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Characterization of Coagulase-Negative Staphylococci and pheno-genotypic beta lactam resistance evaluation in samples from bovine Intramammary infection

[*Caracterização de Staphylococcus coagulase negativa e avaliação fenogenotípica da resistência aos beta-lactâmicos em amostras oriundas de infecção intra-mamária em bovinos*]

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ABSTRACT

This study aimed to identify Coagulase-Negative Staphylococci **(**CoNS) species isolated from bovine mastitis, through phenotypic and PCR-RFLP (Restriction Fragment Length Polymorphism-Polimerase Chain Reaction) methods and to compare both techniques to matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) technique. Among them, the PCR-RFLP method, using a partially conserved sequence of the *gro*EL gene, is a promising alternative, because of its reproducibility and reliability. On the other hand, the proteomic technique MALDI-TOF MS provides an accurate and much faster diagnosis and has been increasingly employed in microbiological identification. The pheno-genotypic profiles of beta-lactam resistance were also investigated, this characterization is important, considering that the use of antimicrobials is a key element for mastitis control in dairy farms. The concordance of the phenotypic, PCR-RFLP and MALDI-TOF MS assays to identify CoNS species was 77,5% (31/40). *S. chromogenes* was the species most frequently isolated. Antibiotic resistance rate was relatively low, registering values of 25.5% to penicillin, 9.6% to oxacillin and 6.2% to cefoxitin. Resistance to imipenem, cephalotin and amoxicillin+clavulanate was not observed. The *mec*A gene and its variant were detected in 7.6% and 4,1% of the isolates respectively. The *bla*Z gene was found in 43.2% of the strains resistant to penicillin.

Keywords: *Staphylococcus* spp., bacterial resistance, PCR–RFLP and MALDI-TOF MS

RESUMO

*Este estudo teve como objetivo identificar isolados de Staphylococcus coagulase-negativa (SCN) isolados de mastite bovina, por meio de métodos fenotípicos e PCR-RFLP (reação em cadeia de polimerase - polimorfismo nos fragmentos de restrição), e compará-los com a técnica de tempo de voo de ionização/desorção por laser assistida por matriz de espectrofotometria de massa (MALDI-TOF MS). O método de PCR-RFLP, que utiliza uma parte conservada da sequência do gene groEL, é uma alternativa promissora, por ser reprodutível e confiável. Por outro lado, a técnica proteômica MALDI-TOF MS permite uma acurácia e um diagnóstico muito mais rápidos e tem sido cada vez mais empregada na identificação microbiológica. Os perfis fenogenotípicos de resistência aos beta-lactâmicos também foram investigados. Essa caracterização é importante, considerando-se que os antimicrobianos são os elementos-chave para o controle da mastite na produção leiteira. A concordância entre os testes fenotípicos, PCR-RFLP E MALDI-TOF MS na identificação foi de 77,5% (31/40). S. chromogenes foi a espécie mais frequentemente isolada. A resistência antimicrobiana foi relativamente baixa, apresentando valores de 25,5% para penicilina, 9,6% para oxacilina e 6,2% para cefoxitina. Resistência ao imipenem, à cefalotina e à amoxacilina + ácido clavulâncico não foi observada. O gene mec*A *e sua variante foram detectados em 7,6% e 4,1% dos isolados, respectivamente. O gene bla*Z *foi encontrado em 43,2% dos isolados resistentes à penicilina.*

Palavras-chave: *Staphylococcus spp., resistência bacteriana, PCR-RFLP, MALDI-TOF espectrofotometria de massa*

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INTRODUCTION

Coagulase negative staphylococci (CoNS) are a heterogeneous group of species with limited but significant impact on dairy farms (Zadoks *et al*., 2011). At least 15 species have been associated with bovine mastitis, but *Staphylococcus chromogenes*, *S. epidermidis*, *S. xylosus* and *S. haemolyticus* are reported as the most prevalent species in herds around the world (Piessens *et al*., 2012; Frey *et al*., 2013). While *S. chromogenes* rarely occurs in humans, *S. epidermidis* commensally inhabits the human skin and mucous membranes. Strains of *S. epidermidis* from bovine intramammary infections (IMIs) frequently exhibit multidrug resistance and there is evidence of its transmission through milkers's hands to the bovine udders (Sawant *et al*., 2009; Sampimon *et al*., 2011). Several molecular approaches have been suggested for the proper identification of CoNS, since phenotypic methods are time-consuming and unreliable for animal samples (Park *et al*., 2011). PCR-RFLP of the *gro*EL or *hsp60* (heat-shock protein) gene has been effectively employed in the identification of CoNS from both bovine and caprine mastitis (Santos *et al.*, 2008; Onni *et al.*, 2012). Recently, the proteomic method MALDI-TOF MS has been increasingly used in microbial identification, due to its reliability, speed, and ease of use. MALDI-TOF MS produces a characteristic spectrum of the analyzed microorganisms, allowing accurate identification and classification of bacterial strains. This method was successfully applied to identify CoNS isolates from clinical and environmental samples (Dubois *et al*., 2010). Lack of susceptibility to penicillin and other beta-lactams is frequently found among CoNS isolated from cattle (Sampimon *et al*., 2011). This resistance is usually caused by the constitutive or induced production of a penicillinase, coded by the *bla*Z gene. Alternatively, it can result from the expression of the *mec*A gene, which encodes PBP2a, a modified PBP (penicillin binding protein) and gives resistance to all beta-lactams, including carbapenems, monobactams and cephalosporins. The aims of this study were to compare the prevalence and antimicrobial susceptibility of CoNS species in bovine mastitis, identified by phenotypical tests, PCR-RFLP and MALDI-TOF MS, and to determine the prevalence of *mec*A and *bla*Z genes in these strains.

MATERIALS AND METHODS

The California Mastitis Test was performed on 680 lactating cows from nine dairy herds, in the Brazilian state of Rio de Janeiro. Positive animals had individual mammary quarter milk samples (10mL) aseptically collected into sterile vials immediately before milking, after discarding the first three milking streams. The 512 milk samples obtained were transported to the laboratory and directly cultured (10µL) on 5% sheep blood agar and mannitol salt phenol red agar plates. The agar plates were incubated at 37ºC for 24h. Ethics committee, (protocol number, CEUA3664040915), certified this research.

A modified scheme based on Cunha *et al*. (2004) was used to identify CoNS isolates, comprising the following tests: fermentation of the sugars xylose, arabinose, sucrose, trehalose, maltose, mannitol, lactose, xylitol, ribose, fructose and mannose; production of hemolysin; presence of urease; and resistance to novobiocin 5mcg (Cefar). Standards strains were used as control. The following type strains were included in the study: *S. simulans* ATCC 27851^{T}, *S. warneri* ATCC 10209^{T}, *S. saprophyticus* ATCC15305 ^{T}, *S. epidermidis* ATCC 12228 ^{T}, *S. hominis* ATCC 27844 ^{T}, *S. xylosus* ATCC 29971^{T}, *S. aureus* ATCC 43300 ^{T} and *S. aureus* ATCC 29213 ^{T}. In addition, three clinical strains, all identified by the conventional biochemical method, were used as positive control strains of *S. sciuri*, *S. chromogenes* and *S. hyicus*. All strains were grown overnight on mannitol salt phenol red agar plates at 37ºC for examination of purity and colony characteristics.

To genomic DNA extraction, the isolates were grown in 5mL of nutrient broth at room temperature for 12-16h with stirring at 150rpm. A 1.5mL aliquot was pelleted by centrifugation at 12000rpm for 5min. The supernatant was discarded and this step was repeated twice. The pellets were resuspended in 600µL of a lysis buffer (200mM Tris-HCl pH 8.0, 25mM EDTA pH 8.0, 25mM NaCl, 1% SDS) followed by incubation at 65ºC for 30min. The aqueous phase was extracted twice with chloroform/isoamyl alcohol (24:1), at 13000rpm for 10min. The

DNA was precipitated with two volumes of 100% ethanol, at -20ºC for 12h. Microtubes were centrifuged at 13000 for 30min and the resulting pellets were washed with 70% ethanol, dried and resuspended in 30µL TE buffer (10mM Tris-HCl, 1 mM EDTA, pH 8.0).

PCR amplification of the *gro*EL gene and PCR-RFLP assay were performed by *gro*EL degenerate primers H279A (5'-GAIIIIGCIGGIGA(TC)GGIACIACI AC-3') and H280A (5'-(TC)(TG)I(TC)(TG)ITCICC(AG) AAICCIGGIGC(TC)TT-3'), previously described (Goh *et al*., 1997), were used to amplify a 550-bp *gro*EL gene fragment as described by Santos *et al*. (2008). To exclude cross-profile with other CoNS, a computational restriction fragment analysis was performed using all staphylococci *hsp*60 sequences stored in the GenBank and the program NEBCutter v2.0 (http://tools.neb.com/NEBcutter2/).

Forty isolates randomly chosen and the 21 which could not be identified by PCR-RFLP were identified at the species level by MALDI-TOF MS. Pure cultures were grown on BHI agar at 37°C for 24h. A small amount from a colony of each pure culture was transferred to a 96-spot target well (96 MSP, Bruker - Billerica, EUA) using a disposable loop, added to 70% formic acid lysis solution (Sigma-Aldrich), and overlaid with 1µL of matrix (α-cyano-4-hydroxycinnamic acid diluted with 50% acetonitrile/2.5% trifluoroacetic acid, Sigma-Aldrich). All samples were processed in positive linear mode (m/z= 2000–20,000), with a 337nm nitrogen laser (FlexControl 3.3 software, Bruker). Each spectrum was analyzed and considered valid when the score matched those of reference spectra of the MALDI Biotyper v2.0 database (Bruker Daltonics GmbH) and was ≥2, according to the criteria proposed by the manufacturer.

All isolates were tested for beta-lactam susceptibility by disk diffusion test (Performance…, 2013a,b). The following antimicrobial disks were used: penicillin G (10UI), cefoxitin (30µg), oxacillin (1µg), amoxicillin+clavulanate (30µg), cephalothin (30µg) and imipenem (10µg).

Genotypic resistance profiles were determined by PCR, targeting genes encoding resistance to beta-lactams, including *mec* genes: *mec*A (Murakami *et al*., 1991), *mec*A variant (Melo *et al*., 2014), and *mec*A1 (*pbp*D) (Couto *et al*., 2010); *mec*I (Lencastre *et al*., 2002), *mec*RI (Rosato *et al*., 2003), and *bla*Z (Rosato *et al*., 2003).

RESULTS

The isolates were identified using different techniques: phenotypical tests, PCR-RFLP (Figure 1A), and MALDI- TOF MS) (Table 1). Twenty-one isolates were unable to amplify the 550bp fragment of the *gro*EL gene, and could not be identified by this technique. Two different patterns were observed for *S. chromogenes* strains, resulting from an intraspecific polymorphism. Sixty-eight isolates identified as *S. chromogenes* showed the characteristic RFLP pattern for this specie, while 16 isolates exhibited an atypical pattern, with only two fragments. Apparently, a restriction site for AluI was lost, and the 75bp and 107bp fragments resulted in a single fragment of approximately 200bp (Figure 1B). Double digestion has been suggested for isolates classified as *S. chromogenes*, to better differentiate this specie from *S. hyicus* and *S. capitis* (Santos *et al.*, 2008). The 84 strains, presumptively classified as *S. chromogenes*, were subjected to PvuII/HindIII endonucleases. All of them showed the same pattern (Figure 1C), confirming the initial identification and indicating that the restriction sites for PvuII/HindIII were not affected by the intraspecific variability.

In total, 95% of the 61 isolates were clearly identified at the species level by MALDI-TOF MS analysis (Table 1). Three isolates (*S. auricularis* and 2 *S. hyicus*) had scores between 1.8 and 2.0, meaning the genus identification was considered secure but the identification was only probable at the species level.

The species identification of the 40 strains previously identified by the PCR-RFLP method was confirmed by the MALDI-TOF analysis, showing 100% concordance. The concordance of the phenotypic, PCR-RFLP and MALDI-TOF MS assays to identify CoNS species was 77,5% (31/40). The 21 isolates which failed to amplify the partial fragment of *gro*EL were also identified by proteomics as CoNS strains. *S. chromogenes* was the most prevalent species (70%).

Table 1. Frequency of distribution of coagulase-negative staphylococci identified by Phenotypic test, PCR-RFLP and MALDI-TOF MS

CoNS species	Phenotypical Identification	Molecular Identification	
		PCR-RFLP	MALDI- TOF MS
S. chromogenes	63.4% (92)	57.9% (84)	70.5% (43)
S. sicuri	6.9% (10)	6.9% (10)	2.5% (4)
s. xylosus	5.5% (8)	6.9% (10)	3.3% (2)
S. lugdunensis	4.8% (7)	1.4% (2)	1.6% (1)
S. simulans	3.4% (5)	1.4% (2)	1.6% (1)
S. hyicus	3.4% (5)	-	3.3% (2)
S. auriculares	3.4% (5)	-	1.6% (1)
S. warneri	2.75% (4)	3.4% (5)	4.9% (3)
S. epidermidis	2.1% (3)	2.75% (4)	3.3% (2)
S. haemolyticus	1.4% (2)	1.4% (2)	-
S. hominis	1.4% (2)	2.1% (3)	3.3% (2)
S. cohnii	0.7% (1)	1.4% (2)	-
S. schleiferi subsp. *Schleiferi*	0.7% (1)	-	-
Not identified	-	14.5%(21/145)	-
Total	145	124	61

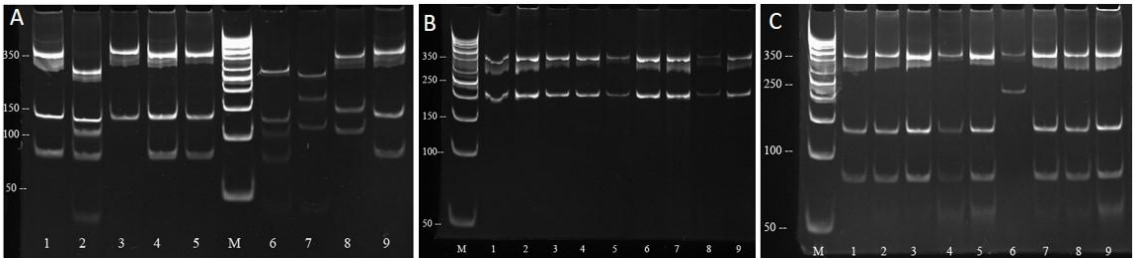

Figure 1. Genotypic identification by PCR-RFLP of the *gro*EL gene. A: (1), (4) e (5) *S. chromogenes* strains; (2) *S. xylosus* strain; (3) *S. haemolyticus* strain; (6) *S. xylosus* ATCC 29971^T, (7) *S. simulans* ATCC 27851^T, (8) *S. warneri* ATCC 10209^T and (9) *S. chromogenes* control strain, (M) 50bp ladder. B: (1-8) *S. chromogenes* isolates; (6) *S. chromogenes* isolate showing an atypical pattern for the species with only two fragments; (9) *S. chromogenes* control strain, (M) 50bp ladder. C: (1-8) *S. chromogenes* isolates after double digestion with HindIII/PvuII; isolates 5, 6, 7 and 8 had shown an anomalous pattern after digestion with AluI; (9) *S. chromogenes* control strain, (M) 50bp ladder.

The biochemical tests showed a concordance of 78.6% with the final identification. Species such as *S. chromogenes*, *S. xylosus* and *S. sciuri* showed a better concordance coefficient, around 90%, but speciation was particularly problematic for *S. simulans* and *S. lugdunensis*.

The genotypic technique showed sensitivity of 85.5%, a consequence of 21 isolates failing to produce amplicons with the primers and PCR conditions used. These could only be identified by proteomics.

Resistance to cefoxitin was found in 6.2% of isolates (9/145). As expected, these strains were also resistant to the other beta-lactams tested. They were identified as *S. chromogenes* (3), *S. epidermidis* (3), *S. hominis* (2) and *S. hyicus* (1), but only 2 *S. epidermidis* were positive for the *mec*A and *mec*RI genes. Another 9 isolates, 6 *S. sciuri* and 3 *S. chromogenes*, were also positive for the *mec*A gene (Murakami *et al*., 1991) and other 6 *S. sciuri* were positives for the *mec*A variant (Melo *et al.*, 2014), but they were susceptible to all antimicrobials. They were also negative for the *mec*I and *mec*RI genes.

Fourteen isolates were resistant to oxacillin, but five of them were susceptible to cefoxitin, indicating a resistance mechanism by beta-lactamase hyperproduction. Resistance to penicillin was encountered in 25.5% isolates (37/145) but only 43.2% of them (16/37) were positive for the *bla*Z gene, most of which were

identified as *S. chromogenes* (13/16). This gene was also found in one isolate each of *S. sciuri*, *S. epidermidis* and *S. auricularis*.

The *mec*A analog (*pbp*D) was found in 19.3% of the CoNS isolates (28/145). This gene was detected in 40% of *S. sciuri* (4/10), 100% of *S. warneri* (6/6), 14% of *S. chromogenes* (14/101) and in one isolate each of *S. xylosus*, *S. lugdunensis*, *S. hominis* and *S. cohnii*. Except for the nine methicillin-resistant CoNS, resistance to cephalotin, imipenem and amoxicillin+clavulanate was not observed.

DISCUSSION

The increasing number of CoNS species and the high frequency in bovine IMIs with which these microorganisms makes diagnosis a challenge (Thorberg *et al.*, 2009). The conventional method of identification, based on laborious biochemical schemes, remained a hindrance to further understanding of their real impact on dairy farms, several molecular techniques were developed in an attempt to assist in CoNS speciation, such as sequencing of housekeeping genes, ribotyping and AFLP fingerprinting, among others (Park *et al*., 2011). These tools helped to clarify to some degree which species were prevalent in bovine mastitis and what kind of resistance profiles they possessed. *S. chromogenes*, *S. simulans*, *S. xylosus* and *S. epidermidis* are usually reported as the most isolated species. S. *epidermidis* is frequently associated with multidrug resistance (Sampimon *et al*., 2011; Waller *et al*., 2011; Frey *et al*., 2013). Among the several genotypic methods suggested for correct CoNS identification, PCR-RFLP of the *gro*EL gene is a reliable option, with good reproducibility. In this study, we used this technique to identify 145CoNS strains. When it was first proposed by Barros *et al.* (2007), polymorphisms in *S. aureus* and S. *lugdunensis* were observed, and this kind of occurrence was not ruled out for other species. Likewise, we detected an intraspecific polymorphism in *S. chromogenes*. Mutations in a restriction site of AluI are the likely cause of the anomalous RFLP pattern. This, however, did not reduce the discriminatory power of the method, as the atypical pattern observed for *S. chromogenes* was not similar to any of the other CoNS. Moreover, the double digestion with PvuII/HindIII suggested for *S. chromogenes* strains confirmed the initial identification. Out of 145 isolates, 124 (85.5%) were accurately speciated by PCR-RFLP. The remaining 21 strains did not produce amplicons, preventing characterization by this method. These strains and another 40 were identified by MALDI-TOF MS. The 21 isolates belonged to the following species: *S. chromogenes* (17/21), *S. hyicus* (2/21), *S. warneri* (1/21) and *S. auricularis* (1/21). The 40 randomly chosen isolates had their identification by the PCR-RFLP of the *gro*EL confirmed by the MALDI-TOF MS. This proteomic method is being increasingly employed in microbiology research, since it is a powerful tool for species identification. The high prevalence of *S. chromogenes* found in this study is in accordance with the observation that this specie, along with *S. simulans*, *S. epidermidis* and *S. xylosus*, is the most prevalent CoNS found in bovine mastitis (Sawant *et al*., 2009; Piessens *et al*., 2012). Still, *S. epidermidis* multidrug resistance is frequently reported, suggesting it is a more problematic pathogen. The fact that humans naturally harbor this microorganism also implies a zooanthroponotic potential (Sampimon *et al*., 2011). Concerning the resistance profiling, according to the CLSI guidelines, the disk diffusion test using cefoxitin is the preferred method for the evaluation of PBP2a production in CoNS isolated from animals. Except for *S. epidermidis*, oxacillin interpretative criteria usually overestimate methicillin resistance for other CoNS. Nonetheless, the most accurate prediction of resistance against beta-lactams, including penicillinase-stable penicillins, include the detection of the *mec*A gene through PCR or screening for PBP2a production, using latex agglutination tests. Lack of susceptibility to antibiotics commonly used in bovine mastitis is reportedly low. Penicillin resistance is the most prevalent and many articles report values around 15% to 30% (Piessens, 2011; Saini *et al*., 2012; Frey *et al*., 2013). Resistance against cefoxitin usually ranges from 2% to 10% in CoNS (Sawant *et al*., 2009; Fessler *et al*., 2010; Waller *et al*., 2011; Sampimon *et al*., 2011; Frey *et al*., 2013). In this study, a similar trend was observed with penicillin and cefoxitin resistance reaching values of 25.5% and 6.2%, respectively. Detection of resistance genes did not correlate well with the phenotypic results. Even though the *mec*A gene was found in 11 strains, only two of them showed resistance to cefoxitin. The *mec*A variant typical from bovine samples, impossible

to detected by routine PCR (Melo *et al*., 2014), was found in 6 *S. sciuri*, that were all susceptible to cefoxitin too. Kim *et al.* (2013) made a study explaining the hetero-resistance to the beta-lactam family of antibiotics in methicillin resistant isolates. Inversely, seven isolates which were resistant in the cefoxitin disk diffusion test were negative for the *mec*A genes, another mechanism could be responsible for this resistance, Garcia-Alvarez *et al.* (2011) described a new homolog of *mec*A, called *mec*C, this gene has been detected in humans and animals, but until the present date it was not found in the Americas.

The *bla*Z gene was found in 43.2% (16/37) of the isolates resistant to penicillin and most of them (13/16) were identified as *S. chromogenes*. Multiresistance was found in three, out of four *S. epidermidis*, and two of them harbored *mec*A and *mec*RI genes. The *mec*A1 analog (*pbp*D) was found in 40% of *S. sciuri* and in other CoNS, such as *S. warneri* and *S. chromogenes*. Although it has been postulated that this gene is the evolutionary precursor of the *mec*A in *S. aureus*, its role as a resistance determinant has not been established (Couto *et al*., 2010; Tsubakishita *et al*., 2010). We did not find any correlation between the presence of *mec*A1 and beta-lactam resistance.

CONCLUSION

MALDI-TOF MS is the best alternative method for CoNS species identification from samples isolated from dairy cows with IMIs, and *S. chromogenes* is the most frequently isolated microorganism. Whereas the poor correlation between phenotypic and genotypic characterization of antimicrobial resistance support the hypothesized that these isolates have higher degrees of variability or may suffer mutations that prevent detection. New mechanisms need to be investigated, to better understand this kind of resistance.

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