrifugation at 14000 rpm for 5 minutes. The obtained pellet was subjected to wash using 500 µL of lysis buffer (20 mM EDTA + 20 mM Tris pH 7.5 + 75 mM NaCl) and again centrifuged. The resulting pellet was resuspended in 300 µL of the same lysis buffer, boiled and cooled twice (two minutes each step). Next, 30 µL of lysozyme (1 mg/mL) were placed into the tube and incubated at 37°C for 1 h, followed by the addition of 33 µL of 10% SDS, incubation at 55°C for 1 h and cooling on ice for 10 minutes. In the next step, 120 µL of 3M sodium acetate were added and the samples were cooled again. Last, the pellet was sequentially washed with chloroform, isopropanol and ethanol, DNA eluted in 30 µL of sterile TE pH 7.5, quantified in NanoDrop spectrophotometer (GE Healthcare Life Science) and kept frozen at -20°C.

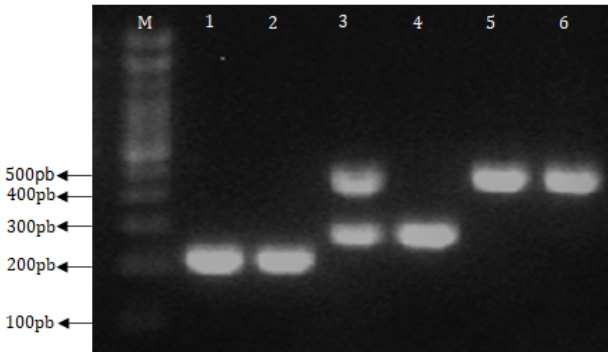
The quality of the genomic DNA extracted was assessed by conventional PCR using the 16S primer pair - F 5'GTA GGT GGC AAG CGT TAT CC 3'- and - R 5'CGC ACA TCA GCG TCA G 3' (MONDAY; BOAHACH, 1999). A specific segment of the genus *Staphylococcus* was amplified by preparing a reaction with a final volume of 30 µL composed of 27 µL of PCR Supermix (Invitrogen, Thermo

Fisher Scientific, Waltham, MA, USA), 1 µL (10 nM) of each primer and 1 µL of template DNA (corresponding to 197 ng of genomic DNA). PCR amplification was carried out following steps of initial denaturation for 5 minutes at 94°C, followed by 36 cycles of denaturation for 45 seconds at 94°C, annealing for 30 seconds at 50.2°C, extension for 30 seconds at 72°C, and a final extension for 10 minutes at 72°C. For duplex PCR, a reaction was prepared with the two primer pairs: mecAF - 5' GTA GAA ATG ACT GAA CGT CCG ATA A 3'/mecAR - 5' CCA ATT CCA CAT TGT TTC GGT CTA A 3' (FONTES et al., 2013) and blaZF – 5' AAG AGA TTT GCC TAT GCT TC 3'/blaZR – 5' GCT TGA CCA CTT TTA TCA GC 3' (SAWANT; GILLESPIE; OLIVER, 2009) with 0.25 µL (25 pM) of each mecA primer, 0.4 µL (40 pM) of each blaZ primer, 2 µL of the multiplex PCR mix (Solis, Biodyne, Tartu, Estonia) and 0.5 µL of template DNA (corresponding to 98.5 ng of genomic DNA). The final volume was adjusted to 10 µL with sterile ultrapure water. Amplification was performed with an initial activation for 12 minutes at 95°C and initial denaturation for 5 minutes at 94°C, followed by 36 cycles as described for the 16S primer reaction. Positive (DNA from the strain *Staphylococcus capitis* subsp. *ureolyticus* K22H/RJ positive for *blaZ* and *mec*A genes) and negative (reaction without DNA) controls were included. The PCR amplicons were separated on 2% agarose gel, stained (SYBR® Safe DNA gel stain, Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), and visualized under UV light (Figure 1).

RESULTS

Among the 46 penicillin-resistant *S. aureus* strains, 44 (95.7%) carried the *blaZ* gene and two (4.3%) did not. Forty-five strains (97.8%) were positive for β-lactamase based on the nitrocefin test. All strains were negative for the *mec*A gene.

Among the 44 *blaZ*-positive strains, 43 (97.7%) were also positive for β-lactamase production and only one (2.3%) was not. The two strains that did not carry the *blaZ* gene were positive for β-lactamase production. The Table 1 shows these results.



Source: Author's collection.

Figure 1. Agarose gel electrophoresis for visualization of the *16S* (228 bp), *blaZ* (517 bp) and *mec*A (310 bp) amplicons. M: molecular weight marker (100 bp); lanes 1-2: *16S* amplicon; lane 3: positive control; lane 4: *mec*A amplicon; lanes 5-6: positive isolates for *blaZ*.

DISCUSSION

Staphylococcus aureus is considered one of the most important and frequent etiologic agents of contagious bovine mastitis. The importance of this agent is due to its high frequency in herds throughout the world and because a large number of strains exhibit determinants of antimicrobial resistance, making antibiotic-based treatment difficult.

The results of the present survey corroborate these statements, as 95.7% of the *S. aureus* strains evaluated carried the *blaZ* gene. Other researchers have reported similar results. Martini et al. (2017), Aslantas; Demir (2016), Yang et al. (2015) and Krewer et al. (2014) evaluating *S. aureus* isolated from bovine mastitis, found high frequencies (97, 100, 94.6 and 93%, respectively) of the *blaZ* gene. Frequencies of positivity for *blaZ* below 50% have also been reported (ELSAYED et al., 2019; HAUBERT et al., 2017; SOUZA et al., 2019).

According to Nobrega et al. (2018), Haubert et al. (2017) and Srednik et al. (2015), the genus *Staphylococcus* maintains resistance determinants in mobile genetic elements, such as transposons and conjugative plasmids, contributing to the transferability of antimicrobial resistance genes among different species or genera, representing a potential risk to human and animal health.

It is noteworthy that 97.7% of the penicillin-resistant strains of *S. aureus* carrying the *blaZ* gene produced β-lactamase. Evaluating *S. aureus* strains isolated from subclinical bovine mastitis, Martini et al. (2017) detected the production of β-lactamase in 86.7% and Robles et al. (2014) in 78% of the *S. aureus* strains investigated. The results of the present investigation and previous studies suggest that the main β-lactam resistance mechanism in *S. aureus* isolated from bovine intramammary infection is enzymatic inactivation and that the enzyme may be actively produced in the mammary gland.

These results also suggest that β-lactam antimicrobials are extensively used in the herds analyzed, which favors the selection of resistant strains. Although there is a consensus regarding

Table 1. Frequency of *blaZ* and β-lactamase production in penicillin-resistant *Staphylococcus aureus* isolated from subclinical bovine mastitis in Garanhuns, PE, Brazil.

Genotype	N (%)	β-lactamase	
		Positive N (%)	Negative N (%)
blaZ^{+}	44 (95.7)	43 (97.7)	1 (2.3)
blaZ^{-}	2 (4.3)	2 (100)	-

blaZ^{+}: strains harboring the gene; blaZ^{-}: strains not harboring the gene

the need for the rational use of antibiotics, including for the treatment of mastitis, in practice, these drugs are employed abusively, particularly β-lactams (MESQUITA et al., 2019; SILVA et al., 2012; YANG et al., 2015).

S. aureus mastitis is the most challenging infectious disease in the veterinary practice of farm animals. The characteristic progression of the disease requires rapid decision-making regarding the establishment of the therapeutic and management protocol, the main objective of which is to eliminate sources of infection and decrease somatic cell counts in bulk milk. However, the indiscriminate use of antibiotics significantly compromises this goal.

The presence of penicillin-resistant and β-lactamase-producing strains of *S. aureus* in dairy cattle, as demonstrated in this study, compromises the success of β-lactam therapy. Moreover, penicillin-resistant strains are more likely to respond poorly to non-β-lactam antibiotics than penicillin-susceptible strains. In addition, the pathogenicity islands where the penicillin resistance genes are located also have virulence factors and the co-expression of these genes contribute to bacterial survival in the presence of an antimicrobial agent (Barkema; Schukken; Zadoks, 2006).

This scenario is very worrisome. In practice, it reduces the chances of a bacteriological cure of staphylococcal mastitis, which, in turn, contributes to the dissemination and persistence of the pathogen in the herd.

In the present study the two penicillin-resistant *S. aureus* strains that did not harbor the *bla*Z gene were positive for β-lactamase production, whereas one strain harboring the *bla*Z gene was negative for β-lactamase production. The first case may be the result of changes in the nucleotide sequence in the primer alignment region, as described for other genes (HAUBERT et al., 2017; SZCZUKA et al., 2016). The second case may be due to an absence of *bla*Z gene expression, as argued by Srednik et al. (2015), but also due to low sensitivity of the nitrocefin disc method. Although nitrocefin method, as used in this study, detected the production of the β-lactamase in 97.7% of the *bla*Z positive *S. aureus* isolates, study conducted by Ferreira et al. (2017) showed a low sensitivity of this test. These authors comparing the sensitivity and specificity of the nitrocefin, disc diffusion, MIC, and zone edge tests for the detection of β-lactamase in *Staphylococcus* spp., found sensitivity as low as 28.9% for the nitrocefin test, although specificity was 100%.

The findings highlighted above underscore the need to use a combination of results from two or more tests to detect β-lactam-resistant strains and confirm a penicillin-sensitive profile (HAUBERT et al., 2017; KAASE et al., 2008). One test should be a molecular method, which is more accurate for detecting the potential for a strain to produce β-lactamase (RUSSI et al., 2015). Although a molecular test should be considered, it is not always feasible in the routine of the veterinary laboratories due to the cost of required reagents and equipment. In these cases, and according to the results demonstrated in the present study, the disc diffusion test as well as the nitrocefin disc method can be used to investigate the potential for a strain of *S. aureus* to produce β-lactamase, since the great majority of the strains demonstrating resistance to penicillin were β-lactamase producers and carried the *bla*Z gene.

The *mec*A gene was not detected in any of the strains investigated in this study. Haubert et al. (2017) and Krewer et al. (2014) also did not detect the *mec*A gene in *S. aureus* isolated from bovine mastitis. However, Souza et al. (2019) and Liu et al. (2017) found frequencies of 30 and 31.25% for the *mec*A gene in strains of *S. aureus* isolated from bovine mastitis and Elsayed et al. (2019) reported a frequency of 75% in strains isolated from the milk of healthy cows and buffaloes as well as those with mastitis.

The low frequency or even absence of the *mec*A gene is expected in animal isolates. According to Virdis et al. (2010), the presence of the *mec*A gene and, consequently, methicillin-resistant strains is low in bacteria of an animal origin. On the other hand, the low frequency or absence of *mec*A could be explained by its genetic instability, which hinders the alignment of primers and, consequently, amplification (HAUBERT et al., 2017; SZCZUKA et al., 2016). In this specific case, homologous genes to *mec*A, such as *mec*B and *mec*C, should be investigated, especially when a high rate of cefoxitin resistance has been detected.

It is noteworthy that the absence of the *mec*A gene in the *S aureus* strains evaluated in the present study, agrees with the profile of susceptibility to oxacillin reported by Silva et al. (2012) for the same isolates. According to these authors, 99 and 1% of the strains were classified in the sensitive and intermediate categories, respectively. Currently, the cefoxitin disk test is recommended to detect methicillin-resistant *Staphylococcus* spp. as it has higher specificity than and equal sensitivity to the oxacillin disk test (CLSI, 2018). However, the results of the present study suggest that oxacillin disk test has high specificity and sensibility, since there was 100% of agreement between this test and PCR.

CONCLUSION

The results reveal that enzymatic β-lactam inactivation is the main antimicrobial resistance mechanism expressed by *S. aureus* in the herds evaluated. The results also indicate that milk produced in the evaluated region represent a risk to public health, since it may transmit antibiotic residues and bacteria carrying resistance genes.

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