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Phenotypic and Genotypic Characteristics and Resistance Profile of *Staphylococcus* spp. from Bovine Mastitis

Érica Chaves Lúcio[1], Gisele Veneroni Gouveia[2], Mateus Matiuzzi da Costa[2], Junior Mário Baltazar de Oliveira[3], Rinaldo Aparecido Mota[3] & José Wilton Pinheiro Junior[3]

ABSTRACT

***Background*:** Bovine mastitis remains one of the health problems that cause the most damage to milk producers. The negative impact of mastitis is due to reduced milk production, early slaughter of females, reduced commercial value of the affected animals, losses in the genetic potential of the herd, expenses with medicines and veterinary medical assistance. *Staphylococcus* spp. stands out as the cause of this disease and has been able to remain in the mammary gland, becoming resistant to several antimicrobials. The aims of the present study were to characterize the phenotypes, genotypes and resistance profiles of *Staphylococcus* spp. isolates from bovine mastitis cases in the state of Pernambuco, Brazil.

***Materials, Methods & Results*:** These isolates were classified according to biochemical tests and the presence of the nuc gene. The polymerase chain reaction (PCR) for amplification of the *mecA* and *blaZ* genes was used to analyze the genetic potentials of antimicrobial resistance. Isolates were also phenotypically tested for resistance to nine antimicrobials (ampicillin, doxicillin, erythromycin, gentamicin, rifampicin, cephalothin, amoxicillin, nalidixic acid and oxacillin). The genetic potentials for biofilm production were evaluated by the amplifications of the *icaD*, *icaA* and bap genes. The phenotypic test of gentian violet was used for biofilm formation analyzes. Ninety-three (93.0%) of the isolates among the *Staphylococcus* spp. samples were classified as *Staphylococcus aureus*. The lowest percentage of sensitivity observed was for amoxicillin (28.0%). All of the isolates were sensitive to erythromycin and gentamicin, and 15 (15%) exhibited sensitivity to all of the drugs tested. All of the isolates were negative for the *mecA* gene, and 36 (36%) were positive for *blaZ*. In the adhesion microplate tests, 44 (44%) of the isolates were capable of biofilm formation. Of these, seven (15.9%) were strong formers, whereas 16 (36.3%) and 21 (47.8%) were moderate and weak formers, respectively. The *icaD* gene was confirmed in 89 (89%) of the isolates. The *icaA* gene was confirmed in 61 (61%) samples, and the *bap* gene in 52 (52%) samples. One of the samples did not possess *icaA*, *icaD* or *bap* and exhibited moderate biofilm formation according to the microplate adherence test. Sixteen isolates simultaneously exhibited the three genes tested for biofilm production (*icaA*, *icaD* and *bap*) and were negative according to the microplate adherence test.

***Discussion*:** The indiscriminate use of antibiotics to treat mastitis is a common practice in the study area, which may have contributed to the high proportion of herds (88.23%; 15/17) with multi-resistant isolates, constituting a selection factor for the dissemination of resistant bacteria among herds. The absence of the *mecA* gene in the present study may be associated with the development of resistant bacteria through another mechanism, such as the overproduction of beta-lactamases. The results demonstrate that antimicrobial resistance occurs in *Staphylococcus* spp. that cause bovine mastitis in herds of Pernambuco and that these isolates have the a great capacity for biofilm formation. It is necessary to sensitize the professionals involved in the milk production chain of Brazil regarding the importance of the adequate use of antimicrobials for the treatment and control of mastitis, since studies in the country indicate the dissemination of resistant bacterial strains.

Keywords: bacterial resistance, biofilm, bovine mastitis, *Staphylococcus* spp.

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[1]School of Veterinary Medicine and Animal Science (EMVZ), Federal University of Bahia (UFBA), Salvador, BA, Brazil. [2]Department of Veterinary Medicine, Federal University of Vale do São Francisco (UNIVASF), Petrolina, PE, Brazil. [3]Laboratory of Infectious Contagious Diseases in Domestic Animals, Recife, PE. CORRESPONDENCE: E.C. Lúcio [erica.chaves@ufba.br]. School of Veterinary Medicine and Animal Science (EMVZ) - UFBA. Av. Adhemar de Barros n. 500. CEP 40170-110 Salvador, BA, Brazil.

INTRODUCTION

Bovine mastitis is an inflammation that results in a complex interaction among the host, environment and infectious agents [27]. Despite several studies on the diagnosis and control, the disease remains a serious problem for dairy farmers [8,30,53]. Among the agents that cause this disease, *Staphylococcus* spp. are prominent because of their resistance or capacity to become resistant to many antibiotics [12,17].

The dissemination of bacterial strains that are resistant to beta-lactam antibiotics has been frequently reported, and the *mecA* and *blaZ* genes are considered to be primarily responsible for the resistance mechanisms of this species. The former gene encodes a modified penicillin-binding protein (PBP) that has a low affinity for penicillin-like antibiotics [1]. The *blaZ* gene is responsible for the expression of beta-lactamase or penicillinases, enzymes that use the beta-lactam ring as a substrate [29].

Biofilm formation is one of the main causes of persistent or chronic bacterial infections [13]. The co-expression of the *icaA* and *icaD* genes is fundamental for the complete synthesis of biofilms [3,39,52]. The *bap* gene encodes a protein that promotes primary fixation to surfaces and intercellular adhesion [15].

Studies aimed at characterizing mastitis-causing bacteria are required for reducing the costs associated with the use of inadequate drugs as well as for avoiding the spread of drug-resistant strains. Therefore, this study aimed to phenotypically and genotypically characterize isolates of *Staphylococcus* spp. obtained from cows with bovine mastitis from the state of Pernambuco, Brazil, and to determine the resistance profiles of these isolates.

MATERIALS AND METHODS

Isolates and bacterial identification

In total, 100 isolates of *Staphylococcus* spp. from the milk samples from cows with clinical or subclinical mastitis were collected from 17 herds (coded from A to Q) in 11 municipalities of the microregion of Garanhuns, in the state of Pernambuco, Brazil. The herds studied represented extensive, semi-intensive or intensive systems, including animals of different breeds, ages and lactation stages. Most of the herds produced more than 500 L of milk per day, which was collected manually in corrals with no veterinary assistance.

The bacterial agents were identified using morphological characteristics (coloration, size, presence or absence of colony hemolysis), staining (Gram staining) and biochemistry: catalase, Voges Proskauer (VP), coagulase, mannitol fermentation and aerobic fermentation of mannitol and glucose anaerobically. *Staphylococcus* spp. were classified based on the demonstration of coagulase production [45] and the presence of the *nuc* gene [23]. The presence of the nuc gene led to classification as *S. aureus*.

Phenotypic test of sensitivity to antimicrobials

The drug sensitivities of the microorganisms were determined using a modified version of the Kirby-Bauer disc diffusion method. The isolates were seeded in Mueller-Hinton[1] broth and incubated at 37ºC until turbidity was attained according to the McFarland scale (0.5). They were then seeded using swabs in Petri dishes containing Mueller-Hinton agar[2] as the culture medium. Discs[3] were impregnated with antimicrobial drugs, including ampicillin (10 µg), doxycycline (30 µg), erythromycin (15 µg), gentamicin (10 µg), rifampicin (30 µg), cephalothin (30 µg), amoxicillin (10 µg), nalidixic acid (30 µg) and oxacillin (1 µg). The dishes were incubated in an incubator for 24 h at 37ºC. The halo areas were then assessed to determine the sensitivity profiles of the isolates [10].

Phenotypic characterization of biofilm formation

To assess biofilm formation, phenotypic characterizations of the isolates were carried out using the gentian violet test for adherence to microplates. The isolated colonies were inoculated in 3 mL of tryptone soya broth with glucose (0.25%)[2] and incubated at 37°C for 24 h. Subsequently, 200 µL of each mixture was inoculated in micro-dilution plates and again incubated at 37°C for 24 h. Next, the samples were washed three times with 200 µL distilled water and dried at room temperature. The dishes were stained with 100 µL crystal violet (0.25%)[4] for 2 to 3 min at room temperature. They were then washed three more times with distilled water. To dissolve the dye, 200 µL alcohol-acetone was used (80:20, v/v). The optical density (OD) was determined by an ELISA microplate reader[5] using a 620 nm filter. All of the samples and the positive and negative controls were analyzed in triplicate. The *Staphylococcus aureus* ATCC25923 strain was used as the positive control because it was able to produce biofilm. A strain of *Staphylococcus epidermidis* that did not produce biofilm was used as the negative control. From the arithmetic

mean of the OD of the isolates calculated from triplicate measurements, it was possible to obtain the OD produced by each isolate (ODi). Based on the mean OD of the negative control (ODc), the microorganisms were classified as follows: non-biofilm former (ODi ≤ ODc) or weak (ODc < ODi ≤ 2.ODc), moderate (ODc < ODi ≤ 4.ODc) or strong (ODi < 4.ODc) biofilm former [35].

DNA extraction

DNA was extracted using an adaptation of the protocols described [6,55]. A fraction of the inoculum was placed in 300 µL Tris-EDTA (TE), and the samples were vortexed[6] for homogenization. Next, 70 µL 10% SDS was added before further homogenization. Then, 100 µL 5 M $NaCl_2$ and 80 µL cetyltrimethylammonium bromide/sodium chloride (CTAB/NaCl) detergent were added. The samples were incubated at 65°C for 20 min. Next, 700 µL chloroform/isoamyl alcohol (24:1, v/v) was added and homogenized by inversion. The samples were centrifuged[7] at 11,750 *g* for 5 min. The first phase was transferred to another tube, and 450 µL of isopropanol8 was added. The tubes were inverted and left on ice for 20 min. Subsequently, the samples were centrifuged at 11,750 *g* for 15 min, the supernatant was discarded, and 500 µL 70% ethanol[8] was added. The samples were then centrifuged at 11,750 *g* for 10 min, the supernatant was discarded, and the micro-tubes were inverted for drying. The samples were resuspended in 80 µL of TE (pH 8.0) and incubated at 65°C for 60 min. Finally, the samples were stored at -20°C.

Polymerase Chain Reaction (PCR)

The specific nuc gene was identified for the molecular characterization of the *Staphylococcus aureus*. The analysis of the genetic potential for resistance to antimicrobials was achieved by the amplification of the *mecA* and *blaZ* genes and the assessment of their association with methicillin (oxacillin) and beta-lactam resistance, respectively. The genetic potential to produce biofilm was assessed by the amplifications of regions of the *icaD*, *icaA* and *bap* genes.

The PCR products were assessed by electrophoresis in an agarose gel (1.5%), staining with ethidium bromide (1.0 mg mL^{-1}) and visualization under ultraviolet light, followed by documentation using an image capturing system[9]. For the positive controls, a strain of *Staphylococcus* spp. resistant to methicillin (MRS) and *S. aureus* ATCC 25923 was used. The primers used are described in Table 1.

nuc Gene. The amplification cycles for the analysis of the nuc gene were carried out as described [23] with a number of modifications, including an initial denaturation at 94°C for 5 min followed by 37 cycles at 94°C for 1 min, primer hybridization at 55°C for 30 s, extension at 72°C for 1 min, and a final extension at 72°C for 7 min.

mecA and *blaZ* Genes. The cycles of amplification of the *mecA* gene involved an initial denaturation at 94°C for 1 min followed by 15 cycles at 94°C for 30 s, 68°C for 30 s and 72°C for 30 s, 20 additional cycles of 30 s at 94ºC, 30 s at 60ºC, and 30 s at 72ºC and a final extension at 72°C for 2 min. The amplification of the *blaZ* gene consisted of an initial denaturation at 94°C for 4 min followed by 30 cycles at 94°C for 1 min, 50.5ºC for 30 s, and 72°C for 30 s and a final extension at 72°C for 5 min [49].

icaD Gene. The amplification of the *icaD* gene consisted of an initial denaturation at 94°C for 2 min followed by 30 cycles at 92ºC for 45 s, 49.8ºC for 45 s, and 72ºC for 1 min and a final extension at 72°C for 7 min [52].

icaA Gene. The amplification of the *icaA* gene was performed as described [52] with a number of modifications, including an initial denaturation at 94°C for 2 min followed by 30 cycles at 92ºC for 45 s, 58.6ºC for 45 s and 72ºC for 1 min and a final extension at 72°C for 7 min.

bap Gene. The amplification of the bap gene was performed as described [15] with a number of modifications, including an initial denaturation at 94°C for 2 min followed by 35 cycles at 94ºC for 45 s, 56.5ºC for 45 s, and 72ºC for 50 s and a final extension at 72°C for 5 min.

Statistical analysis

The kappa coefficient (K) was used to assess the agreement between the presence of the *blaZ* gene and the presence of beta-lactam resistance, as well as the agreements between microplate adhesion and the presences of the *icaA*, *icaD* and *bap* genes. The conventional interpretation of the K values was as follows: 0.00 - 0.20 = weak agreement; 0.21 - 0.40 = regular agreement; 0.41 - 0.60 = moderate agreement; 0.61 - 0.80 = good agreement; and 0.81- 1.00 = very good agreement. Negative values were interpreted as 0.00 [26]. Bioestat version 5.010 was used for the calculations.

Table 1. Primers used to amplify the genes.

Gene	Primer	Sequence (5´-3´)	Fragment amplified	Reference
nuc	*nuc F*	GCGATTGATGGTGATACGGTT	279 bp[a]	[23]
	nuc R	AGCCAAGCCTTGACGAACTAAAGC		
mecA	SA - 1	AAAATCGATGGTAAAGGTTGGC	214 bp	[37]
	SA - 2	AGTTCTGCAGTACCGGATTTGC		
blaZ	*blaZ F*	AAGAGATTTGCCTATGCTTC	517 bp	[49]
	blaZ R	GCTTGACCACTTTTATCAGC		
*Ica*D	*icaD F*	AAGCCCAGACAGAGGCAATATCCA	381 bp	[52]
	IcaD R	AGTACAAACAAACTCATCCATCCGA		
*Ica*A	*icaA F*	CCTAACTAACGAAAGGTAG	1315 bp	[52]
	IcaA R	AAGATATAGCGATAAGTGC		
bap	*bap F*	AAGCCCAGACAGAGGCAATATCCA	971 bp	[15]
	bap R	AGTACAAACAAACTCATCCATCCGA		

[a]base pairs.

RESULTS

Ninety-three out of 100 isolates (93%) were characterized as *Staphylococcus aureus* by the presence of the *nuc* gene. Among the 7 that were not classified as *S. aureus*, 4 (57.1%) were coagulase negative, and 3 (42.9%) were coagulase positive. Of the samples evaluated, only 1 (1%) was associated with clinical mastitis, which the etiological agent involved being *S. aureus*, the other 99 (99%) samples were associated with sub-clinical mastitis.

Among the *Staphylococcus* spp., the lowest percentage of sensitivity to antimicrobials was observed for amoxicillin (28%), followed by ampicillin (29%), nalidixic acid (46%), cephalothin (83%), oxacillin (83%), rifampicin (84%) and doxycycline (86%). All of the isolates were sensitive to erythromycin and gentamicin, and 15 (15%) exhibited sensitivity to all of the drugs tested. The isolates resistance profile in herds are reported in Table 2.

All of the isolates studied were negative for the mecA gene, whereas the *blaZ* gene was detected in 36 isolates (36%). Table 3 displays the *blaZ*-positive samples in each study herd.

The microplate adherence test showed that 44 (44%) of the *Staphylococcus* spp. isolates were capable of forming biofilms. Of these, 7 (15.9%) were strong formers, whereas 16 (36.3%) and 21 (47.8%) were moderate and weak formers, respectively. The four coagulase-negative samples were among the 16 with moderate biofilm-formation capacity.

The *icaD* gene was confirmed in 89 (89%) of the isolates studied and the *icaA* gene in 61 (61%). Two (50%) of the CPN that were not classified as *S. aureus* were confirmed as possessing these two genes. The presence of the *bap* gene was confirmed in 52 (52%) of the *Staphylococcus* spp. samples analyzed, and of them, two were coagulase-negative staphylococci (CNS) (Table 4).

In this study, one CNS sample was positive for the bap gene but negative for *ica* locus genes, exhibiting moderate biofilm production according to the phenotypic test.

One of the samples did not possess *icaA*, *icaD* or *bap* and exhibited moderate biofilm formation according to the microplate adherence test. Sixteen isolates simultaneously exhibited the 3 genes tested for biofilm production (*icaA*, *icaD* and *bap*) and were negative according to the microplate adherence test.

The 3 CPS samples were negative for all resistance genes and biofilm formation as well as the phenotypic adhesion test microplate.

The results of the analysis of agreement between the presence of the *blaZ* gene and the presence of antimicrobial resistance, as well as between microplate adherence and the presences of the *icaA*, *icaD* and *bap* genes, are displayed in Table 5. Only weak agreement was observed between the phenotypic and genotypic test results.

Table 2. Resistance profiles of *Staphylococcus* spp. associated with bovine mastitis in herds from the microregion of Garanhuns, Pernambuco, Brazil.

Herd	Resistance profile								
	AMO[a]	AMP[b]	NAL[c]	CEP[d]	OXA[e]	RIF[f]	DOX[g]	ERY[h]	GEN[i]
A (n=24)	21	21	11	12	10	11	2	0	0
B (n=7)	7	7	6	2	2	2	1	0	0
C (n=8)	4	6	7	1	1	1	1	0	0
D (n=1)	1	0	1	0	0	0	0	0	0
E (n=2)	2	2	2	0	0	0	1	0	0
F (n=3)	3	3	3	0	0	0	2	0	0
G (n=1)	1	1	1	0	0	0	1	0	0
H (n=3)	3	3	3	0	0	0	3	0	0
I (n=1)	1	1	1	0	0	0	1	0	0
J (n=2)	2	2	2	0	0	0	0	0	0
K (n=7)	5	4	5	1	2	1	1	0	0
L (n=7)	4	1	3	0	1	0	0	0	0
M (n=6)	2	2	2	0	0	0	0	0	0
N (n=6)	0	1	2	1	1	1	0	0	0
O (n=19)	13	14	4	0	0	0	1	0	0
P (n=1)	1	1	1	0	0	0	0	0	0
Q (n=2)	2	2	0	0	0	0	0	0	0
Total	72	71	54	17	17	16	14	0	0

[a]amoxicillin; [b]ampicillin; [c]nalidixic acid; [d]cephalothin; [e]oxacillin; [f]rifampicin; [g]doxycycline; [h]erythromycin; [i]gentamicin.

Table 3. The presence of the *blaZ* gene in isolates of *Staphylococcus* spp. associated with bovine mastitis in herds from the microregion of Garanhuns, Pernambuco, Brazil.

Herd	Positive *blaZ* samples
A (n=24)	8 (33.33%)
B (n=7)	4 (57.14%)
C (n=8)	4 (50.00%)
D (n=1)	1 (100%)
E (n=2)	1 (50.00%)
F (n=3)	-
G (n=1)	-
H (n=3)	-
I (n=1)	1 (100%)
J (n=2)	1 (50.00%)
K (n=7)	4 (57.14%)
L (n=7)	1 (14.28%)
M (n=6)	4 (66.66%)
N (n=6)	2 (33.33%)
O (n=19)	4 (21.08%)
P (n=1)	1 (100%)
Q (n=2)	-

Table 4. The biofilm formation potentials of the samples showing positive results on the phenotypic microplate adherence tests and the presences of the associated genes.

Microplate adherence	*ica*D	*ica*A	*bap*
Strong (n=7)	7 (100%)	6 (85.71%)	3 (42.85%)
Moderate (n=12)	12 (100%)	8 (66.66%)	8 (66.66%)
Weak (n=21)	21 (100%)	16 (76.19%)	14 (66.66%)
Negative (n=56)	47 (83.92%)	30 (53.57%)	25 (44.64%)

Table 5. Agreement between the phenotypic and genotypic tests in relation to the biofilm production capacities and antimicrobial resistance of the isolates.

Phenotypic test	Genotypic test			
	*ica*D	*ica*A	*bap*	*blaZ*
Microplate adherence	0.40	0.16	0.16	
Beta-lactam resistance				0.08

DISCUSSION

With respect to antimicrobial sensitivity, previous study demonstrated a sensitivity of 100% for gentamicin among staphylococci isolated from bovine mastitis cases from the same region as that of the present study [40]. However, it was also reported 42% sensitivity to gentamicin among the CPS and associated this finding with the intense use of the drug in the areas studied [17].

In the present study, antimicrobial resistance to three to five drugs was recorded for 45 (45%) *Staphylococcus* spp., obtaining similar results to another study, with a 48.6% strength for bacteria analyzed [38]. However, were also analyzed isolates of *Staphylococcus* spp. from herds in the state Pernambuco, Brazil, and it was found that 65.6% were resistant to three or more drugs [24]. In another region of the country had been identified resistance to more than one class of antimicrobials at approximately half of the *Staplylococcus* spp. studied [46]. The indiscriminate use of drugs to treat mastitis is a common practice in the study area, which may have contributed to the high proportion of herds (88.23%; 15/17) with multi-resistant isolates, constituting a selection factor for the dissemination of resistant bacteria among herds.

All of the isolates studied were negative for the mecA gene, including those that exhibited phenotypic resistance according to the tests conducted. It was also found a lack of *mecA* in 250 samples of *Staphylococcus* spp. from herds in Rio de Janeiro, stating that there is a need for further studies to investigate the processes of regulation and gene transcription, as well as the product of expression, the PBP2a protein [34]. However, in another study in Rio de Janeiro, the *mecA* gene was found in cows milk samples in 21 (70%) isolates [43]. Already in the Federal District (Brasília) evaluated 60 samples of *Staphylococcus* spp. in clinical cases and sub-clinical cases of bovine and bubaline mastitis and found the *mecA* gene in one (3.30%) of the bubaline samples and three (10%) of the bovine samples [21].

The absence of the *mecA* gene in the present study may be associated with the development of resistant bacteria through another mechanism, such as the overproduction of beta-lactamases [19]. It was suggested that the resistance of *S. aureus* to methicillin (MRSA) is more common in humans than in domestic animals [20,28,49]. However, a number of studies have reported the presence of MRSA and MRS in clinical and sub-clinical cases of bovine mastitis, and the associated strains are generally multi-resistant [25].

The *blaZ* gene was detected in 36 isolates (36%), higher number than previously found by other authors who studied isolates from mastitis [34]. It was emphasized the increasing number of reports of isolation producing bacteria of beta-lactamases in animals, and that bacterial isolates resistant to beta-lactams are increasing on a global scale [29]. It is known that the gene responsible for the expression of beta-lactamases is *blaZ*.

In the present study, high resistence values were identified for beta-lactam, even though all of the samples were negative for the *mecA* gene. This finding corroborates the current notion regarding the participation of other mechanisms of resistance, such as the presence of the homologous gene *mecC* (previously $mecA_{LGA251}$), of other types of PBPs (such as PBP3 and PBP4), or mutations in PBPs [5,7,22,42]. It is generally thought that many factors are associated with resistance to beta-lactams, and more detailed investigations, including the identification of different genetic markers of resistance and the regulation of the expression of the *mec* system, are required for understanding the real value of the presence of this gene in predicting beta-lactam resistance [18,34]. The high percentage of resistant isolates is disconcerting because it indicates that the currently available antibiotics are inefficient, which would hinder or prevent the treatment of mastitis, resulting in higher expenses and risks for dairy owners [17].

With regard to the capacity to produce biofilm, the results of the present study (microplate adherence tests) demonstrated the potential of the isolates to cause infections that are difficult to treat. It was reported that this phenotypic test is one of the most commonly used methods for quantifying biofilm formation by *Staphylococcus* spp. and that it can function as an indicator of the pathogenicity of microorganisms [50].

The molecular techniques that are used to identify the ica locus by codifying the synthesis of slime are important tools for the accurate identifications of virulent strains that form biofilms [3]. High frequencies of isolates positive for the *icaD* gene have previously been reported in studies of *S. aureus* obtained from bovine milk [31,52].

The presence of the *bap* gene was confirmed in 52 (52%) of the *Staphylococcus* spp. samples analyzed; of them, two were CNS. This result was far bigger than that found in 350 *S. aureus* samples from cows with mastitis and reported that only 5% possess the *bap* gene in association with the strong biofilm production, as determined by phenotypic testing [14]. The presence of the *bap* gene has previously been described in *S. aureus* and in several CNS species in association with mastitis, including *S. epidermidis*, *S. chromogenes* and *S. xylosus* [51].

A study conducted in Brazil, with samples of milk from cows and heifers from the state of São Paulo, detected by PCR the *icaA* and *icaD* genes in 98% and 100% of the isolates of *S. aureus*, respectively [9]. Later, the *icaA* and *icaD* genes were identified in all *S. aureus* isolates from 3 regions of the country, and the *bap* gene in 95.6% of them [47].

In this study, one CNS sample was positive for the *bap* gene and negative for the *ica* locus genes, which exhibited moderate biofilm production according to the phenotypic test.

This result corroborates previous findings where it was reported that *Staphylococcus* spp. that possess the *bap* gene are strong producers of biofilm, even though most of them do not contain the *ica* locus genes [51].

A sample classified as CNS that did not possess the *icaA*, *icaD* and *bap* genes exhibited moderate biofilm formation according to the microplate adherence test. Thus, there may be other untested markers that are also associated with the formation and maintenance of biofilms, how the genes *fnbA* and *fnbB*, IS257 and the locus *agr* [4,11,16,32,54]. In addition, 16 isolates simultaneously exhibited the three genes tested for biofilm production (*icaA*, *icaD* and *bap*) and were negative according to the microplate adherence test. This can be explained by the subjectivity of phenotypic data. Indeed, it is known that the production of biofilms by bacteria *in vitro* can be influenced by growth conditions and different mechanisms of adhesion. Furthermore, bacteria grown *in vitro* may not face the environmental challenges that stimulate the production of biofilms [41].

The analysis of the agreement between the genotypic and phenotypic tests performed on the *Staphylococcus* spp. isolates showed that all of the genotypic tests were more sensitive than the phenotypic tests. This result may be explained by the subjectivity of the phenotypic data, the absence of environmental challenges that would induce specific expression patterns [41,44].

The lack of availability of laboratory diagnostic resources to agricultural professionals can lead to increasing bacterial resistance because antimicrobials are often administered unnecessarily [36]. The prophylactic management is the most effective means of preventing mastitis, the formation of biofilms, and, consequently, high levels of resistance in microorganisms. Thus, to facilitate the control of mastitis, further studies should be conducted to determine those agents that form biofilms and their associated adhesive mecha-

nisms [33]. The resistant bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA), also reflect a problem for public health, because they can be transmitted to humans by direct contact with infected animals or contaminated food, such as milk [2,48].

In conclusion, our results demonstrate that antimicrobial resistance occurs frequently in *Staphylococcus* spp. that cause bovine mastitis in herds of Pernambuco and that these isolates have a great capacity for biofilm formation. It is necessary to sensitize the professionals involved in the milk production chain of Brazil regarding the importance of the adequate use of antimicrobials for the treatment and control of mastitis, since studies in the country indicate the dissemination of resistant bacterial strains.

MANUFACTURERS

[1]Kasvi Produtos para Laboratórios. Curitiba, PR, Brazil.

[2]HiMedia Laboratories Pvt. Ltd. Mumbai, Maharashtra, India.

[3]Newprov Produtos para Laboratórios. Pinhais, PR, Brazil.

[4]Proquimios Comércio e Indústria Ltda. Rio de Janeiro, RJ, Brazil.

[5]Asys. Cambridgeshire, Cambridge, England.

[6]Phoenix Inc. Phoenix, AZ, USA.

[7]Labnet International Inc. Edison, NJ, USA.

[8]Fmaia Indústria e Comércio Ltda. Cotia, SP, Brazil.

[9]Loccus Biotecnologia. Cotia, SP, Brazil.

[10]Instituto de Desenvolvimento Sustentável Mamirauá. Maraã, AM, Brazil.

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