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β-lactam resistance in coagulase-negative *Staphylococcus* isolated from subclinical goat mastitis

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Abstract – The objective of this work was to evaluate resistance mechanisms to β-lactam antimicrobials in 251 strains of coagulase-negative *Staphylococcus* (CoNS) isolated from subclinical goat mastitis, as well as to determine the sensitivity and specificity of the nitrocefin and disk diffusion methods to detect penicillin resistance, in comparison with the polymerase chain reaction (PCR). The isolates were evaluated for the presence of the *bla*Z and *mec*A genes, β-lactamase production, and susceptibility to penicillin. Of the total isolates, 228 (91%) carried the *bla*Z gene and, among these, 144 (63%) were positive for β-lactamase production. Resistance to penicillin was observed in 125 of the isolates, of which 96.8% carried the *bla*Z gene. The sensitivity of the phenotypic methods to detect β-lactamase production was low, but their specificity was high; the Kappa coefficient showed a poor agreement between the phenotypic methods and PCR. The *mec*A gene was detected in only 3% of the isolates, which were identified as belonging to the species: *S. capitis* subsp. *ureolyticus*, *S. caprae*, *S. warneri*, *S. sciuri*, *S. simulans*, and *S. cohnii* subsp. *urealyticum*. Coagulase-negative *Staphylococcus* are important mastitis-causing pathogens in goat and harbor the *blaZ* and *mecA* genes related to resistance to β-lactam antimicrobials. The sensitivity of the nitrocefin and disk diffusion methods to detect penicillin resistance is low in relation to that of PCR.

Index terms: *Staphylococcus*, antimicrobial resistance, β-lactamase, *bla*Z, *mec*A.

Resistência a β-lactâmicos em *Staphylococcus* coagulase-negativa isolados de cabras com mastite subclínica

Resumo – O objetivo deste trabalho foi avaliar os mecanismos de resistência aos antimicrobianos β-lactâmicos em 251 amostras de *Staphylococcus* coagulase-negativa (CoNS) isolados de cabras com mastite subclínica, bem como determinar a sensibilidade e a especificidade dos testes de nitrocefina e difusão em disco para detectar a resistência à penicilina, em comparação à reação em cadeia da polimerase (PCR). As amostras foram avaliadas quanto à presença dos genes *bla*Z e *mec*A, à produção de β-lactamase e à susceptibilidade à penicilina. Do total de isolados, 228 (91%) apresentaram o gene *bla*Z e, dentre estes, 144 (63%) foram positivos para β-lactamase. A resistência à penicilina foi observada em 125 isolados e, destes, 96,8% apresentaram o gene *bla*Z. A sensibilidade dos testes fenotípicos para detectar a produção de β-lactamase foi baixa, mas a sua especificidade foi alta; o coeficiente Kappa mostrou leve concordância entre os testes fenotípicos e a PCR. O gene *mec*A foi detectado

em apenas 3% dos isolados, que foram identificados como pertencentes às espécies: *S. capitis* subsp. *ureolyticus*, *S. caprae*, *S. warneri*, *S. sciuri*, *S. simulans* e *S. cohnii* subsp. *urealyticum*. *Staphylococcus* coagulase-negativa são importantes agentes etiológicos da mastite caprina e apresentam genes *blaZ* e *mec*A de resistência aos antimicrobianos β-lactâmicos. A sensibilidade dos testes de nitrocefina e difusão em disco para detectar a resistência à penicilina é baixa em relação à da PCR.

Termos para indexação: *Staphylococcus*, resistência antimicrobiana, β-lactamase, *blaZ*, *mec*A.

Introduction

Although *Staphylococcus aureus* is considered the main bacterial cause of mastitis in dairy animals, species of the coagulase-negative *Staphylococcus* (CoNS) group are also commonly isolated from goat intramammary infections either in clinical or subclinical form (Akter et al., 2020). This group of bacteria can cause a persistent intramammary infection, an increase in somatic cell counts, and a decrease in the production and quality of milk and other dairy products (Klimiene et al., 2016). For the treatment of mastitis caused by bacterial infections, intramammary antimicrobials are recommended, particularly β-lactam agents (Krewer et al., 2015). However, their use may contribute to the selection, emergence, and dissemination of multidrug-resistant strains, as reported in several studies (Bhargava & Zhang, 2012; Klimiene et al., 2016; Nobrega et al., 2018).

The mechanisms of resistance to penicillin and other β-lactams in staphylococci are mediated by the *blaZ* and SCC*mec* genes. The *blaZ* gene codes four different types of β-lactamases (types A–D) that hydrolyze the β-lactam ring (Ferreira et al., 2017), and the staphylococcal cassette chromosome *mec* (SCC*mec*) codes a low-affinity penicillin-binding protein and confers broad-spectrum β-lactam resistance (Klimiene et al., 2016).

Although raw or processed goat milk is used to manufacture cheese in various parts of the world and could possibly transmit resistant bacterial strains and antibiotic residues to consumers (Cantekin et al., 2019), there is still a scarcity of data in the literature on antimicrobial resistance in bacterial isolates from goats, especially with regards to gene coding for resistance mechanisms. Therefore, the detection of antimicrobial-resistant CoNS strains is of clinical importance to both animal and human health, as it can assist in the selection of more effective drugs and is a valuable epidemiological tool for monitoring the emergence and dissemination of multidrug-resistant bacteria at a farm level (Bhargava & Zang, 2012).

The objective of this work was to evaluate resistance mechanisms to β-lactam antimicrobials in 251 strains of coagulase-negative *Staphylococcus* isolated from subclinical goat mastitis, as well as to determine the sensitivity and specificity of the nitrocefin and disk diffusion methods to detect penicillin resistance, in comparison with polymerase chain reaction (PCR).

Materials and Methods

A total of 251 CoNS strains were isolated from the milk of goats with subclinical mastitis. The strains originated from the collection of the Laboratory of Animal Microbiology of Universidade Federal do Agreste de Pernambuco, located in the municipality of Garanhuns, in the state of Pernambuco, Brazil. Milk collection and bacteriological analysis were performed in previous studies (Silva et al., 2004; Lima et al., 2015). The used milk samples were obtained from herds located in the Southeastern and Northeastern regions of Brazil, and the number of strains selected for analysis were: 102, 78, 39, and 32 from the states of Rio de Janeiro, Pernambuco, Paraíba, and Ceará, respectively.

The identification of CoNS isolates at the species level was carried out as described by Silva et al. (2004). Briefly, the isolates were grouped according to their susceptibility profile towards 5 µg Novobiocin (CECON, São Paulo, SP, Brazil), and the resistant group was subjected to tests for sucrose, D-mannose, D-cellobiose, D-xylose, L-arabinose and raffinose fermentation, reduction of nitrate, and urease activity. The susceptible group was tested for arginine utilization, urease activity, and D-trehalose, maltose, D-mannitol, D-mannose, and sucrose fermentation.

The production of β-lactamase was detected on Cefinase paper discs (Becton, Dickinson and Company, Franklin Lakes, NJ, USA), following the manufacturer's instructions. Strain *Staphylococcus aureus* ATCC 29213 was used as a positive control. The susceptibility to penicillin was determined by disk diffusion, using Penicillin G 10U (CECON, São

Paulo, SP, Brazil); an inhibition zone ≤ 28 mm was considered a resistance profile (CLSI, 2018).

Genomic DNA extraction was performed using the heating method (Hassanzadeh et al., 2016) with some modifications. Briefly, the CoNS isolates were cultured in tryptone soya broth (Oxoid Limited, Hampshire, United Kingdom) and incubated overnight at 37°C. A total of 1 mL of this culture was centrifuged at 18620 *g* for 5 min. After discarding the supernatant, the pellet was washed with 500 µL lysis buffer (20 mmol L^{-1} EDTA + 20 mmol L^{-1} Tris pH 7.5 + 75 mmol L^{-1} NaCl) and centrifuged. The resulting pellet was resuspended in 300 µL of the same lysis buffer, boiled, and cooled twice – each step lasted 2 min. Then, 30 µL lysozyme (1 mg mL^{-1}) were placed in a tube and incubated for 1 hour at 37°C, followed by the addition of 33 µL 10% sodium dodecyl sulfate, incubation for 1 hour at 55°C, and cooling on ice for 10 min. Subsequently, 120 µL 3M sodium acetate were added and the samples were cooled again. After centrifugation, the pellet was subjected to consecutive chloroform, isopropanol, and ethanol-wash steps. DNA was eluted, quantified, and kept frozen at -20°C.

A conventional PCR with 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 & Bohach, 1999) – was performed to assess the quality of the DNA samples. A specific segment of the genus *Staphylococcus* was amplified using the PCR SuperMix kit (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), and the reaction was prepared according to the manufacturer's instructions. The amplification cycles were: initial denaturation at 94°C for 5 min, followed by 36 cycles of denaturation at 94°C for 45 s, annealing at 50.2°C for 30 s, and extension at 72°C for 30 s, with a final extension at 72°C for 10 min. For Multiplex PCR, a reaction was prepared with 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 et al., 2009), with 0.25 µL (25 pmol L^{-1}) of each mecA primer, 0.4 µL (40 pmol L^{-1}) of each blaZ primer, 2 µL of the Multiplex PCR mix (Solis BioDyne, Tartu, Estonia), and 0.5 µL of template DNA. The final volume was adjusted to 10 µL with sterile ultrapure water. Amplification was performed with the following cycles: initial activation at 95°C for 12 min and initial denaturation at 94°C for 5 min, followed by 36 cycles of denaturation at 94°C for 45 s, annealing at 50.2°C for 30 s, and extension at 72°C for 30 s, with a final extension at 72°C for 10 min. The following positive and negative controls were included: DNA from the strain *Staphylococcus capitis* subsp. *ureolyticus* K22H/RJ, positive for the *blaZ* and *mec*A genes; and reaction without DNA. The PCR products were separated on 2% agarose gel, stained, and visualized under UV light.

The frequency (%) of the *blaZ* and *mec*A genes, β-lactamase production, and penicillin resistance was determined, as well as the frequency of the genes according to the origin of the CoNS isolates. The sensitivity and specificity of the phenotypic methods to detect β-lactamase production were also obtained. The Kappa index was used to calculate the agreement rate between PCR, nitrocefin disk, and disk diffusion; in this case, PCR was used as a gold standard, and the index was calculated using the online VassarStats (2020) software.

Results and Discussion

Of the 251 isolates studied, 59% were positive for β-lactamase production (β-lactamase$^+$), and the PCR analysis showed that 91% of the total evaluated isolates carried the *blaZ* gene (*blaZ*$^+$) (Figure 1 and Table 1).

The presence of the *blaZ* gene in CoNS causing mastitis had already been observed at rates ranging from 6 to 66.6% (Srednik et al., 2015; Klimiene et al., 2016; Haubert et al., 2017; Nobrega et al., 2018; Cantekin et al., 2019). In the present study, the frequency of detection of the *blaZ* gene was high (91%). Moreover, the production of the enzyme β-lactamase was detected in the majority of the *blaZ*$^+$ isolates, making the *blaZ*$^+$/β-lactamase$^+$ profile the most prevalent and suggesting the potential production of this enzyme within the mammary gland. As previously pointed out by Olsen et al. (2006), the presence of the *blaZ* gene in many CoNS isolates, as detected in this and in other works, strongly suggests its high frequency in the assessed bacterial group.

Almost 50% of the CoNS isolates showed in vitro resistance to penicillin. Other studies also reported high rates of β-lactam-resistant CoNS isolates, particularly with resistance to penicillin. Bhargava &

Zhang (2012) evaluated 87 CoNS strains isolated from different agricultural animals and found a 91.9% rate of penicillin resistance. Salaberry et al. (2016) and Lima et al. (2018) assessed CoNS from goat mastitis and reported 40.4 and 83% rates of resistance to penicillin, respectively.

CoNS are the main agents of subclinical mastitis in dairy goats, and the use of intramammary β-lactams is one of the most widespread measures for the treatment of mastitis caused by these and other staphylococci (Krewer et al., 2015; Klimiene et al., 2016). However, the excessive use of these antimicrobial agents has contributed to the emergence of resistant strains, particularly to penicillin derivatives (Klimiene et al., 2016; Ferreira et al., 2017). The results of the present study corroborate this argument, indicating a probable overuse of β-lactams in the evaluated herds.

Figure 1. Agarose gel electrophoresis for detection of the *16*S (228 bp), *bla*Z (517 bp), and *mec*A (310 bp) genes by polymerase chain reaction. M, molecular weight marker (100 bp); lanes 1–2, *16*S fragment; lane 3, positive isolates for both the *bla*Z and *mec*A genes; lanes 4–5, positive isolates for *mec*A; and lanes 6–7, positive isolates for *bla*Z.

It was observed that the most penicillin-resistant isolates harbored the *bla*Z gene and produced β-lactamase, suggesting that the enzymatic inactivation of β-lactam antimicrobials is the most predominant resistance mechanism expressed by the assessed CoNS. As shown in previous studies, staphylococci are key reservoirs of β-lactam resistance markers, which are normally present in mobile DNA, such as conjugative plasmids and transposons, contributing to the transferability of these antimicrobial resistance genes between different species or genera, which is extremely important to human and veterinary medicine (Srednik et al., 2015; Haubert et al., 2017; Nobrega et al., 2018).

The high frequency of penicillin-resistance and β-lactamase-producer CoNS found in the present work is quite worrisome since it has been shown that bacterial strains with this phenotype are more likely to respond poorly to non β-lactam antimicrobials, compared with penicillin-susceptible strains (Barkema et al., 2006; Ferreira et al., 2017). According to Barkema et al. (2006), this response pattern may be due to the localization of penicillin resistance genes in pathogenicity islands, especially since these islands also have genes of several virulence factors, which contribute to the survival of the bacterium in the presence of an antimicrobial agent.

The frequency of the isolates that were *bla*Z$^+$ differed according to the states in which the herds were located: 39% in Rio de Janeiro, 32.9% in Pernambuco, 14.5% in Paraíba, and 13.6% in Ceará (Table 2). These results suggest that CoNS from goat mastitis harboring *bla*Z are present in several goat herds in Brazil, possibly due to a non-prudent use of antibiotics in the

Table 1. Total frequencies of *bla*Z, *mec*A, β-lactamase, as well as penicillin profile, in coagulase-negative *Staphylococcus* isolated from subclinical goat mastitis (N=251).

Variable	Category	*blaZ* gene		Total	Penicillin		Total
		Presence	Absence		Resistant	Sensitive	
β-lactamase	Positive	144	4	148	117	31	148
	Negative	84	19	103	8	95	103
*mec*A gene	Presence	7	1	8	-	-	-
	Absence	221	22	243	-	-	-
Penicillin	Resistant	121	4	125	-	-	-
	Sensitive	107	19	126	-	-	-

evaluated herds, which would increase the selection of CoNS harboring antimicrobial resistance genes. This type of use, together with the potential for the genetic transfer of resistance genes, could be associated with the dissemination of antimicrobial resistance at a farm level (Klimiene et al., 2016).

In the present work, only 3% of the CoNS carried the *mec*A gene (*mec*A$^+$). Most of these methicillin-resistant CoNS (MRCoNS) were isolated from four herds located in the state of Rio de Janeiro and were identified as *S. capitis* subsp. *ureolyticus*, *Staphylococcus caprae*, and *Staphylococcus warneri*. The remaining three MRCoNS were isolated from herds in Ceará (*Staphylococcus sciuri*), Pernambuco (*Staphylococcus simulans*), and Paraíba (*Staphylococcus cohnii* subsp. *urealyticum*). These results are similar to those obtained by Moura et al. (2018), who reported a frequency of 1.25% for the *mec*A gene in CoNS isolated from goat mastitis. Contrarily, Cantekin et al. (2019), Nobrega et al. (2018), Bhargava & Zang (2012), and Klimiene et al. (2016) found rates of 6.6, 17, 60, and 67.8% in CoNS isolated from different animal origins.

The low frequency of *mec*A in this and other studies could be explained by the instability of the gene. According to Haubert et al. (2017) and Szczuka et al. (2016), insertions, deletions, and other genetic rearrangements could alter the nucleotide sequence of the primer alignment region of the *mec*A gene, impairing its amplification. However, the low frequency of the *mec*A gene could also be due to another phenomenon: some methicillin-resistant *S. aureus* isolates do not carry the *mec*A gene but rather its homologues *mec*C (García-Álvarez et al., 2011). The presence of the *mec*C gene has been reported in *S. aureus* isolated from different hosts (García-Álvarez et al., 2011; Ariza-Miguel et al., 2014), as well as in CoNS isolated from wild and domestic animals (Harrison et al., 2014; Loncaric et al., 2019).

Although the rates of MRCoNS found in the present study were low, at least one strain was isolated from each herd located in all the previously mentioned Brazilian states. This result is especially important to veterinary and human medicines since there is a strong evidence to support the hypothesis that MRCoNS strains are reservoirs of the *mec*A gene for *S. aureus* and commensal bacteria, which poses a serious animal and public health risk (Bhargava & Zhang, 2012; Fluit et al., 2013). Furthermore, some of the MRCoNS identified in the present study, e.g., *S. sciuri*, *S. simulans*, *S. cohnii* subsp. *urealyticum*, and *S. warneri*, are also important human pathogens (Razonable et al., 2001; Garza-González et al., 2011; Ferreira et al., 2017). Therefore, as the farming of dairy goats keeps these animals in close contact with humans and raw goat milk is used to produce gourmet cheese in several parts of the world, there is a potential risk of the direct zoonotic transmission of MRCoNS species to humans.

The sensitivity and specificity of the nitrocefin method were 63.2 and 82.6%, respectively, at a 95% confidence interval (95% CI) of 56.9–69.4 and 67.1–98.1. For disk diffusion, sensitivity and specificity were 53.1 and 82.6%, respectively, at a 95% CI of 46.6–59.5 and 67.1–98.1. The Kappa index for the agreement of the nitrocefin and disk diffusion methods with PCR was 0.18 (95% CI of 0.04–0.32) and 0.12 (95% CI of -0.004–0.24), respectively.

Compared with PCR, the sensitivity of the nitrocefin and disk diffusion methods to detect β-lactamase production, an indicator of penicillin resistance, was low. This means that when these assays are performed, the percentage of strains considered penicillin sensitive is quite high (Table 1). Besides this low accuracy, the Kappa coefficient also showed a poor agreement between these methods and PCR. A low sensitivity of the nitrocefin and disk diffusion methods was also reported in previous studies. When comparing the sensitivity and specificity of the nitrocefin, disk diffusion, dilution, and zone edge assays for the detection of β-lactamase in *Staphylococcus* spp., Ferreira et al. (2017) found a sensitivity of 28.9 and 72.2% for nitrocefin and disk diffusion, respectively. Kaase et al. (2008) also observed a low sensitivity of 35.7% for nitrocefin, in comparison with four other phenotypic methods used to detect β-lactamase

Table 2. Absolute frequency of the *bla*Z and *mec*A genes according to the origin of coagulase-negative *Staphylococcus* isolates.

Origin (Brazilian states)	N	Absolute frequency		
		*bla*Z	*mec*A	*bla*Z+*mec*A
Rio de Janeiro	102	89	01	04
Pernambuco	78	75	-	01
Paraíba	39	33	-	01
Ceará	32	31	-	01
Total	251	228	01	07

in *S. aureus* isolates. According to these authors, the sensitivity of nitrocefin depends on the disc manufacturer and on how the assay is performed. Therefore, it is necessary to evaluate the available assays, in order to compare their performances and choose the one with a greater sensitivity and a lower variation in different situations.

In relation to disk diffusion, according to Ferreira et al. (2017) and Kaase et al. (2008), the limitation of the test may be due to the current zone diameter of ≤ 28 mm for penicillin G, as recommended by the CLSI (2018), since these authors observed the *bla*Z gene in staphylococci isolates whose zone diameter was > 28 mm. Therefore, when using disk diffusion as the only phenotypic assay to detect resistance to β-lactams, which is the usual practice in veterinary medicine, laboratories may misidentify resistant isolates as sensitive ones, which would negatively affect decision making regarding the use of antibiotic therapy.

The obtained results suggest that two or more assays should be combined to ensure the detection of β-lactam resistance or potentially resistant strains. For Kaase et al. (2008) and Haubert et al. (2017), a genetic assay is ideal, since an evaluation at the genetic level is necessary to understand the bacterial resistance phenomenon, as well as to confirm a penicillin-sensitive profile. However, it is important to emphasize that the genetic approach is not always available for all veterinary laboratories, due to the cost of molecular biology techniques. In this case, the laboratory must select the most sensitive, easy-to-perform, and low-cost phenotypic assay available.

It is also worth highlighting that the low sensitivity of the phenotypic methods used in the present study may be due to the non-expression of the *bla*Z gene, as pointed out by Srednik et al. (2015).

Conclusions

1. The group coagulase-negative *Staphylococcus* (CoNS) causing goat mastitis harbors and expresses the *bla*Z gene at a frequency of 91%, and the enzymatic inactivation encoded by this gene is the major resistance mechanism expressed by CoNS isolated from dairy goat herds.

2. The *mec*A gene is present in coagulase-negative *Staphylococcus* isolated from goat mastitis at a rate of 3%.

3. The disk diffusion technique should not be the only phenotypic assay used to detect resistance to β-lactam antimicrobials in CoNS due to a possible misidentification of resistant isolates as sensitive.

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