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REGULAR ARTICLES

Resistance to antimicrobials and biofilm formation in *Staphylococcus* spp. isolated from bovine mastitis in the Northeast of Brazil

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Abstract Mastitis is the principal disease affecting dairy herds worldwide. The aim of the present study was to characterize phenotypic and genotypic features associated with resistance to antimicrobials in *Staphylococcus* spp. isolated from 2064 milk samples of 525 lactating cows in the Northeast of Brazil. Of the 218 isolates analyzed, 57.8 % were characterized as *Staphylococcus aureus*, 28 % as coagulase-positive staphylococci other than *S. aureus* (oCPS), and 14.2 % as coagulase-negative staphylococci (CNS). The test for susceptibility to antimicrobials showed amoxicillin (32.6 %) to be the less effective drug in vitro, and the multidrug resistance (MDR) rate for beta-lactams varied from 0 to 0.75. The genotypic characterization showed that 93.1 % of the samples were tested positive for the *blaZ* gene, while none amplified *mec*A. The antibiotic efflux mechanism was observed in 0.9 % of isolates. The biofilm formation was found in 3.7 and 96.3 % of samples, respectively, on Congo red agar and on the microplate adhesion test, while the *ica*D gene was present in 92.2 % of *Staphylococcus* spp. The high frequency of *blaZ* gene observed in this study was associated with the resistance of most *Staphylococcus* spp. to one or more of the beta-lactams tested, which are routinely used in Brazilian herds for mastitis treatment. The biofilm formation was also detected in the isolates analyzed being an important characteristic for pathogenicity and antimicrobial resistance of bacteria.

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Introduction

Bovine mastitis is the disease that causes most economic damage to the dairy industry worldwide and also poses a potential health risk for consumers (Kumar et al. 2010). *Staphylococcus aureus* is one of the main microorganisms isolated from intramammary infections in dairy cows, and cases are usually subclinical and difficult to treat (Taponen and Pyörälä 2009). The toxins produced by this species may also be present in the milk and its derivatives and frequently cause food poisoning in humans (Loncarevic et al. 2005). Coagulase-negative staphylococci (CNS) are regarded as emerging pathogens and normally give rise to slight to moderate inflammation of the mammary glands (Waller et al. 2011).

The indiscriminate use of antimicrobials to combat mastitis has led to the selection of resistant strains of *Staphylococcus* spp., undermining the efficacy of treatment. Beta-lactam antibiotics are routinely used to treat intramammary infections (Haveri et al. 2005), and the most common reason for their ceasing to be effective is the production of beta-lactamase enzyme, which is coded by the *blaZ* gene and gives rise to hydrolysis of the beta-lactam ring of penicillins (Olsen et al. 2006). The resistance of *Staphylococcus* spp. to methicillin/oxacillin, however, is associated with alteration of the site of action of the drug by synthesis of a low-affinity penicillin-binding protein (PBP_{2a}) and is mediated by the *mec*A gene (Sawant et al. 2009). The presence of efflux pumps in the bacteria's plasma membrane enables extrusion of toxic compounds to the external medium and is the mechanism responsible for the resistance of microorganisms to different classes

of antimicrobials of clinical interest (Webber and Piddock 2003).

Control of the disease may also be hampered by persistence of microorganisms in the mammary tissue, which may be associated with the formation of biofilms. These structures are made up of bacteria that adhere tenaciously to the surface and are enveloped in a matrix of organic polymers (Melchior et al. 2009). These facilitate the adhesion of *Staphylococcus* spp. to epithelial surfaces and the colonization of the mammary epithelium, diminishing the immunological response of the host in the elimination of pathogens (Simojoki et al. 2012). Biofilm formation is also associated with antimicrobial resistance, because of the difficulty antibiotics have in diffusing through the polysaccharide matrix and reduced metabolic activity of bacteria inside the multilayers (Melchior et al. 2006). The role of biofilms in the pathogenesis of chronic infections has aroused interest in the characterization of the genes involved in their formation, of which the *ica* locus is the best understood, as it is present in most *Staphylococcus* spp. isolated from bovine mastitis (Vasudevan et al. 2003).

Studies in Brazil and around the world have reported an increase in the resistance to antimicrobials of *Staphylococcus* spp. isolated from mastitis, which raises the cost of medication and also has serious implications for animal and human health (Sawant et al. 2009; Medeiros et al. 2011). Monitoring of in vitro sensitivity to drugs and the detection of the biofilm formation may aid in the identification of resistance mechanisms and in the establishment of effective measures for controlling the disease. The aim of this study is thus to characterize phenotypic and genotypic features associated with resistance in *Staphylococcus* spp. isolated from bovine mastitis in the Northeast of Brazil.

Materials and methods

Bacterial isolates

An examination was carried out of 218 *Staphylococcus* spp. isolated from 2064 milk samples of 525 lactating cows from eight farms in the States of Bahia and Pernambuco, seven located in the lower middle São Francisco Valley and one in the Agreste region of Pernambuco, Brazil. From the milk samples analyzed, 53 (2.6 %) and 584 (28.2 %) were taken from animals with clinical and subclinical mastitis, respectively, and 527 (25.5 %) were associated with intramammary infections.

The farms were chosen randomly, and the herds studied were managed using an extensive (pasture feeding management), semi-intensive (food management based on grazing and feed supplementation), or intensive (food management based on feed supplementation and zero grazing) production system and comprised animals of different breeds (Holstein, Jersey, Gyr and crossbreed) ages (2–12 years) and stages of lactation (1–7 lactations).

Sample collection, isolation, and identification of bacteria

The milk sample collection was performed in all lactating cows from each farm studied. First, a physical examination of the animals was carried out to evaluate the presence of udder inflammation signs (redness, swelling, warmness, painfulness) and occurrence of alterations on milk (clots, blood, or color changes). The subclinical mastitis was detected in animals that showed absence of udder inflammation signs or alterations on milk and that demonstrated positive results on California Mastitis Test (CMT) (Schalm and Noorlander 1957), which were scored as 1+, 2+, or 3+ depending on the intensity of the reaction. An average of 5 mL of milk was collected from each animal from all four quarters individually, regardless of the reaction to the CMT, as recommended by the National Mastitis Council (National Mastitis Council 1999).

Aliquots of 10 μL of cow's milk were streaked onto 5 % ovine blood agar (Himedia, India), and the plates were then incubated at 37 °C for 48 h. The bacterial agents were identified by way of morphological characteristics (coloring, size, presence or absence of colony hemolysis), Gram staining, and biochemical tests. The milk samples that showed isolation of three or more different microorganisms were considered contaminated (662 samples; 32.1 %) (National Mastitis Council 1999) and not used in this study. One *Staphylococcus* spp. isolate was investigated per milk sample, and microorganisms of this genus were characterized as Gram-positive cocci, catalase-positive (Vetec Química Fina Ltda, Brazil), and facultative anaerobes (fermentative). The complete biochemical identification involved tests for the production of coagulase (Newprov, Brazil), acetoin (Vetec Química Fina Ltda, Brazil), urease (Himedia, India) and DNAse (Himedia, India); fermentation of glucose (Himedia, India), maltose (Himedia, India), and mannitol (Himedia, India); and hydrolysis of esculin (Vetec Química Fina Ltda, Brazil), (Quinn et al. 1994).

As recommended by the National Mastitis Council, *S. aureus* was differentiated from other coagulase-positive staphylococci (oCPS) species by Voges-Proskauer test, since *S. aureus* is acetoin positive while oCPS do not produce acetoin. In addition, *S. aureus* was characterized by the production of hemolysis on blood agar, what does not occur for *Staphylococcus hyicus* strains (National Mastitis Council 1999).

Resistance to antimicrobials

The resistance profile of *Staphylococus* spp. was determined using the disc diffusion method. Isolated colonies selected from a 24-h Tryptic Soy Agar (Himedia, India) plate were transferred to Mueller Hinton broth (Himedia, India), and the

suspension was adjusted until it achieved a turbidity equivalent to a 0.5 McFarland standard. Using a cotton swab, the inoculum suspension was streaked onto a Mueller Hinton agar (Himedia, India) plate (CLSI 2012). Discs (Newprov, Brazil) were used with the following antimicrobials: amoxicillin (10 μg; susceptible zone diameter ≥29 mm), ampicillin (10 μg; ≥29 mm), cephalexin (30 μg; ≥18 mm), ciprofloxacin (5 μg; ≥21 mm), doxycycline (30 μg; ≥16 mm), enrofloxacin (5 μg; ≥21 mm), erythromycin (15 μg; ≥23 mm), streptomycin (10 μg; ≥15 mm), gentamicin (10 μg; ≥15 mm), lincomycin (2 μg; ≥17 mm), oxacillin (1 μg; ≥13 mm), penicillin (10 units; ≥29 mm), rifampicin (5 μg; ≥20 mm), trimethoprim-sulfamethoxazole (25 μg; ≥16 mm), and tetracycline (30 μg; ≥19 mm). After 16 to 18 h of incubation at 35±2 °C, each plate was examined and the results were interpreted in accordance with CLSI breakpoints for all drugs (CLSI 2008, 2012), except for cephalexin and lincomycin which followed the manufacturer's recommendations. The quality of the technique and of the discs used was controlled using the *S. aureus* ATCC 25923 strain. The multi-drug resistance (MDR) rate for the beta-lactams was calculated by dividing the number of beta-lactam drugs each isolate is resistant to by the total of beta-lactam drugs tested. Since amoxicillin and ampicillin are both aminopenicillins; the resistance to at least one of them was considered for the MDR rate calculation.

Phenotypic detection of antibiotic efflux mechanism

The *Staphylococcus* spp. isolates were streaked onto Mueller Hinton agar (Himedia, India) containing ethidium bromide (0.5 μg/mL) (Promega, USA). After incubation of plates at 37 °C for 24 h, the reading was carried out using a transilluminator under ultraviolet light. Colonies that did not fluoresce were taken to be positive for the efflux pump (Bjorland et al. 2005).

Phenotypic detection of biofilm formation

Congo red agar

The *Staphylococcus* spp. isolates were streaked onto Congo red agar (Vetec Química Fina Ltda, Brazil) and then incubated at 37 °C for 24 h. Colonies capable of forming a biofilm showed up black, while those that showed up red were considered not to be capable of forming this structure (Greco et al. 2008).

Microplate adhesion method

The detection of the biofilm formation using this method was carried out as adapted by Merino et al. (2009). The optical density (OD) was determined using a microplate reader and measured at a wavelength of 620 nm. All the isolates were tested in triplicate, using the *S. aureus* ATCC 25923 and *Staphylococcus epidermidis* ATCC 12228 control strains. The OD produced by each isolate (OD_i) was obtained using the arithmetic mean of the OD of isolates in triplicate. Taking the mean OD of the negative control (OD_c) as the basis, the microorganisms were classified as not forming a biofilm ($OD_i \leq OD_c$), weak ($OD_c < OD_i \leq 2.OD_c$), moderate ($2.OD_c < OD_i \leq 4.OD_c$), or strong ($OD_i > 4.OD_c$) forming a biofilm (Stepanovic et al. 2000).

Genotypic characterization of *Staphylococcus* spp.

After extraction of DNA from the isolates as described by Wade et al. (2005), PCR was carried out to study *nuc* (specific for *S. aureus*), *bla*Z (resistance to penicillins), *mec*A (resistance to methicillin), *msr*A (resistance to macrolides), and *ica*D (biofilm formation) genes. The sequences of primers are listed in Table 1, and the conditions used for each reaction were determined in previous studies by Murakami et al. (1991), Vasudevan et al. (2003), Sawant et al. (2009), and Kateete et al. (2010). The PCR products underwent electrophoresis in 1.5 % agarose (Sigma-Aldrich, USA) gel colored with ethidium bromide and were viewed under ultraviolet light and documented using an image capture system. For positive controls for reactions, *S. aureus* ATCC 25923 and a human clinical isolate of methicillin-resistant *S. aureus* (MRSA 231) previously confirmed by molecular tests and DNA sequencing were used.

Results

Of the 218 *Staphylococcus* spp. investigated, 126 (57.8 %) were characterized as *S. aureus* by way of biochemical tests and the presence of *nuc* gene. Apart from these, 61 (28 %)

Table 1 Primers used for genotypic characterization of *Staphylococcus* spp. isolates from bovine origin

Gene	Sequence 5'-3'	Product (bp[a])	Reference
*bla*Z	AAGAGATTTGCCTATGCTTC GCTTGACCACTTTTATCAGC	517	Sawant et al. 2009
*ica*D	AAACGTAAGAGAGGTGG GGCAATATGATCAAGATAC	381	Vasudevan et al. 2003
*mec*A	CGGTAACATTGATCGCAACGTTCA CTTTGGAACGATGCCTAATCTCAT	214	Murakami et al. 1991
*msr*A	TGGTACTGGCAAAACCACAT AAACGTCACGCATGTCTTCA	1000	Sawant et al. 2009
nuc	GCGATTGATGGTGATACGGTT AGCCAAGCCTTGACGAACTAAGC	279	Kateete et al. 2010

[a] Base pairs

Table 2 Multi-drug resistance (MDR) rate to beta-lactam antimicrobials and detection of antimicrobial resistance genes in *Staphylococcus* spp. isolated from bovine mastitis on farms in Pernambuco and Bahia, Brazil

MDR rate[a]	*S. aureus*[b] (*n*=126)	oCPS[c] (*n*=61)	CNS[d] (*n*=31)	Total of isolates
0	32	16	20	68 (31.2 %)
0.25	4	1	1	6 (2.7 %)
0.5	90	42	9	141 (64.7 %)
0.75	0	2	1	3 (1.4 %)
Genes[e]				
blaZ	123	55	25	203 (93.1 %)
icaD	122	57	22	201 (92.2 %)
msrA	1	0	1	2 (0.9 %)

[a] Resistant isolates to none (MDR rate=0), one (MDR rate=0.25), two (MDR rate=0.5), or three (MDR rate=0.75) of the beta-lactams tested

[b] *Staphylococcus aureus*

[c] Coagulase-positive staphylococci other than *S. aureus*

[d] Coagulase-negative staphylococci

[e] Polymerase chain reaction results: number of positive isolates for *blaZ*, *icaD*, and *msrA* genes

were classified as oCPS and 31 (14.2 %) as CNS. Four *S. aureus* and three oCPS were obtained from cases of clinical mastitis, and 112 (51.4 %) of the isolates came from mammary quarters that showed 1+ (39.3 %), 2+ (33 %), or 3+ (27.7 %) scores on CMT. *S. aureus* (59 %) and oCPS (56.9 %) demonstrated a higher number of positive reactions on CMT than CNS (22.5 %).

The lowest percentage of susceptibility of microorganisms to antimicrobials was for amoxicillin (32.6 %), followed by ampicillin (33 %), penicillin (34 %), tetracycline (82.6 %), streptomycin (88.1 %), doxycycline (88.6 %), trimethoprim-sulfamethoxazole (97.8 %), erythromycin (98.2 %), lincomycin (98.2 %), oxacillin (98.2 %), ciprofloxacin (99.1 %), cephalexin (99.5 %), enrofloxacin (99.5 %), and gentamycin (99.5 %). All the isolates were susceptible to rifampicin, and 61 (28 %) showed susceptibility to all the drugs tested. The MDR rate to beta-lactams varied from 0 to 0.75, with 150 (68.8 %) isolates showing resistance to one or more antimicrobials of this class (MDR rate ≥0.25). Moreover, 68 (31.2 %) isolates were susceptible to all beta-lactams evaluated (Table 2).

Genotypic characterization revealed that 203 (93.1 %) of *Staphylococcus* spp. had the *blaZ* gene (Table 2). The *mecA* gene amplification was not observed in any of the isolates tested.

The efflux mechanism was detected by the phenotypic test in two (0.9 %) *S. aureus*. The *msrA* gene was also present in two (0.9 %) isolates (one *S. aureus* and one CNS) (Tables 2 and 3), although these did not test positive using the phenotypic method.

In the Congo red agar, eight (3.7 %) isolates (three *S. aureus*, three oCPS, and two CNS) exhibited black colonies, while, in the microplate adhesion test, 210 (96.3 %) of *Staphylococcus* spp. were capable of forming a biofilm. Of these, 54 (25.7 %) were characterized as strong biofilm formers, and 79 (37.6 %) and 77 (36.7 %) showed moderate and weak formation, respectively. The formation of a strong and moderate biofilm was found in 72.2 and 57.4 % of the

Table 3 Detection of antimicrobial resistance genes and multi-drug resistance (MDR) rate to beta-lactam antimicrobials in *Staphylococcus* spp. isolated from bovine mastitis on farms in Pernambuco and Bahia, Brazil

	S. aureus[a]				oCPS[b]				CNS[c]				Total
PCR results[d]/MDR rate[e]	0	0.25	0.5	0.75	0	0.25	0.5	0.75	0	0.25	0.5	0.75	
blaZ	2	0	1	0	2	0	1	0	3	0	3	0	12 (5.5 %)
icaD	2	0	0	0	5	0	0	0	3	0	0	0	10 (4.6 %)
blaZ, *icaD*	27	4	88	0	9	1	41	1	11	0	6	1	189 (86.7 %)
blaZ, *icaD*, *msrA*	0	0	1	0	0	0	0	0	1	0	0	0	2 (0.9 %)
Negative[f]	1	0	0	0	0	0	0	1	2	1	0	0	5 (2.3 %)
Total	32	4	90	0	16	1	42	2	20	1	9	1	
Total %	25,4	3,2	71,4	0	26,2	1,6	68,9	3,3	64,6	3,2	29	3,2	

[a] *Staphylococcus aureus*

[b] Coagulase-positive staphylococci other than *S. aureus*

[c] Coagulase-negative staphylococci

[d] Polymerase chain reaction results: number of positive isolates for *blaZ*, *icaD*, and *msrA* genes

[e] Resistant isolates to none (MDR rate=0), one (MDR rate=0.25), two (MDR rate=0.5), or three (MDR rate=0.75) of the beta-lactams tested

[f] Negative isolates to all genes tested

S. aureus and oCPS analyzed, respectively, while 77.4 % of the CNS produced a weak structure or did not form one (Table 4). Only six (2.8 %) isolates tested positive and seven (3.2 %) negative for both Congo red agar and microplate adhesion test techniques. The *ica*D gene was detected in 201 (92.2 %) samples analyzed (Table 2), among which 143 (71.1 %) were resistant to one or more beta-lactams (MDR rate ≥0.25). Only five (2.3 %) isolates were negative for *bla*Z, *mec*A, *ica*D and *msr*A genes simultaneously (Table 3).

Discussion

The eight farms studied had a higher frequency of *S. aureus* and oCPS compared to the number of CNS identified. Considering the high sensitivity and specificity of genotypic techniques when compared to phenotypic methods for bacterial identification (Kateete et al. 2010), *S. aureus* confirmation was performed by amplification of *nuc* gene, which encodes for a specific thermostable nuclease and has been used to detect this bacteria in previous studies (Palomares et al. 2003; Costa et al. 2005; Asfour and Darwish 2011). Although *S. aureus* is one of the most important pathogens in the etiology of bovine mastitis, other coagulase-positive species, as *Staphylococcus intermedius*, have been isolated from cases of clinical and subclinical infections around the world (Bradley et al. 2007; Arslan et al. 2009; Asfour and Darwish 2011).

As in previous studies (Haftu et al. 2012), most (96.8 %) *Staphylococcus* spp. analyzed came from subclinical cases. According to Taponen and Pyörälä (2009), the increase in the somatic cell count in *S. aureus* infections is markedly higher than in CNS mastitis, since the species capable of coagulating blood plasma are considered potentially more pathogenic due to the production of an enzyme that enables the microorganism to persist in the presence of a host immune response. Some milk samples analyzed were negative on CMT but positive for *Staphylococcus* spp. This result reinforces the fact that even when triage tests are used on the dairy farms, there are animals that may harbor mastitis-causing agents.

Regarding the susceptibility to antimicrobials, amoxicillin, ampicillin, and penicillin were the less effective drugs in vitro. The resistance of *Staphylococcus* spp. to penicillins is a well-known phenomenon worldwide, and 10 to 70 % of *S. aureus* in cows may be resistant to these drugs, depending on geographical location (Vintov et al. 2003). In this study, 67 and 66 % of the isolates were resistant to amoxicillin and ampicillin, and to penicillin, respectively, being these rates higher than those found by Rajala-Schultz et al. (2004) and Alian et al. (2012) in *Staphylococcus* spp. from mastitic milk.

The beta-lactamase production is the main resistance mechanism of *Staphylococcus* spp. to penicillins and aminopenicillins. Among the positive samples for *bla*Z gene, 148 (72.9 %) were resistant to one or more (MDR rate ≥0.25) beta-lactams tested. Furthermore, the highest frequencies for this gene were found in *S. aureus* (97.6 %) and oCPS (90.1 %) (Table 2), of which 73.1 and 76.3 %, respectively, showed resistance to two beta-lactams tested. On the other hand, 36 % of the CNS testing positive for *bla*Z had a MDR rate of 0.5. These results are different from those obtained by Asfour and Darwish (2011), who found a proportion of *S. aureus* (0.4 %) producing beta-lactamases lower than in CNS (32 %).

In this study, the higher indices of resistance of *S. aureus* (74.6 %) to one or more beta-lactams when compared to the CNS resistance (32.2 %) may be associated with the higher prevalence of this species in all the dairy herds examined, with CNS being minor pathogens for the etiology of intramammary infections. *S. aureus* is an important contagious pathogen that

Table 4 Biofilm formation by microplate adhesion test and detection of *ica*D gene in *Staphylococcus* spp. isolated from bovine mastitis on farms in Pernambuco and Bahia, Brazil

	S. aureus[a]				oCPS[b]				CNS[c]			
Microplate adhesion test[d]/PCR results[e]	S	M	W	A	S	M	W	A	S	M	W	A
*ica*D +	33	54	34	1	14	20	20	3	3	2	15	2
*ica*D-	2	2	0	0	0	1	3	0	2	0	5	2
Total of isolates	35	56	34	1	14	21	23	3	5	2	20	4
Total of isolates %	27.7	44.5	27.0	0.8	23.0	34.4	37.7	4.9	16.1	6.4	64.5	13.0

S strong biofilm formation, *M* moderate biofilm formation, *W* weak biofilm formation, *A* absence of biofilm formation

[a] *Staphylococcus aureus*

[b] Coagulase-positive staphylococci other than *S. aureus*

[c] Coagulase-negative staphylococci

[d] Microplate adhesion test

[e] Polymerase chain reaction results: number of positive (*ica*D +) and negative (*ica*D-) isolates for *ica*D gene

colonizes the mammary gland, teat canal, and teat lesions of dairy cows, and it is transmitted between animals via fomites especially at the time of milking. This bacterium primarily causes subclinical infections that are not easily diagnosed by farmers and often become chronic, being spread considerably in herds without adoption of adequate procedures for mastitis control (National Mastitis Council 1999; Sommerhäuser et al. 2003; Barkema et al. 2006). Other researches also have found *S. aureus* as the main pathogen isolated from cows with mastitis around the world (Arslan et al. 2009; Haftu et al. 2012; Keane et al. 2013).

Although resistance to oxacillin was found in three oCPS and one CNS, the *mec*A gene was not detected in any of the isolates tested. The expression of phenotypic resistance to this drug may be associated with variations in growth conditions, alterations in the production of PBP subtypes, or overproduction of beta-lactamases (Moon et al. 2007), since two of the oCPS resistant to oxacillin showed the *bla*Z gene. According to Guérin Faublée et al. (2003), methicillin-resistant *S. aureus* (MRSA) are more common in human than veterinary medicine. MRSA and methicillin-resistant *Staphylococcus* spp. have been reported in clinical and subclinical cases of bovine mastitis, which are multi-resistant and potentially zoonotic (Kumar et al. 2010; Vanderhaeghen et al. 2010).

The presence of transporter proteins in the bacterial cell membrane is one of the processes involved in resistance to macrolides and tetracyline (Bjorland et al. 2005). Most of the isolates evaluated exhibited sensitivity to these drugs, and this is related to the low rate of detection of the efflux mechanism using the triage technique. Only two *S. aureus* and two oCPS were resistant to erythromycin, although none exhibited *msr*A or tested positive using the phenotypic test. It is known that the most important resistance mechanism for erythromycin in *S. aureus* occurs through ribosomal alterations regulated by *erm*A, B, or C genes and less frequently by way of active efflux (Gatermann et al. 2007).

The characterization of biofilm formation by phenotypic methods showed divergent results between Congo red agar and microplate adhesion test techniques. It has already been described in previous studies and may be associated with the sensitivity of phenotypic expression of the biofilm to in vitro conditions (Knobloch et al. 2002; Vasudevan et al. 2003). Furthermore, formation of a strong and moderate biofilm was found in 72.2 and 57.4 % of the *S. aureus* and oCPS analyzed, respectively, while 77.4 % of the CNS produced a weak structure or did not form one. In a study conducted by Arciola et al. (2001), *S. aureus* also formed a stronger biofilm, helping these pathogens to persist in the udder, facilitating chronic intramammary infections and making them difficult to treat. Coagulase-positive staphylococci (CPS) and CNS are very similar in terms of the capacity to adhere, while *S. aureus* appears to be more invasive (Taponen and Pyörälä 2009).

In the biofilm formation, the *ica* locus is responsible for the formation of the intracellular adhesion polysaccharide in *Staphylococcus* spp. (Arciola et al. 2001). The high frequency (92.2 %) of isolates testing positive for the *ica*D gene observed has been reported in other studies examining *S. aureus* from cow's milk (Vasudevan et al. 2003; Melchior et al. 2009). Furthermore, 92.8 % of samples that showed adhesion in microplates presented the *ica*D gene. Some strains (6.9 %) in which the gene was not detected were capable of forming a biofilm phenotypically, and this may be attributed to the fact that other markers not tested are also related to the formation of this structure, such as the *ica*A and *bap* genes (Cucarella et al. 2004).

Of the 195 isolates considered biofilm formers by the microplate adhesion method and genotypic test, 146 (74.8 %) showed resistance to one or more of the antimicrobial drugs tested, especially beta-lactams and tetracycline. Furthermore, the two isolates characterized as not forming biofilm by both techniques were susceptible to all the drugs tested. According to Olson et al. (2002), bacteria growing in a biofilm can become 10–1000 times more resistant to the effects of antimicrobial agents as compared to planktonic growing bacteria of the same strain. The polysaccharide matrix impairs the access of antibiotics to the bacterial cells, and the slow growth of the microorganisms inside the multilayers contributes for the decreased susceptibility to antimicrobial agents requiring growing cells for their bactericidal effects (e.g., penicillins and cephalosporins) (Melchior et al. 2006). "Persister" cells may remain even after administration of antibiotics and are considered to be responsible for the survival of the bacterial population. The physiological adaptive changes in these cells seem to be the key to the survival properties of biofilms (Lewis 2001).

Since genotyping was not performed in this study, the possibility that a same strain of *Staphylococcus* spp. has been isolated from different animals or quarters and repeatedly tested cannot be eliminated. It could justify the similarity between the profiles of resistance to beta-lactams and the high frequency of *bla*Z+*ica*D genes found in isolates from the same herds analyzed.

S. aureus was the main pathogen isolated from cows with mastitis in Northeast of Brazil. The high frequency of *bla*Z gene observed in this study was associated with the resistance of most *Staphylococcus* spp. to one or more of the beta-lactams tested — especially penicillin and aminopenicillins — which are routinely used in Brazilian herds for mastitis treatment. The biofilm formation was also detected in the isolates analyzed being an important characteristic for pathogenicity and antimicrobial resistance of bacteria.

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