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VETERINARY MICROBIOLOGY - RESEARCH PAPER

Diversity and emergence of multi-resistant *Staphylococcus* spp. isolated from subclinical mastitis in cows in of the state of Piauí, Brazil

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Abstract

This research aimed to identify the diversity of bacterial species of the genus *Staphylococcus* spp. in subclinical mastitis in dairy herds in the state of Piauí, Northeastern Brazil, and to evaluate the phenotypic and genotypic resistance profile. Samples were obtained from a total of 17 dairy farms, amounting to 321 positive samples in the *California Mastitis Test*. *Staphylococcus* spp. were identified by matrix-assisted laser desorption ionization time-of-flight mass spectroscopy. Subsequently, an antibiogram was performed, and a polymerase chain reaction was carried out to screen for resistance genes in the isolates. Among all the isolates, 59.45% (110/185) belonged to the *Staphylococcus* genus. Moreover, the following *Staphylococcus* spp. were identified *Staphylococcus aureus*, 68.1% (75/110); *Staphylococcus chromogenes*, 12.7% (14/110); *Staphylococcus epidermidis*, 5.4% (6/110); *Staphylococcus sciuri*, 4.5% (5/110); *Staphylococcus warneri*, 2.7% (3/110); *Staphylococcus haemolyticus*, 1.8% (2/110); *Staphylococcus hominis*, 1.8% (2/110); *Staphylococcus arlettae*, 0.9% (1/110); *Staphylococcus capitis*, 0.9% (1/110); and *Staphylococcus gallinarum*, 0.9% (1/110). The antibiogram showed a high frequency of resistance to penicillin and ampicillin, 70.0% (77/110) and 61.8% (68/110), respectively, and a low frequency of resistance to gentamicin and vancomycin, 10.9% (12/110) and 11.8% (13/110), respectively. In the genotypic tests for the different species of *Staphylococcus* spp., the occurrence of the *blaZ* gene was observed in 60.9% (67/110) of the isolates, followed by *tet*L and *tet*M, both with 20.0% (22/110) each, and the *mec*A and *van*B genes were detected in 0.9% (1/110) of the samples. The identification of all *Staphylococcus* species isolated from subclinical mastitis cases and the phenotypic and genotypic resistance characterization in these isolates is of great importance for dairy farming in the state of Piauí, as well as for public health.

Keywords Antimicrobial resistance · Dairy cattle · Infection · MALDI-TOF MS

Introduction

Bovine mastitis, characterized by an inflammatory reaction in the mammary glands, is considered one of the major diseases that affect dairy cows. It can cause several productive and economic losses, such as reduced production of milk, increased expenses for the treatment, and in some cases, early disposal of animals [1–4].

Regarding the causative agents of bovine mastitis, the genus *Staphylococcus* is one of the most researched pathogens regarding the etiology of bovine mastitis, especially to subclinical mastitis. The importance of this genus is associated with its high resistance to antimicrobials, enterotoxaemia cases in humans, and disease development in animals [5–7]. Initially, studies on bovine mastitis had focused on *S. aureus*. However, recently, there has been an increase in research related to resistant coagulase-negative staphylococci (CoNS), with emphasis on *Staphylococcus epidermidis* and *Staphylococcus chromogenes* [8–12].

In recent years, antimicrobial resistance has become a major problem for the treatment of both animal and human

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diseases, as it is already possible to identify several species of bacteria carrying genes that confer resistance to some antimicrobials, such as the *bla*Z, *mec*A, and *mec*C (resistance to β-lactams) [13–15], *tet*L and *tet*M (tetracycline resistance) [13, 16], and *van*A and *van*B (vancomycin resistance) [17]. For example, gram-positive bacteria, such as *Staphylococcus* spp., can transmit these genes horizontally to other *Staphylococcus* spp. and/or other gram-positive bacteria, whether of human or animal origin [16].

Several studies have investigated the etiology of bovine mastitis and the resistance profile of the causative agents in various Brazilian states, such as Rio de Janeiro [18], Pará [19], São Paulo [20], Minas Gerais [13], Pernambuco [21, 22], Mato Grosso [23], and Paraná [10]. However, there are still various lacunes in other states, such as Piauí state, where there are no recordings of the said illness in the dairy region, nor records of the resistance profile of bacteria causing bovine mastitis. Likewise, this research aimed to identify the diversity of bacterial species of the genus *Staphylococcus* spp. in subclinical mastitis in dairy herds in the state of Piauí, Northeastern Brazil, and to evaluate the phenotypic and genotypic resistance profile.

Material and methods

Sampling and sample collection

Farms were chosen non-probabilistically for convenience. Milk samples were collected from 17 farms, being 3 in the municipality of Luiz Correia, 3 in Buriti dos Lopes and 11 in the municipality of Parnaíba, both municipalities located in the dairy basin of Piauí, Northeast Brazil. Initially, the *California Mastitis Test* (CMT) was performed on 680 breast quarters (170 Girolando cows) as per the protocol provided by Schalm and Noorlander [24]. The teats had been previously sanitized for subsequent testing. Milk samples were collected from the glands that displayed two (++) or three (+++) crosses in the CMT result, totaling 321 positive samples. Subsequently, these samples were stored in sterile bottles, placed in isothermal boxes, refrigerated, and sent for microbiological and molecular analyses in the Laboratory of Animals Infectious Diseases of the Federal Rural University of Pernambuco.

Microbiological isolation and identification of Staphylococcus spp.

Milk samples were cultivated in Base Agar supplemented with 7% sheep blood. The plates were incubated in a bacteriological incubator at 37 °C for 72 h and evaluated every 24 h. In post bacterial growth, the colonies were characterized by their morphology and morphotintorial characteristics via Gram staining, followed by biochemical tests of catalases in *Staphylococcus* spp. Subsequently, species identification was performed by the matrix-assisted laser desorption ionization time-of-flight mass spectroscopy (MALDI-TOF MS) (Bruker Daltonics) of the Institute of Pharmacology and Molecular Biology of the Federal University of São Paulo, as described by Wolters et al. [25].

Evaluation of antimicrobial resistance in Staphylococcus spp.

The phenotypic profile of antimicrobial resistance in *Staphylococcus* spp. was determined using the agar disk diffusion method [26]. The antimicrobials used were ampicillin (10 µg), penicillin G (10 UI), cefoxitin (30 µg), gentamicin (10 µg), oxacillin (1 µg), tetracycline (30 µg), erythromycin (15 µg), and vancomycin (30 µg), as per the recommendations in the Clinical and Laboratory Standards Institute guidelines [27].

Evaluation of resistance genes in Staphylococcus spp.

To obtain bacterial DNA, *Staphylococcus* spp. colonies were subjected to the DNA extraction method described by Fan et al. [28]. The genotypical resistance profile was evaluated for the genes *tet*M and *tet*L for tetracycline, *bla*Z for penicillin, *van*A and *van*B for vancomycin, and *mec*C and *mec*A for methicillin (Table 1).

Conventional polymerase chain reaction (PCR) was employed to amplify specific regions of these genes according to their thermal profiles with some modifications in the reagent concentrations. To this end, the final volume of each reaction was 12.5 µl, containing 100 ng of template DNA, 10 pmol of forward and reverse primers, and 6.25 µl of Go-TaqGreen Master Mix (Promega). Bacterial strains harboring these genes were used as a positive control, and ultrapure Milli-Q water was used as a negative control. The PCR products were stained with Blue Green (LGC Biotechnology) and subjected to electrophoresis in 1.5% agarose gel for 50 min at 100 V. The separated DNA bands were visualized and photographed by a photo documenter under ultraviolet light.

Statistical analysis

The results of microbiological analysis, polymerase chain reaction, and disk diffusion technique were expressed in relative and absolute frequencies [35].

Results

In this study, 57.63% of the CMT-positive samples (185/321) were also positive in the microbiological examination, and 59.45% (110/185) of the positive isolates

Table 1 Genes, oligonucleotide sequences, size of amplified fragments, and reference

Gene	Sequence (5′ – 3′)	Fragment size (pb)	References
*bla*Z	F: AAGAGATTTGCCTATGCTTC R: GGCAATATGATCAAGATAC	517	[29]
*tet*L	F: TCGTTAGCGTGCTGTCATTC R: GTATCCCACCAATGTAGCCG	267	[30]
*tet*M	F: GTGGACAAAGGTACAACGAG R: CGGTAAAGTTCGTCACACAC	406	[30]
*mec*A	F: TGGTATGTGGAAGTTAGATTGGGAT R:CTAATCTCATATGTGTTCCTGTATTGGC	155	[31]
*mec*C	F: CATTAAAATCAGAGCGAGGC R: TGGCTGAACCCATTTTTGAT	188	[32]
*van*A	F: GGGAAAACGACAATTGC R: GTACAATGCGGCCGTTA	732	[33]
*van*B	F: GTGACAAACCGGAGGCGAGGA R: CCGCCATCCTCCTGCAAAAAA	430	[34]

obtained belonged to *Staphylococcus* spp. The MALDI-TOF MS analysis detected the following *Staphylococcus* spp. among the positive isolates: *S. aureus*, 68.1% (75/110); *S. chromogenes*, 12.7% (14/110); *S. epidermidis*, 5.4% (6/110); *Staphylococcus sciuri*, 4.5% (5/110); *Staphylococcus warneri*, 2.7% (3/110); *Staphylococcus haemolyticus*, 1.8% (2/110); *Staphylococcus hominis*, 1.8% (2/110); *Staphylococcus arlettae*, 0.9% (1/110); *Staphylococcus capitis*, 0.9% (1/110); and *Staphylococcus gallinarum*, 0.9% (1/110).

The results of the phenotypic resistance test, evaluating the resistance of *Staphylococcus* spp. to antimicrobials, demonstrated that 70.0% (77/110) and 61.8% (68/110) of *Staphylococcus* spp. isolates were resistant to penicillin and ampicillin, respectively. On the other hand, only 10.9% (12/110) and 11.8% (13/110) of the isolates were resistant to gentamicin and vancomycin, respectively. The distribution of phenotypic resistance of *Staphylococcus* species against each antimicrobial tested is described in Fig. 1.

The genotypic resistance test in *Staphylococcus* spp. revealed the presence of the *bla*Z gene in 60.9% (67/110) of the isolates and the *tet*L and *tet*M in 20% (22/110) of the isolates, each. Additionally, 0.9% (1/110) of the isolates presented the *mec*A and *van*B genes, while none of them had the *mec*C and *van*A genes (Fig. 2).

Of the samples carrying the *bla*Z gene, 83.5% (56/67) showed phenotypic resistance to penicillin; 63.6% (14/22) of the *tet*L positive samples were resistant to tetracycline in the disc-diffusion test, and 59.0% (13/22) of the positive *tet*L samples were also phenotypically resistant to tetracycline. One positive *mec*A sample (0.9%; 1/110) also presented phenotypical resistance to oxacillin and to cefoxitin, and one *van*B positive sample (0.9% 1/110) was resistant to vancomycin in the phenotypical test.

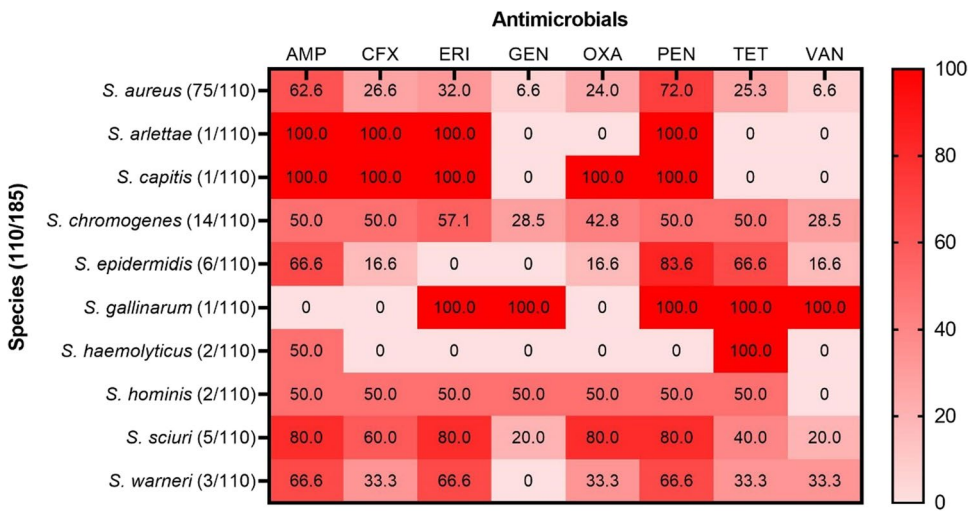

Fig. 1 Relative frequencies of phenotypic antimicrobial resistance for different species of *Staphylococcus*. Color variations show different percentages. 0 = no resistant samples, (AMP) ampicillin, (CFX) cefoxitin, (ERI) erythromycin, (GEN) gentamicin, (OXA) oxacillin, (PEN) penicillin, (TET) tetracycline, (VAN) vancomycin

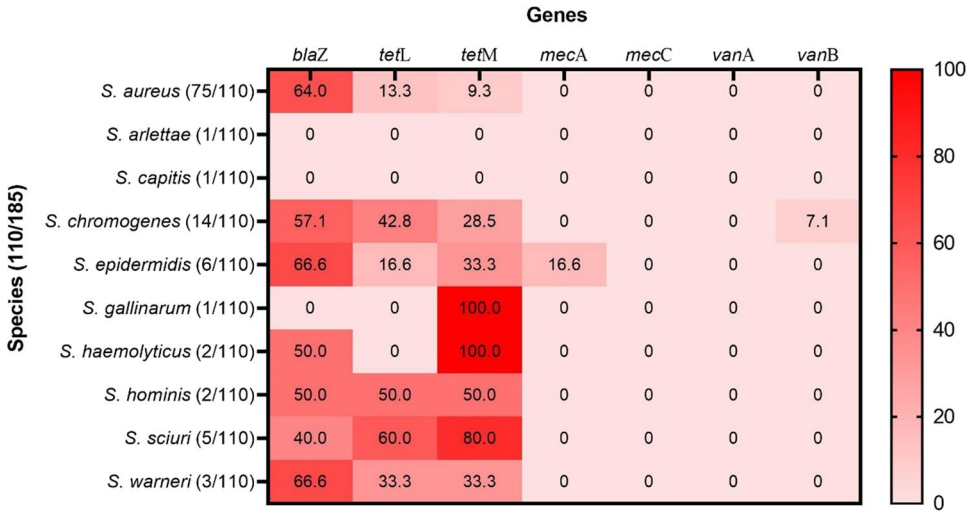

Fig. 2 Relative frequency of resistance genes for different species of *Staphylococcus*. Color variations show different percentages. 0 = absence of resistance gene

Among the *Staphylococcus* spp. isolates, 10.9% (12/110) had both tetracycline resistance genes (*tet*L and *tet*M) and 7.2% (8/110) presented multiple genotypical resistance, presenting three resistance genes (*blaZ*, *tet*L, and *tet*M).

Discussion

This is the first study evaluating the occurrence of bovine subclinical mastitis caused by *Staphylococcus* spp. and investigating the phenotypic and genotypic resistance profile of these bacteria in Piauí, Brazil. Our findings concurred with that of most etiological studies (those conducted in Brazil and globally) on bovine subclinical mastitis that *Staphylococcus* spp. were predominant (59.45%; 110/185) in the milk samples extracted from cows affected with subclinical mastitis [7, 8, 12, 19, 21, 36–39]. This study can be considered the most widespread study in identifying all *Staphylococcus* spp. (*S. arlettae*, *S. capitis*, *S. chromogenes*, *S. gallinarum*, *S. haemolyticus*, *S. hominis*, *S. sciuri*, and *S. warneri*) responsible for causing bovine subclinical mastitis in the Northeast region of Brazil; most studies have only identified *S. aureus* and coagulase-negative staphylococci (CoNS) as the causative agents in this region [21, 22, 36, 39]. These species, while never identified in Northeast Brazil, have been identified in the South and Southeast regions of Brazil [38]. Additionally, our findings emphasize the importance of the MALDI-TOF MS technique in investigating the etiology of bovine subclinical mastitis, as it is quick, cost-efficient, and easily operatable [40].

Post identifying the bacterial species, we generated the phenotypic resistance profile of the *Staphylococcus* spp. isolates using the disk diffusion test. We observed that resistance to penicillin and ampicillin was above 60.0%. This observation can be attributed to the wide-scale use of these drugs in the treatment of this disease [21, 41]. On the other hand, less than 40.0% of the isolates displayed resistance against other antimicrobials.

Several factors are pointed out as possible causes of the emergence of bacterial isolates resistant to antimicrobials in the agricultural production environment, among them the high use of antimicrobials or their indiscriminate use stand out [42, 43]. Both characterize the acquired form of resistance, in which a bacterial population that was naturally susceptible to the antimicrobial becomes resistant due to mutations in chromosomal genes or due to the acquisition of external genetic determinants of resistance [44]. Particularly for beta-lactams (such as penicillin and ampicillin), antimicrobials with the highest percentage of resistant isolates detected in this study, two mechanisms responsible for resistance are frequently reported. The first is the production of enzymes that inactivate antimicrobials, resulting in the destruction of the beta-lactam ring; and the second is the modification of the antimicrobial target, causing a decrease, or total loss, of the affinity between the drug and its binding site [15, 45, 46]. These mechanisms were studied and detected in the present study, demonstrating their presence in *Staphylococcus* spp. that causes of subclinical mastitis in the state of Piaui.

Regarding gentamicin, 10.9% of the isolates were resistant, this low resistance of the *Staphylococcus* species isolated in this study may be related to its little use in dairy cattle, due to its toxic potential for animals and the prolonged residual power in milk [47, 48] and according to Awandkar et al. [49], the low preference to gentamicin in veterinary therapy may be the reason behind this high sensitivity.

Regarding phenotypic resistance and the search for penicillin resistance genes, 70% (77/110) of *Staphylococcus* spp. were resistant in the disk-diffusion test, and of these, 87.0% (67/77) carried the *blaZ* gene, being 64.0% (48/75) of the *S. aureus* species and 45.7% (16/35) of the CoNS group.

This high number of *bla*Z-carrying *Staphylococcus* spp. have already been observed in studies conducted in Brazil, such as Krewer et al. [50], with 93.1% (203/218), Martini et al. [13] with 97.7% (88/90), Santos et al. [21] with 68.9% (111/161), and Silva et al. [22] with 74.07% (20/27), and in other regions of the world such as the USA, with 53.48% (46/86) by Ruegg et al. [51] and in China, with 94.6% (35/37) by Yang et al. [52]. Only *S. arlettae*, *S. capitis*, and *S. gallinarum* did not presented the *bla*Z gene. The gene *bla*Z increases the production of β-lactamases in a cell; thus, inactivating β-lactams and conferring resistance against these compounds in bacteria [53]. Resistance to β-lactams can be mainly attributed to the indiscriminate use of these antimicrobials in mastitis treatment [21] and the increased occurrence of the *bla*Z gene in several *Staphylococcus* spp. as was observed in this study.

Concerning the genes that confer resistance against tetracycline, 20.0% (22/110) of the *Staphylococcus* spp. isolates were positive for the *tet*L gene and 20.0% (22/110) for the *tet*M gene. In *S. aureus*, the frequency was 13.3% (10/75) for *tet*L and 9.3% (7/75) for *tet*M. A higher frequency was observed in the CoNS group, with 35.2% (12/35) for *tet*L and 42.8% (15/35) for *tet*M, with emphasis on *S. chromogenes* and *S. sciuri*. This study is the first in reporting the occurrence of tetracycline resistance genes *tet*L and *tet*M in CoNS in bovine subclinical mastitis isolates in the Northeast region of Brazil. Presently, there are only a few studies reporting these in *S. aureus*; however, they are restricted to the Southeast region of Brazil [13, 16], where frequencies of occurrence of these genes were observed to be 8.8% for *tet*L and 2.2% for *tet*M [13] and 1.61% for *tet*L and 3.22% for *tet*M [16]. It is important to highlight that the *tet*L and *tet*M have distinct mechanisms, one, caused by the *tet*L gene, is an antimicrobial efflux system, and the other, caused, by the *tet*M gene, caused ribosome protection, this portrays the bacterial versatility regarding resistance acquisition [30].

We found that only one (1/110) isolate of *S. epidermidis* harbored the *mec*A gene. To date, only one related study has demonstrated the presence of this gene in *S. aureus* and CoNS isolates from milk, environmental, and human samples in mastitis cases in the Northeast region of Brazil (Pernambuco) [14]. Moreover, studies have highlighted that methicillin-resistant CoNS are globally recognized as a major cause of persistent infections in humans and animals, particularly *S. epidermidis*, *S. haemolyticus*, and *Staphylococcus lugdunensis* [54–56].

In the present study, no *Staphylococcus* spp. presented the *mec*C gene, although this gene has been the target of several studies in Brazil, there are only two studies with positive samples [15, 57]. In the Americas, one of the first reports of finding this gene in a case of bovine mastitis was in an isolate of *Staphylococcus saprophyticus* in Argentina [58]. However, in Brazil, there are only two reports on the occurrence of this gene in a *Staphylococcus* spp. isolate from a case of bovine mastitis; the first in the state of Pernambuco in the northeast of the country [15] and the second in the Southeast region of Brazil [57], both reports identified the *mec*C gene in *S. aureus* isolates.

Regarding the presence of vancomycin resistance genes (*van*A and *van*B), the presence of the *van*B gene was observed in one isolate (1/110), a *Staphylococcus chromogenes*, and the absence of the *van*A gene in all isolates. There are reports of the occurrence of the *van*A (15/178) and *van*B (1/178) genes in Brazil in *Staphylococcus* species isolated from the milk of goats with mastitis [59], but until the completion of this research, there are no reports of the occurrence of these genes in *Staphylococcus* species or in other species of bacteria isolated from bovine mastitis in Brazil. This finding is unprecedented in Brazil and is alarming for public health issues, configuring as the first record of this gene (*van*B) in a bacteria isolated from bovine mastitis.

The findings of *mec*A and *van*B positive bacteria in bovine mastitis samples if of major impact, especially for public health, since, consuming milk that has not been correctly processed may cause infection in humans. The presence of *mec*A and *van*B carrying bacteria is indicative of possible horizontal transmission of resistant bacteria from humans to animals, since, methicillin and vancomycin are not used in the treatment of animal infection. Another worrying fact is the occurrence of both *mec*A and *van*B genes in the same study since vancomycin is considered the first choice of antibiotics used in treatment against methicillin-resistant *Staphylococcus* (MRS) [16, 60–66].

In late years, strains have been identified carrying two or more resistance genes, showcasing that bacterium may present different resistance mechanisms [13, 52]. It was noted that some bacteria in our study also harbored more than one resistance gene (*bla*Z, *tet*L, and *tet*M). The *mec*A and *van*B genes were not associated with other genes; however, it was observed that both were expressed in the phenotypical resistance test.

Some strains carrying these genes did not display similar behavior in the disk-diffusion test, since bacterial strains may carry a resistance gene but may not express this gene; the phenotypic expression of the gene depends on several factors such as environmental conditions and the genetic context [67].

Conclusion

The identification of all *Staphylococcus* species in the present study related to mastitis cases, as well as its characterization of the phenotypic and genotypic resistance profile of these isolates for some classes of antimicrobials, has a

high impact on the dairy region, since it will allow for the elaboration of control measures against this disease. Also, it is worrying that the circulation of antimicrobial-resistant samples in dairy farming, considering that antimicrobials, such as vancomycin and methicillin, are not used in the treatment of animal infections in Brazil.

Author contribution RPO: study design, collections, processing, identification of isolates, PCR analysis, and writing. JGS: idealization of the study, processing, and identification of isolates. BBA: sample processing and gene identification by PCR. RGC and MAJ: identification of isolates by MALDI-TOF MS. JF: identification of *tet* and van genes, yielding controls and sending them for analysis. MPOF: idealization of the study and collection of samples. RAM: idealization of the study, processing, identification of grants, and writing. All authors were essential for the study, thank you all.

Data availability Data sharing not applicable, all data generated are described in this study.

Declarations

Ethics approval This study was approved by the Animal Use Ethics Committee of the Federal Rural University of Pernambuco under license number 79/2018.

Consent to participate No humans participated in the study.

Consent for publication The authors are giving their consent to the publisher to publish their manuscript upon acceptance.

Conflict of interest The authors declare no competing interests.

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