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Characteristics of resistance and virulence factors in different species of coagulase-negative staphylococci isolated from milk of healthy sheep and animals with subclinical mastitis

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ABSTRACT

Coagulase-negative staphylococci (CNS) are among the main responsible agents for mastitis in sheep. Cure rates can be reduced due to several causes, such as those related to virulence factors presented by microorganisms. This study aims at characterizing the virulence and resistance factors to antimicrobial agents in different CNS species isolated from sheep milk. After collecting milk samples, the samples were analyzed and the CNS species were identified. After identification, the susceptibility-sensitivity profile was examined using the disk diffusion technique for 10 antimicrobial agents. The DNA was extracted to detect the presence of the *mecA* gene, biofilm (*icaADBC*, *bap*, and *bhp*) and toxin genes (*sea*, *seb*, *sec*, *sed*, *tst*, and *luk*-PV) by PCR. Samples carrying toxin genes had their expression assessed using the reverse-transcription PCR technique. The biofilm production was assessed using the adherence method on a polystyrene plate. One hundred twelve CNS samples were isolated, 53 (47.3%) from animals with subclinical mastitis and 59 (52.7%) from healthy animals. Drugs tested have shown to be efficient for most CNS samples. The largest resistance percentage of CNS was found for the penicillin (17.0%) and tetracycline (10.7%) and 4 samples carried the *mecA* gene. As for the biofilm genes, the *icaADBC* operon was found in 10 (8.9%) samples, the *bap* gene was found in 16 (14.3%), and the *bhp* gene was found in 3 (2.7%). In addition, 69 (61.6%) samples produced biofilm. The survey of toxin genes has shown that 70 (62.5%) samples showed some toxin-encoding gene. However, none of the samples has expressed any of the genes from those toxins studied.
Key words: sheep mastitis, coagulase-negative staphylococci, resistance, biofilm, toxin

INTRODUCTION

Among the diseases that affect dairy herds compromising milk quality, mastitis is the one that stands out as one of the most important with regard to economic terms and public health (Pyörälä, 2002). Among the etiologic agents of sheep mastitis, *Staphylococcus* spp. is the main isolated microorganism (Bergonier and Berthelot, 2003).

In the *Staphylococcus* group, the CNS stand out, which have long been considered contaminants (Taponen and Pyörälä, 2009). However, the role of this group of microorganisms has been revised and they are currently considered the most important etiologic agents of sheep mastitis (Leitner et al., 2003), its relevance is strengthened because they are among the most frequently etiologic agents found in cases of subclinical mastitis, ranging from 25 to 93% of the isolates (Bergonier et al., 2003).

The CNS are classified as opportunistic microorganisms for being present in the milking environment, equipment, and teat surface, causing infectious mastitis when they reach the teat canal (Radostits et al., 2007). These microorganisms can cause persistent infections leading to an increased number of somatic cells, changes in milk composition, and reduction of production (Luthje and Schwarz, 2006; Pyörälä and Taponem, 2009), which will consequently lead to a reduced development of lambs and high mortality rates (Fthenakis and Jones, 1990; Ebrahimi et al., 2007).

Coagulase-negative staphylococci have not only been reported to have a negative effect on udder health; the possibility that CNS IMI or even teat apex colonization by CNS has a positive effect on udder health is an intriguing idea that has been around for a long time (Matthews et al., 1991). Indeed, among the most important conclusions of a recent meta-analysis of existing literature by Reyher et al. (2012) is that challenge studies [experimental studies where the udder is chal-

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lenged with (a) major pathogen(s)] show strong and significant protective effects of preexisting infectious mastitis by minor pathogens. This was particularly apparent in studies where major pathogens were introduced into the mammary gland by methods bypassing the teat end. Moreover, the protective effect was especially present for CNS (as opposed to *Corynebacterium bovis*). Yet, the same meta-analysis found observational studies to show no protective effect of preexisting infectious mastitis with minor pathogens.

Another study showed *Staphylococcus epidermidis*, *Staphylococcus simulans*, *Staphylococcus hominis*, *Staphylococcus saprophyticus*, and *Staphylococcus arlettae* isolates from milk to produce antimicrobial substances inhibiting the growth of some major mastitis pathogens, including *Staphylococcus aureus* (dos Santos Nascimento et al., 2005). It must be acknowledged that ascribing a beneficial effect to the CNS as a group is probably inaccurate; such effect will rather be situated at the species or even strain level. Moreover, elucidation of the possible protective role of CNS in relation to udder health should also include the mechanisms behind it, which might eventually lead to a reconsideration of the role of CNS in udder health (Vanderhaeghen et al., 2014).

Due to the heterogeneity of this group, which contains 52 species and 28 subspecies described to date (http://www.bacterio.net), sheep mastitis caused by CNS is still poorly understood and is not usually identified at species level, which makes it difficult to control infection because a great diversity of species have their own characteristics, some of which are more virulent than others, or may have different clinical characteristics (Taponen and Pyörälä, 2009).

The identification of CNS is usually based on phenotypic biochemical reactions, and misidentification may happen due to the variable expression of some phenotypic traits and because the identification schemes were designed to identify CNS from humans (Irlinger, 2008; Zadoks and Watts, 2009). Thus, different methods based on molecular biology are available and have been successfully used to identify *Staphylococcus* species, such as PCR of the 16S rRNA gene (Heikens et al., 2005), internal transcribed spacer (**ITS**)-PCR (Couto et al., 2001), and sequencing-based identification systems of the 16S rRNA, *hsp*60, *tuf*, *sodA*, and *rpoB* genes (Kwok et al., 1999; Vannuffel et al., 1999; Poyart et al., 2001; Mellmann et al., 2006; Irlinger, 2008). Typing methods such as random amplification of polymorphic DNA-PCR, amplified fragment length polymorphism, and pulsed field gel electrophoresis have emerged as promising technologies to increase our understanding of the spread and possible differences in clinical characteristics of mastitis caused by different CNS species (Piessens et al., 2011; Mello et al., 2016).

One of the main concerns regarding mastitis control is the resistance of etiologic agents to antimicrobials. Success in therapy is hampered by the increasing number of drug-resistant strains that are used in veterinary medicine. The CNS are more resistant to antimicrobials in relation to *Staphylococcus aureus* and may even present a characteristic of multidrug resistance (Taponen and Pyörälä, 2009).

In addition to resistance mechanisms, staphylococci may have several virulence factors. One of the virulence factors of great importance is related to the ability that these microorganisms have to produce biofilms (Aguilar et al., 2001), which protects the bacteria from the action of the immune system components, for hindering the action of phagocytes (Fox et al., 2005), in addition to working as a barrier that hinders the penetration of antimicrobial agents (Stewart, 1996).

Besides biofilm, staphylococci are capable of producing toxins, especially enterotoxins that are secreted into the food, and because they are thermostable, they are not destroyed at high temperatures, which makes them primarily responsible for food poisoning cases (Argudín et al., 2010). Much is known about the enterotoxins and the toxigenic potential of *S. aureus*; however, few studies are available on the toxigenic potential of CNS (Zell et al., 2008). Thus, because of the relevance of CNS in the etiology of sheep mastitis, this study aimed at characterizing the virulence and resistance factors to antimicrobials in different species of CNS isolated from milk of animals with subclinical mastitis and healthy animals.

MATERIALS AND METHODS

Origin of Samples

Sheep milk samples were derived from 242 animals from herds of the Santa Inês and Bergamacia breed, located within the state of São Paulo, Brazil. Milk samples were collected from all animals (2 samples collected from each animal, one sample from each teat) to perform the microbiological testing for CNS isolation.

Subclinical cases were identified just before collecting the milk samples for microbiological diagnosis of mastitis, through the California Mastitis Test (**CMT**), according to Schalm and Noorlander (1957), and confirmed by electronic SCC performed in samples collected into bottles with Bronopol using the electronic device Somacount 300 (Bentley Instruments, Chaska, MN). Subclinical mastitis was confirmed when mammary glands presented positive scores to California mastitis

test or SCC (>3.0 × 10^5 cells/mL of milk; McDougall et al., 2001) and were bacteriologically positive.

Isolation and Identification of CNS

Milk samples were cultured on blood agar (Oxoid, Basingstoke, UK) and incubated at 37°C for 48 h. Suspected bacterial colonies were stained by the Gram method for morphological visualization. Colonies characterized as clustered gram-positive cocci were subjected to catalase and coagulase testing. *Staphylococcus* genus was differentiated from *Micrococcus*, based on the evidence of oxidation and fermentation of glucose and by its resistance to bacitracin (0.04 U), and by sensitivity to furazolidone (100 mg; Oxoid; Baker, 1984).

Identification of CNS Species

Identification of CNS was performed using primers *G1* GAAGTCGTAACAAGG 16S and *L1* CAAGGCATCCACCGT 23S of conserved sequences adjacent to the 16S and 23S genes by ITS-PCR as described by Couto et al. (2001). In order to control results, the following international references were used: *Staphylococcus epidermidis* (ATCC 12228), *Staphylococcus epidermidis* (ATCC 35983), *Staphylococcus haemolyticus* (ATCC 29970), *Staphylococcus hominis* (ATCC 27844), *S. hominis* ssp. *novobiosepticus* (ATCC 700237), *Staphylococcus lugdunensis* (ATCC 700328), *Staphylococcus saprophyticus* (ATCC 15305), and *Staphylococcus warneri* (ATCC 10209).

Samples that could not be identified by this method were identified by sequencing of *rpoB* gene, according to the protocol described by Mellmann et al. (2006). This region encodes the B subunit of RNA polymerase (nucleotides 1444–1928).

Sensitivity to Antimicrobials. Isolated CNS were subjected to in vitro sensitivity testing using the disk diffusion technique according to recommendations of the Clinical Laboratory Standards Institute (CLSI, 2009), with 10 antimicrobials: rifampicin (5 μg), linezolid (30 μg), vancomycin (30 μg), clindamycin (2 μg), erythromycin (15 μg), penicillin (10 IU), tetracycline (30 μg), gentamicin (10 μg), ciprofloxacin (5 μg), and cotrimoxazole (25 μg; Oxoid).

DNA Extraction and PCR Amplification to Detect the mecA Gene, Biofilm (icaADBC, bap, and bhp), and Toxin Genes (sea, seb, sec, sed, tst, and luk-PV)

Total nucleic acid was extracted from isolates cultured on blood agar followed by enrichment on brain-heart infusion (Oxoid) broth at 37°C for 24 h. Extraction was carried out with Kit Illustra blood genomic Prep mini spin (GE Healthcare, Chalfont, UK) with previous digestion of staphylococcal cells using lysozyme (10 mg/mL) and proteinase K (20 mg/mL). After that, 500 μL of lysis solution was added to the mixture and it was centrifuged at 5,000 × *g* for 1 min at 4°C. Subsequently, the supernatant was transferred to the column and the kit protocol was followed as described in the insert, starting in the "Genomic DNA Binding" item. The purified DNA was stored at −20°C.

Detection of the *mecA* gene, biofilm (*icaADBC*, *bap*, and *bhp*), and toxins (*sea*, *seb*, *sec*, *sed*, *tst*, and *luk-PV*) was performed using the primers shown in Table 1 and following the parameters as recommended by the following authors: Johnson et al. (1991), Murakami et al. (1991), Lina et al. (1999), Arciola et al. (2001), Cucarella et al. (2001), and Qin et al. (2007).

Agarose Gel Electrophoresis

Electrophoresis gels were prepared with 2.0% of agarose Ultrapure (Invitrogen, Carlsbad, CA) diluted in 1× Tris borate/EDTA and stained with SYBR Safe DNA Gel Stain (Thermo Fisher Scientific). The DNA bands were visualized under UV light transilluminator. The PCR reactions that presented amplified fragments greater than 1,000 bp were subjected to agarose gel electrophoresis to 0.8%. The ITS-PCR bands were visualized after electrophoresis.

Reverse-Transcription PCR

The expression of enterotoxins, toxic shock syndrome toxins (*TSST-1*), and Panton–Valentine leukocidin (*PVL*) was analyzed by the reaction with of reverse transcriptase, followed by reverse-transcription PCR. The CNS samples presenting encoding genes for enterotoxins (*sea*, *seb*, *sec*, *sed*), toxin 1 of toxic shock syndrome (*tst*), or leukocidin *luk*-PV were subjected to reverse-transcription PCR to check the expression of these genes.

RNA Extraction

Total RNA was extracted from isolates cultured on blood agar followed by enrichment on brain-heart infusion broth at 37°C for 24 h. An Illustra RNAspin Mini RNA Isolation Kit (GE Healthcare Life Sciences, Buckinghamshire, UK) was used for extracting according to the manufacturer's instructions. Initially, 200 μL of *Staphylococcus* cultures was transferred to a sterile 1.5-mL microtube and centrifuged at 10,000 × *g* for 1 min at 4°C. The whole supernatant was discarded

Table 1. Primers used in PCR

Gene	Nucleotide sequence 5′ to 3′	Amplified product (bp)	Reference
icaA	ACAGTCGCTACGAAAAGAAA GGAAATGCCATAATGAGAAC	103	Arciola et al., 2001
icaC	TAACTTTAGGCGCATATGTTT TTCCAGTTAGGCTGGTATTG	400	Arciola et al., 2001
icaD	ATGGTCAAGCCCAGACAGAG CGTGTTTTCAACATTTAATGCAA	198	Arciola et al., 2001
icaB	CTGATCAAGAATTTAAATCACAAA AAAGTCCCATAAGCCTGTTT	302	Arciola et al., 2001
bap	CCCTATATCGAAGGTGTAGAATTGCAC GCTGTTGAAGTTAATACTGTACCTGC	971	Cucarella et al., 2004
bhp	ATGAAAAATAAACAAGGATTTC GCCTAAGCTAGATAATGTTTG	1,278	Qin et al., 2007
sea	TTGGAAACGGTTAAAACGAA GAACCTTCCCATCAAAAACA	120	Johnson et al., 1991
seb	TCGCATCAAACTGACAAACG GCAGGTACTCTATAAGTGCC	478	Johnson et al., 1991
sec	GACATAAAAGCTAGGAATTT AAATCGGATTAACATTATCC	257	Johnson et al., 1991
sed	CTAGTTTGGTAATATCTCCT TAATGCTATATCTTATAGGG	317	Johnson et al., 1991
tst	ATGGCAGCATCAGCTTGATA TTTCCAATAACCACCCGTTT	350	Johnson et al., 1991
luk-PV	ATCATTAGGTAAAATGTCTGGACATGATCC GCATCAATTGTATTGGATAGCAAAAGC	433	Lina et al., 1999
mecA	ATCGATGGTAAAGGTTGG AGTTCTGCAGTACCGGATTTG	533	Murakami et al., 1991

and 100 μL of Tris and EDTA buffer containing 2 mg/mL of lysozyme was added, followed by incubation at 37°C for 10 min. For cell lysis, 350 μL of buffer RA1 and 3.5 μL of β-mercaptoethanol (Sigma, Dorset, UK) were added together to this microtube. The solution was transferred into the RNAspin Mini Filter unit and centrifuged at 11,000 × *g* for 1 min at 4°C. Filters were discarded after centrifugation, and the microtube containing the filtrate had its binding conditions adjusted by the addition of 350 μL of 70% ethanol. This adjusted filtrate was transferred to RNAspin Mini Column and centrifuged at 8,000 × *g* for 30 s at 4°C. For membrane binding, 350 μL of membrane desalting buffer was added and centrifuged at 11,000 × *g* for 1 min at 4°C. Washing of the samples was done in 2 phases. For the first wash, 600 μL of buffer RA3 was added to the column and centrifuged at 11,000 × *g* for 1 min at 4°C. For the second wash, 250 μL of RA3 was added in the column and centrifuged at 11,000 × *g* for 2 min at 4°C. Then, the column was placed into a new 1.5-mL Eppendorf for eluting the RNA, which was performed with 45 μL of H_2O RNA free and 5 μL of RNA guard and centrifuged at 11,000 × *g* for 1 min at 4°C. The DNase treatment for complete removal of possible DNA residues was accomplished by adding 2 μL of buffer, then adding 2 μL of Stop DNase and incubated for 1 h at 37°C. After that, 2 μL of Stop DNase was added, and incubated at 65°C for 10 min for inhibiting the DNase enzyme. The RNA was immediately brought to the temperature of −80°C.

Obtaining cDNA

Samples were prepared at the same time using 2 mixes. Mix 1 was composed of 14 μL of RNA (aliquoted and treated with DNase), 1 μL of random primer, 1 μL of deoxynucleotide triphosphates, and 4 μL of nuclease free water (extraction kit). Mix 2 was composed of 4 μL of 5× first-strand buffer, 1 μL of dithiothreitol (0.1 *M*), and 1 μL of Superscript III (200 U/μL; Invitrogen). Mix 1 was brought to the thermocycler at 65°C for 5 min. It was then removed from the thermocycler and placed immediately on ice for approximately 5 min. After that, mix 2 was added (6 μL) to mix 1 and the sample was placed in the thermocycler again, continuing the program with cycles of 65°C for 5 min, 25°C for 5 min, 50°C for 60 min, 70°C for 15 min, and ending at 20°C. Finally the cDNA was frozen at −80°C.

PCR of cDNA

From the cDNA obtained, the first PCR reaction was performed to check the expression of toxin genes using the primers described in Table 1. Then, electrophoresis

was performed for viewing the amplified products as described in the item above.

Detection of Biofilm Production by the Adherence Method on Polystyrene Plates

The detection method of biofilm production on culture plates proposed by Christensen et al. (1985) was used with modifications proposed by Oliveira and Cunha (2010). This method is based on spectrophotometric features and on the reading of the optical density of the adhesive material produced by the bacterium.

Cultures with 24 h on tryptic soy broth (**TSB**) were used, diluted at the proportion of 1:1 of TSB (Oxoid) supplemented with 2% of glucose. The tests were performed in 96-microwell flat-bottom plates (Costar, model 3599 manufactured by Corning Inc., Corning, NY), in quadruplicate. The wells were filled with 200 μL of each diluted culture. For all tests, we applied the international reference lineages: for *S. aureus* ATCC 29213 (biofilm producer) as positive control and ATCC 33591 (not a producer) as negative control; for CNS *S. epidermidis* ATCC 35983 (biofilm producing) as positive control and *S. epidermidis* ATCC 12228 (not a producer) as negative control. Wells with sterile TSB were also used as negative control. Plates were incubated for 24 h at 37°C. The content of each well was carefully removed using a multichannel pipette and followed by 4 times of washing using 200 μL of PBS, pH 7.2. Plate drying was performed at room temperature for 1 h. Wells were stained for 1 min with 200 μL of crystal violet at 2%. All the volume was removed by aspiration and the excess of dye was washed with distilled water using a multichannel pipette. Plate drying was carried out at room temperature for 60 min and the optical density was read in a 540 nm filter using absorbance microplate reader Multiskan EX (Labsystems, Dickinson, TX). The samples were classified as negative when the cutoff point value of absorbance corresponded to nonadherent controls, and as positive when the cutoff point value corresponded to low adherent or high adherent controls.

Statistics

Statistical analysis was carried out in the software GraphPad Prism (La Jolla, CA) using Fisher's exact test or chi-squared distribution with corrections, when appropriate. Comparison was made between the species, frequencies were determined as well as the measures of association of each one. The results were considered significant when $P < 0.05$ and only significant results have been described in this study.

RESULTS

Identification

Two samples were collected, one of each udder from 231 animals, and only one sample was collected from 11 animals, due to the feasibility of a single udder, for a total of 473 samples. Out of the total of samples analyzed, 112 CNS from 112 animals (one CNS sample per animal) were isolated. Out of the 112 CNS samples, 53 (47.3%) were isolated from animals with subclinical mastitis, whereas the remaining 59 (52.7%) were isolated from healthy animals, considered colonizing agents. Table 2 shows CNS species isolated from milk samples of healthy sheep and animals with subclinical mastitis.

Eleven CNS species were identified, and the most isolated species was *S. simulans* in both animals with subclinical mastitis and in healthy animals; however, *S. haemolyticus* was the only species that showed a significant association between the groups (P-value = 0.02), and it was more present in the group of sick animals. Other species appeared in similar numbers in both groups; however, they did not show statistically significant differences.

Table 2. CNS species isolated from milk samples of healthy sheep and animals with subclinical mastitis

CNS species	Mastitis [no. (%)]	Healthy [no. (%)]	Total [no. (%)]
S. simulans	19 (35.9)	14 (23.7)	33 (29.5)
S. haemolyticus	11 (20.6)	5 (8.4)	16 (14.3)
S. warneri	4 (7.5)	9 (15.2)	13 (11.6)
S. xylosus	5 (9.4)	8 (13.6)	13 (11.6)
S. devriesei	4 (7.5)	8 (13.6)	12 (10.7)
S. epidermidis	1 (1.9)	7 (11.9)	8 (7.1)
S. chromogenes	5 (9.4)	3 (5.1)	8 (7.1)
S. auriculares	3 (5.7)	3 (5.1)	6 (5.4)
S. caprae	—	1 (1.7)	1 (0.9)
S. arlettae	1 (1.9)	—	1 (0.9)
S. hominis	—	1 (1.7)	1 (0.9)
Total	53 (47.3)	59 (52.7)	112 (100)

Antimicrobial Resistance and Detection of the mecA Gene

The 10 drugs tested were effective against most CNS samples in the in vitro antimicrobial sensitivity test. Samples were resistant to penicillin (17.0%), tetracycline (10.7%), clindamycin (6.25%), erythromycin (2.7%), cotrimoxazole (2.7%), gentamycin (0.9%), ciprofloxacin (0.9%), and rifampicin (0.9%). All samples were sensitive to vancomycin and linezolid. Among the resistant samples, 7 (6.25%) were resistant to more than one antimicrobial agent, of which 5 were *S. xylosus*, 1 was *S. epidermidis*, and 1 was *S. haemolyticus*. Table 3 shows the results of antimicrobial sensitivity profile for each species of the 112 CNS samples.

The *mecA* gene was found in 2 *S. sumulans*, 1 *S. epidermidis* and 1 *S. haemolyticus*. The 4 samples were isolated from animals with subclinical mastitis. All samples with the *mecA* gene were resistant to penicillin, but in addition to these drugs, samples of *S. epidermidis* and *S. haemolyticus* showed multidrug resistance, because *S. epidermidis* was also resistant to gentamicin, clindamycin, cotrimoxazole, and rifampin; and *S. haemolyticus* to erythromycin, clindamycin, and cotrimoxazole.

Research on Biofilm Genes by PCR and Biofilm Production on Microplate

Operon *icaADBC* was found in 10 (8.9%) CNS samples, 6 of which were isolated from subclinical mastitis cases (2 for *S. simulans*, 3 for *S. haemolyticus*, and 1 for *S. epidermidis*).

The *bap* gene (biofilm-associated protein) was detected in 16 (14.3%) samples and was most prevalent in *S. xylosus*, with 9 (8.0%) samples. Out of the 16 samples with the gene, 8 (7.1%) were isolated from subclinical mastitis cases (3 for *S. xylosus*, 2 for *S. devriesei*, 2 for *S. simulans*, and 1 for *S. warneri*). The *bhp* gene (*bap* homologous protein) was found in 3 (2.7%) samples, all for *S. epidermidis* isolated from healthy animals. Thus, a total of 29 (25.9%) samples carried a gene for biofilm production. In Table 4, we can see the biofilm genes found in CNS species.

Biofilm production occurred in 69 (61.6%) CNS samples; 33 (29.5%) were isolated from mastitis cases and 36 (32.1%) from healthy animals. Among these biofilm-producing CNS samples, 29 (25.9%) samples suffered from any of the aforementioned genes for biofilm production.

Considering only the isolates from mastitis cases, 62.3% produced biofilm. Likewise, considering only the isolated samples of healthy animals, 61.0% produced biofilm.

Biofilm-producing species were found in descending order: *S. simulans* with 24 (21.4%) samples, followed by 12 (10.7%) *S. haemolyticus*, 8 (7.1%) *S. xylosus*, 8 (7.1%) *S. warneri*, 8 (7.1%) *S. epidermidis*, 5 (4.5%) *S. chromogenes*, and 4 (3.6%) *S. devriesei*. Results of biofilm production by CNS in both animal groups are found in Table 5.

Research on Toxin-Encoding Genes by PCR and Expression by RT-PCR

A survey on toxin genes has shown that out of the 112 CNS samples, 70 (62.5%) had some toxin-encoding gene. Among the genes detected, *sea* was the most prevalent, being present in its isolated form or concomitantly with other toxin gene in 56 (50.0%) samples. The *seb* gene present in 19 samples (17.0%) was the second toxin gene most found, followed by *sec* in 15 samples (13.4%).

The *tst* gene was found in only one sample of the *S. haemolyticus* species. The PVL gene (*luk*-PV) was not found in the samples. Table 6 shows the research results on toxin genes found in 112 CNS samples.

Out of the 70 samples with any enterotoxin-encoding gene, 32 (28.6%) were found in CNS samples isolated from milk samples of animals with subclinical mastitis and 38 (33.9%) of healthy animals. With regard to the assessment of the expression of toxin genes by RT-PCR, none of the samples was positive for the expression of toxin genes found by PCR.

DISCUSSION

The relevance of CNS for the udder health has been studied for many years with regard to its effect of milk quality and quantity, as well as its SCC and potential for causing persistent infectious mastitis. Our study first reports the isolation of the species *S. devriesei* of ovine, found in 10.7% of samples, whereas 4 (7.5%) of isolates were associated with mastitis. This species was first described by Supré et al. (2010) when isolated in 9 samples of the cows' teats and one sample isolated from cow milk.

Other CNS species were found, and the species *S. simulans*, *S. haemolyticus*, *S. warneri*, *S. xylosus*, *S. epidermidis* and *S. chromogenes* were associated with colonization and infection. The species *S. epidermidis*, *S. xylosus*, *S. chromogenes*, and *S. simulans* are the most frequently isolated from mastitis cases in ovines (Bergonier et al., 2003); however, the *S. haemolyticus* species is more frequently isolated in bovines (Onni et al., 2010). Studies have shown differences among the CNS species (Simojoki et al., 2011; Supré et al., 2011),

Table 3. Antimicrobial sensitivity profile (ASP) of CNS isolated from sheep milk for 10 antimicrobial agents[1]

CNS species (no. of samples)	ASP[2]	Tet [no. (%)]	Pen [no. (%)]	Gen [no. (%)]	Eri [no. (%)]	Cip [no. (%)]	Lin [no. (%)]	Clin [no. (%)]	Cotri [no. (%)]	Rif [no. (%)]	Van [no. (%)]
S. simulans (33)	S	31 (94.0)	31 (94.0)	33 (100)	33 (100)	33 (100)	33 (100)	33 (100)	33 (100)	33 (100)	33 (100)
	R	2 (6.0)	2 (6.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
S. haemolyticus (16)	S	15 (93.8)	14 (87.5)	16 (100)	15 (93.8)	16 (100)	16 (100)	11 (68.8)	15 (93.8)	16 (100)	16 (100)
	R	1 (6.2)	2 (12.5)	0 (0.0)	1 (6.2)	0 (0.0)	0 (0.0)	5 (31.2)	1 (6.2)	0 (0.0)	0 (0.0)
S. warneri (13)	S	12 (92)	12 (92.3)	13 (100)	13 (100)	13 (100)	13 (100)	13 (100)	13 (100)	13 (100)	13 (100)
	R	1 (7.7)	1 (7.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
S. xylosus (13)	S	7 (53.8)	6 (46.2)	13 (100)	13 (100)	13 (100)	13 (100)	12 (92.3)	12 (92.3)	13 (100)	13 (100)
	R	6 (46.2)	7 (53.8)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (7.7)	0 (0.0)	0 (0.0)	0 (0.0)
S. devriesei (12)	S	11 (91.7)	12 (100)	12 (100)	12 (100)	11 (91.7)	12 (100)	12 (100)	12 (100)	12 (100)	12 (100)
	R	1 (8.3)	0 (0.0)	0 (0.0)	0 (0.0)	1 (8.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
S. epidermidis (8)	S	7 (87.5)	3 (37.5)	7 (87.5)	8 (100)	8 (100.0)	8 (100.0)	7 (87.5)	6 (75.0)	12 (92.3)	8 (100)
	R	1 (12.5)	5 (62.5)	1 (12.5)	0 (0.0)	0 (0.0)	0 (0.0)	1 (12.5)	2 (25.0)	1 (7.7)	0 (0.0)
S. chromogenes (8)	S	8 (100)	7 (87.5)	8 (100.0)	7 (87.5)	8 (100)	8 (100)	8 (100)	8 (100)	8 (100)	8 (100)
	R	0 (0.0)	1 (12.5)	0 (0.0)	1 (12.5)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
S. auriculares (6)	S	6 (100.0)	6 (100.0)	6 (100.0)	6 (100.0)	6 (100.0)	6 (100.0)	6 (100.0)	6 (100.0)	6 (100.0)	6 (100.0)
	R	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
S. caprae (1)	S	1 (100.0)	1 (100.0)	1 (100.0)	1 (100.0)	1 (100.0)	1 (100.0)	1 (100.0)	1 (100.0)	1 (100.0)	1 (100.0)
	R	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
S. arlettae (1)	S	1 (100.0)	1 (100.0)	1 (100.0)	1 (100.0)	1 (100.0)	1 (100.0)	1 (100.0)	1 (100.0)	1 (100.0)	1 (100.0)
	R	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
S. hominis (1)	S	1 (100.0)	0 (0.0)	1 (100.0)	0 (0.0)	1 (100.0)	1 (100.0)	1 (100.0)	1 (100.0)	1 (100.0)	1 (100.0)
	R	0 (0.0)	1 (100)	0 (0.0)	1 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)

[1]Tet = tetracycline; Pen = penicillin; Gen = gentamicin; Eri = erythromycin, Cip = ciprofloxacin; Lin = linezolid; Clin = clindamycin; Cotri = cotrimoxazole; Rif = rifampicin; Van = vancomycin.

[2]S = sensitive; R = resistant.

Table 4. Biofilm genes found in CNS species isolated from sheep milk

CNS species	Total samples [no. (%)]	*icaADBC* [no. (%)]	*bap* [no. (%)]	*bhp* [no. (%)]
S. simulans	33 (29.5)	3 (2.7)	2 (1.8)	—
S. haemolyticus	16 (14.3)	3 (2.7)	—	—
S. warneri	13 (11.6)	1 (0.9)	1 (0.9)	—
S. xylosus	13 (11.6)	1 (0.9)	9 (8.0)	—
S. devriesei	12 (10.7)	—	3 (2.7)	—
S. epidermidis	8 (7.1)	2 (1.8)	—	3 (2.7)
S. chromogenes	8 (7.1)	—	1 (0.9)	—
S. auriculares	6 (5.4)	—	—	—
S. caprae	1 (0.9)	—	—	—
S. arlettae	1 (0.9)	—	—	—
S. hominis	1 (0.9)	—	—	—
Total	112 (100)	10 (8.9)	16 (14.3)	3 (2.7)

with *S. chromogenes*, *S. simulans*, and *S. xylosus* causing a statistically significant increase in the elevation of CCS, like *S. aureus* (Supré et al., 2011), suggesting that these species are more relevant for udder health than others.

In this study, *S. chromogenes*, *S. simulans*, and *S. xylosus* were isolated from mastitis cases, with high SCC, and healthy animals. However, although *S. simulans* has been the most isolated species, *S. haemolyticus*, the species described as unusual in ovines (Onni et al., 2010), was the most isolated species in mastitis cases, and for this reason, it was significantly associated with the disease. *Staphylococcus haemolyticus* seems to be a versatile microorganism that can occupy different habitats. In an ecological context, *S. haemolyticus* seems to inhabit several habitats (animal, human, and environmental), and in the epidemiological context, *S. haemolyticus* seems to act as an opportunistic pathogen (Bexiga et al., 2014). The ecology of *S. simulans* and the main sources of intramammary infection are still unclear (Vanderhaeghen et al., 2015). The infection caused by *S. simulans* may be linked to environmental reservoirs (Piessens et al., 2012), but some accounts say that *S. simulans* can cause contagious mastitis (Taponen et al., 2008).

Staphylococcus chromogenes seems to be a commonly found species in mastitis cases, and the infectious mastitis is usually caused by the opportunistic species. *Staphylococcus xylosus* also seems to be a versatile organism, but little is known about its epidemiology in mastitis (Vanderhaeghen et al., 2015).

The species *S. warneri* was also found in this study, but more associated with colonization (15.2%). This species has been described as a minor pathogen of IMI (Gillespie et al., 2009; Waller et al., 2011), and according to Mørk et al. (2012), the species cannot survive in the udder for a long period of time and, therefore, causes no persistent mastitis.

Eleven CNS species were identified in this study, and accurate studies with phenotypic identification have shown that approximately 25 species of CNS cause mastitis (Park et al., 2011; Piessens et al., 2011; Supré et al., 2011). The CNS is a heterogeneous group of microorganisms, composed of a great diversity of species,

Table 5. Biofilm production by the microplate method for the 112 CNS samples isolated from healthy sheep and animals with subclinical mastitis[1]

	Sheep with mastitis (53 samples)		Healthy sheep (59 samples)	
CNS species	B+ [no. (%)]	B– [no. (%)]	B+ [no. (%)]	B– [no. (%)]
S. simulans	13 (24.5)	6 (11.3)	11 (18.6)	3 (5.1)
S. haemolyticus	9 (17.0)	2 (3.8)	3 (5.1)	2 (3.4)
S. warneri	3 (5.7)	1 (1.9)	5 (8.5)	4 (6.8)
S. xylosus	2 (3.8)	3 (5.7)	6 (10.2)	2 (3.4)
S. devriesei	1 (1.9)	3 (5.7)	3 (5.1)	5 (8.5)
S. epidermidis	1 (1.9)	0 (0.0)	7 (11.9)	0 (0.0)
S. chromogenes	4 (7.5)	1 (1.9)	1 (1.7)	2 (3.4)
S. auriculares	0 (0.0)	3 (5.7)	0 (0.0)	3 (5.1)
S. caprae	—	—	0 (0.0)	1 (1.7)
S. arlettae	0 (0.0)	1 (1.9)	—	—
S. hominis	—	—	0 (0.0)	1 (1.7)
Total	33 (62.3)	20 (37.7)	36 (61.0)	23 (39.0)

[1]B+ = positive for biofilm production; B− = negative for biofilm production.

Table 6. Toxin genes found in CNS isolated from sheep milk

CNS species	No. of samples	No. of samples with genes	Toxin gene[1]					
			sea	*seb*	*sec*	*tst*	*sea*+*seb*	*sea*+*sec*
S. simulans	33	24	11	1	5	—	2	5
S. haemolyticus	16	10	5	2		1	2	—
S. warneri	13	7	3	—	1	—	3	—
S. xylosus	13	8	6	1	—	—	1	—
S. devriesei	12	6	5	—	—	—	—	1
S. epidermidis	8	3	3	—	—	—	—	—
S. chromogenes	8	4	—	—	1	—	1	2
S. auriculares	6	6	1	2	—	—	3	—
S. caprae	1	1	—	—	—	—	1	—
S. arlettae	1	1	1	—	—	—	—	—
S. hominis	1	0	—	—	—	—	—	—
Total	112	70	35	6	7	1	13	8

[1]*sea*, *seb*, *sec*, *tst* = genes of toxins A, B, C, and toxic shock syndrome toxin (TSST-1).

and each one of them has its own characteristics, which may differ in virulence characteristics; some species are more virulent (Taponen and Pyörälä, 2009) or epidemiologic than others in the increase (Sampimon et al., 2009) or in the persistence (Taponen et al., 2006) of SCC. Thus, the correct identification of CNS species is essential for epidemiological investigations as well as for mastitis control.

Different commercial kits based on specific biochemical reactions have been developed for identifying CNS species; however, such methods based on phenotypic reactions often fail at identifying CNS species (Heikens et al., 2005). Thereby, new identification methods based on molecular biology are being continuously tested and developed (Couto et al., 2001; Skow et al., 2005). Several genetic targets are being used for molecular identification of CNS species, including the 16S rRNA genes (Heikens et al., 2005), ITS-PCR (Couto et al., 2001), the software *gap* (Yugueros et al., 2000), *hsp*60 (Kwok et al., 1999), *sod*A (Poyart et al., 2001), *fem*A (Vannuffel et al., 1999), and *rpoB* (Mellmann et al., 2006). In this study, 2 genotypic methods were used for identification. The ITS-PCR has proven to be appropriate for identifying the CNS species in ovines; however, because the standard strains (ATCC) of all species needed to be identified, it was necessary to sequence the *rpoB* gene to identify the species *S. devriesei*, *S. chromogenes*, *S. auriculares*, and *S. arlettae* because of the lack of standard strains for identification with ITS-PCR.

The intramammary therapy aimed at curing sheep with mastitis has been carried out with different antimicrobials (Naccari et al., 2003). Among the drugs approved for use in the mastitis treatment, β-lactams such as penicillin and cephalosporin (Pyörälä and Taponen, 2009) stand out. In this study, most CNS samples were sensitive to the tested antimicrobial agents, whereas penicillin (17.0%) and tetracycline (10.7%) showed the highest resistance percentages. Resistance to penicillin was also reported by other authors (Lollai et al., 2008; Kunz et al., 2011).

This resistance seems to be more common in CNS than in *S. aureus*; in this study, the *mecA* gene was found in 4 (3.6%) CNS samples, all of which were resistant to penicillin. Onni et al. (2011) analyzed the resistance profile of samples of *S. epidermidis* isolated from sheep mastitis and found 2 positive samples for the *mecA* gene, and these samples were resistant to 5 of the 13 drugs tested. In this study, the positive sample of *S. epidermidis* and *S. haemolyticus mecA* also showed resistance to 6 and 5 drugs, respectively.

Another important factor that can enable maintenance of CNS in host agents is the ability to form biofilm. In staphylococci, a major contributor to the production of biofilms is the polysaccharide called polysaccharide of intercellular adhesion (**PIA**), which has its synthesis controlled by the product of the *ica* gene (intercellular adhesion) corresponding to 4 genes (*icaA*, *icaD*, *icaB*, and *icaC*) that are organized in an operon (Arciola et al., 2001; Götz, 2002). In this paper, operon *icaADBC* was found in 10 samples; however, in addition to the polysaccharide PIA, proteins are also involved in the production of biofilms, such as the Bap and Bhp proteins.

The results of this study show that biofilm from a protein source is much more important in biofilm formation in CNS isolated from sheep milk than PIA, and it was found in 17% of samples (14.3% *bap* and 2.7% *bhp*), whereas operon *ica* was found in 8.9% of CNS. The study by Piessens et al. (2012) confirms these results, because when analyzing CNS samples isolated from environmental and cow mastitis, *bap* was found in 11.2% of samples, whereas the *icaA* gene was found in only 5.2%. In a study by Zuniga et al. (2015) with the purpose to assess the presence of adhesin genes and

bap genes in coagulase-positive staphylococci and CNS, the *bap* gene was found more frequently in CNS than in coagulase-positive staphylococci.

It is, therefore, important to notice that biofilm production occurred in more than half of CNS samples (61.6%), but only 25.0% carried the surveyed genes (operon *ica*, *bap*, or *bhp*), suggesting that other mechanisms, besides these ones, are involved in biofilm formation in CNS (Cucarella et al., 2004; Fredheim et al., 2009). Other proteins can act on the initial phase of biofilm formation and in the accumulation phase, working as intercellular adhesin: *embp*, encoding the extracellular matrix binding protein (Christner et al., 2010); *spa*, encoding protein A (Merino et al., 2009); *fnbA* and *fnbB*, encoding fibrinogen-binding proteins A and B, respectively (O'Neill et al., 2008); *sasG*, encoding surface protein G (Corrigan et al., 2007); *atlE*, encoding the bifunctional adhesin and autolysin AtlE (Heilmann et al., 1996); and some others (Otto, 2013). Many others genes are associated with biofilm formation, but their function and involvement in the process are not yet completely elucidated (Stevens et al., 2009).

As for the toxigenic potential of CNS, the pathogenic role of CNS and its enterotoxin in the development of food poisoning has not been well established yet. Recent studies have provided strong evidence for the presence and location of enterotoxins coding elements in CNS genome and enterotoxin production (Podkowik et al., 2013). In this paper, we found genes for toxins in 62.5% of CNS samples, which were found in samples isolated from milk of both sick animals and healthy animals. However, despite the presence of genes, no sample has presented such toxin genes by RT-PCR.

Furthermore, although the expression of toxin genes in CNS sample has not been observed in the CNS samples of this study, other authors have already reported the expression of toxin genes by CNS (Valle et al., 1990; Orden et al., 1992; Zell et al., 2008), which reinforces the importance of studying the toxigenic potential in this group.

The PVL toxin gene was not found in the CNS samples. The occurrence of CNS species carrying the *luk-PV* gene is unclear because it is little investigated in samples of both human and animal origin.

CONCLUSIONS

Several CNS species were isolated from both sick sheep and healthy sheep, and a low percentage of samples were resistant to the antibiotics tested. Also, the samples did not produce toxins, despite the large presence of genes in isolates; however, the biofilm was the main virulence factor found, because over half of the samples were producing biofilm, including samples isolated from healthy animals. Knowledge of CNS at the species level and the resistance profile to antimicrobials, in addition to virulence factors, particularly the ability to produce biofilm that is directly linked to bacteria maintenance and invasiveness in the mammary gland, are essential for the control and prevention of sheep mastitis caused by CNS.

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