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Relationship between virulence factor genes in coagulase-negative *Staphylococcus* spp. and failure of antimicrobial treatment of subclinical mastitis in sheep[1]

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ABSTRACT.- Zafalon L.F., Cunha M.L.R.S., Brandão H.M., Mosqueira V.C.F., Santana R.C.M., Barioni Júnior W., Martins K.B. & Pilon L.E. 2018. **Relationship between virulence factor genes in coagulase-negative *Staphylococcus* spp. and failure of antimicrobial treatment of subclinical mastitis in sheep**. *Pesquisa Veterinária Brasileira 38(4):579-585.* Embrapa Pecuária Sudeste, Rodovia Washington Luiz Km 234, Cx. Postal 339, São Carlos, SP 13560-970, Brazil. E-mail: luiz.zafalon@embrapa.br

Coagulase-negative *Staphylococcus* spp. (CNS) are the main microorganisms involved in ovine mastitis. Treatment at the end of lactation can contribute towards cure and prevention of subclinical cases during the subsequent lactation. However, virulence factors and resistance mechanisms presented by CNS can decrease cure rates. The aims of the study were to identify the species of CNS in milk of mastitic ewes with and without antimicrobial treatment, and to investigate the presence of genes relating to resistance of β-lactam antimicrobials, formation of biofilms, production of enterotoxins and production of the toxic shock syndrome toxin. Cases of failure in the treatment were related with the presence/absence of the respective genes. Sixty sheep were divided into three groups: G1, without treatment; G2, animals treated via the intramammary route with 100mg of cloxacillin during drying off; and G3, sheep treated via the intramammary route with 50 mg of nanoparticulate cloxacillin. Milk samples were gathered during drying off and 15 and 30 days after the parturition of the subsequent lactation. The analyses to identify the species of CNS were carried out by means of the internal transcribe spacer technique and the investigation of the genes responsible for the virulence factors and resistance to oxacillin was performed using the polymerase chain reaction (PCR) technique. No sample was positive for the *mec*A gene. The only gene relating to production of enterotoxins was *sec*. Among the genes relating to production of biofilm, *ica*D was the only one identified in the three experimental groups. *Staphylococcus warneri* was the main species of CNS isolated during the pre and post-partum periods of the sheep. The species carrying genes relating to production of enterotoxins and biofilms were present in uncured sheep.

INDEX TERMS: Virulence factor genes, coagulase-negative, *Staphylococcus* spp., antimicrobial treatment, mastitis, sheep, biofilms, enterotoxins, bacterioses.

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RESUMO.- [Relação entre genes de fatores de virulência em *Staphylococcus* spp. coagulase-negativos e a falha do tratamento antimicrobiano da mastite subclínica ovina.] *Staphylococus* spp. coagulase-negativos (SCN) estão entre os principais micro-organismos envolvidos na mastite ovina. O tratamento ao final da lactação pode contribuir com a cura e a prevenção de casos subclínicos durante a lactação seguinte. Todavia, fatores de virulência e mecanismos de resistência apresentados por SCN podem reduzir as taxas de cura. Os objetivos desse estudo foram identificar as espécies de SCN no leite de ovelhas com mastite com e sem tratamento antimicrobiano e investigar a presença de genes relacionados com resistência a antibióticos beta lactâmicos, formação de biofilmes, produção de enterotoxinas e produção da toxina da síndrome do choque tóxico. Casos de falhas no tratamento foram relacionados com a presença/ausência dos respectivos genes. Sessenta ovelhas foram divididas em três grupos: G1, sem tratamento; G2, animais tratados via intramamária com 100mg de cloxacilina antes da secagem; e G3, ovelhas tratadas via intramamária com 50 mg de cloxacilina nanoparticulada. Amostras de leite foram obtidas durante a secagem e 15 e 30 dias depois do parto na lactação seguinte. As análises para identificar as espécies de SCN foram conduzidas por meio da técnica de *Internal transcribe spacer* e a investigação dos genes responsáveis pelos fatores de virulência e resistência à oxacilina foi realizada usando a técnica reação em cadeia da polimerase. Nenhuma amostra foi positiva para o gene *mecA*. O único gene relacionado com a produção de enterotoxinas foi o *sec*. Dentre os genes relacionados com a produção de biofilme, *icaD* foi o único identificado nos três grupos experimentais. *Staphylococcus warneri* foi a principal espécie de SCN isolada durante o pré e pós-parto. As espécies que apresentaram genes relacionados com a produção de enterotoxinas e biofilmes estavam presentes nas ovelhas não curadas.

TERMOS DE INDEXAÇÃO: Genes, fatores de virulência, *Staphylococcus* spp., coagulase-negativos, tratamento antimicrobiano, mastite subclínica, ovinos, biofilmes, enterotoxinas, bacterioses.

INTRODUCTION

Mastitis is among the main sanitary problems in ovine breeding. The disease can be classified as clinical or subclinical. Unlike the clinical form, subclinical mastitis does not present macroscopic alterations of the mammary gland and is strongly related to financial losses for producers (McDougall et al. 2001). Its etiology is quite broad, although the pathogens of greatest occurrence are *Staphylococcus* spp., especially coagulase-negative *Staphylococcus* spp. (CNS) (Bolsanello et al. 2009).

Intramammary therapy to control mastitis in sheep can be performed using different antimicrobials. However, excessive and indiscriminate use of antimicrobials increases the resistance of the microorganisms. The appearance of CNS that is resistant to oxacillin is determined by alteration of the enzyme targeted by β-lactam antibiotics, encoded by the *mec*A gene (Diekema et al. 2001). Virulence factors also favor maintenance of CNS in hosts, and these factors include production of biofilm, encoded through the genes *ica*A, *ica*C, *ica*D, *bap* and *bhp*, and production of enterotoxins and the toxin responsible for toxic shock syndrome, encoded by the genes *sea*, *seb*, *sec*, *sed* and *tsst-1* (Balaban & Rasooly 2001).

The aims of the present study were to identify the species of CNS in milk of mastitic ewes with and without antimicrobial treatment, and to investigate the presence of genes relating to resistance of β-lactam antimicrobials, formation of biofilms, production of enterotoxins and production of the toxic shock syndrome toxin. Cases of failure in the treatment were related with the presence/absence of the respective genes.

MATERIALS AND METHODS

The study was conducted using an experimental herd of 60 sheep of the Santa Inês and Morada Nova breeds, located in São Carlos, São Paulo, Brazil. The animals underwent a general clinical examination and a specific examination of their mammary glands, following the routine of the farm. The sheep selected for inclusion in the experimental groups did not present any diseases or signs of clinical mastitis.

Milk samples were gathered approximately 15 days before weaning and on the 15th and 30th day after parturition of the subsequent lactation. The mammary glands that presented somatic cell count (SCC) >$2.5x10^5$ cells/mL of milk (Pengov 2001) and microbiological isolation, were considered to be positive for subclinical mastitis. A case of mastitis was defined when one or more colonies equal (the same morphology and size, pigmentation and type of hemolysis), until two different types were identified in the two samples (Harmon et al. 1990). Milk samples were collected for SCC after storage in plastic containers containing bronopol conservative, and were sent to a reference laboratory within the Brazilian Milk Quality Network, where counting procedures were performed using an electronic device (Somacount 300; Bentley Instruments®).

Milk aliquots of 100µL were plating on blood agar with 5% defibrinated ovine blood and then incubated at 37°C for up to 72 hours, with readings every 24 hours. Genotype identification of CNS species was performed using conserved sequence primers for the genes 16S and 23S through the internal transcribed spacer polymerase chain reaction technique (ITS-PCR): *G1* "GAAGTCGTAACAAGG" 16S and *L1* "CAAGGCATCCACCGT" 23S (Couto et al. 2001).

The presence of genes *mec*A (Murakami et al. 1991), *ica*A, *ica*C, *ica*D, *bap* and *bhp* (Cucarella et al. 2004, Arciola et al. 2001, Qin et al. 2007), *sea*, *seb*, *sec*, *sed* and *tsst-1* (Johnson et al. 1991, Cunha et al. 2004) were assessed (Table 1).

Antimicrobial treatments were carried out before weaning, with two formulations of cloxacillin in a single dose: one in an oil-based formulation (100mg) and the other using a nanoparticulate structure (50mg), in water vehicle. The animals were treated after *in vitro* confirmation of susceptibility of the microorganisms towards the active ingredient. The sheep were randomly distributed into three experimental groups, while maintaining homogeneity of the groups according to weight, age and parity: G1 (n=21), composed of sheep with subclinical mastitis without intramammary antimicrobial treatment; G2 (n=19), sheep with subclinical mastitis treated with 100mg of intramammary cloxacillin–benzathine; and G3 (n=20), sheep with subclinical mastitis treated with 50 mg of nanoparticulate intramammary cloxacillin-benzathine, in accordance with the methodology described in patent WO2011150481A1 (Mosqueira et al. 2011).

Before the treatments, the hands of the person responsible for gathering the samples and the ostia of the animals' teats underwent antisepsis with 70% isopropyl alcohol. The intramammary infusions were performed using a 20-gauge intravenous catheter (1.1mm in caliber x 48mm in length). Mammary halves were considered cured when there was complete absence of microorganism isolation during the subsequent lactation. On the other hand, mammary halves were classified as "not cured" when the same pathogen identified before treatment was again isolated, or in cases of reinfection. The cure

was evaluated in combination with the SCC results, as previously mentioned.

The frequency distributions of the cases of subclinical mastitis were compared using the chi-square test. Significant values that were close to those of the significance reference were adjusted in accordance with Yates's correction for continuity (p=0.05) (Sampaio 1998). The multiple correspondence analysis was carried out to determine the relationship between the classes of variables. It was also considered in this analysis the experimental three groups.

The experiment was approved by the Embrapa Southeast Livestock Ethics Committee on the Experiments with animals and register under the number PRT 04/2015.

RESULTS AND DISCUSSION

Table 2 shows the distribution of animals into the different experimental groups before weaning, i.e. before treatment, and also 15 and 30 days after the parturition of the subsequent lactation. All the animals presented subclinical mastitis in one of the mammary halves, while the other was healthy.

Three microorganisms isolated in the ewes before the treatment in G2 were not recovered after storage under frozen conditions, which made it impossible to conduct analyses later on, to identify the species. As shown in Table 2, 15 days after parturition, the ewes of G3 treated with a half of antibiotic dose presented greater occurrence of cure than ewes treated with 100mg of cloxacillin-benzathine (G3) and then the untreated sheep (G1) (p=0.0192). This finding was probably due to the efficiency of the nanoparticulate antimicrobial administered during the drying off, among the sheep with subclinical mastitis. The nanoparticulate system used in this experiment can deliver the antibiotic to polymorph nuclear cell compartment (Mosqueira et al. 2011). In addition, Brownian motion influence the small particle diffusivity (Uma et al. 2011), that favored the mammary biodistribution of the nonoestructured cloxacillin when compared to oil-based cloxacillin.

Table 3 shows the species of CNS isolated before weaning the distributed among the three experimental groups. The species of greatest occurrence in the milk of these sheep with subclinical mastitis before drying-off treatment were in agreement with those isolated by other authors, also from subclinical cases (Martins 2013, Pilon et al. 2014). Among the agents involved in the infectious etiology of ovine subclinical mastitis, CNS is the most prevalent agent and for this reason was chosen for investigation in the present study. Veríssimo et al. (2010)

Table 1. Primers used in detection of genes relating to resistance to oxacillin and to encoding for biofilms, enterotoxins and the toxin responsible for toxic shock syndrome, in strains of *Staphylococcus* spp. isolated from ovine milk

Gene	Nucleotide sequence (5'-3')	Amplified product (bp)
*mec*A	AAAATC GAT GGT AAA GGT TGG	533
	AGT TCT GCA GTA CCG GAT TTG	
*ica*A	ACA GTC GCT ACG AAA AGA AA	103
	GGA AAT GCC ATA ATG AGA AC	
*ica*C	TAA CTT TAG GCG CAT ATG TTT	400
	TTC CAG TTA GGC TGG TAT TG	
*ica*D	ATG GTC AAG CCC AGA CAG AG	198
	CGT GTT TTC AAC ATT TAA TGCAA	
bap	CCCTATATCGAAGGTGTAGAATTGCAC	971
	GCTGTTGAAGTTAATACTGTACCTGC	
bhp	ATGAAAAATAAACAAGGATTTC	1300
	GCCTAAGCTAGATAATGTTTG	
sea	TTG GAA ACG GTT AAA ACG AA	120
	GAA CCT TCC CAT CAA AAA CA	
seb	TCG CAT CAA ACT GAC AAA CG	478
	GCA GGT ACT CTA TAA GTG CC	
sec	GAC ATA AAA GCT AGG AAT TT	257
	AAA TCG GAT TAA CAT TAT CC	
sed	CTA GTT TGG TAA TAT CTC CT	317
	TAA TGC TAT ATC TTA TAG GG	
tsst-1	ATG GCA GCA TCA GCT TGA TA	350
	TTT CCA ATA ACC ACC CGT TT	

Table 2. Distribution of sheep with subclinical mastitis into different groups according to whether treatment was implemented, before weaning and 15 and 30 days after parturition

Periods	Groups					
	G1		G2		G3	
	N	%	N	%	N	%
Before weaning	21	40.4	19	44.2	20	57.1
15 days post-partum	16[a]	30.8	13[a]	30.2	7[b]	20.0
30 days post-partum	15[a]	28.8	11[a]	25.6	8[a]	22.9
TOTAL	52	100.0	43	100.0	35	100.0

G1 = Sheep that did not receive intramammary antimicrobial, G2 = sheep that were treated with conventional antimicrobial (100mg of cloxacillin–benzathine), and G3 = sheep that were treated with nanoencapsulated antimicrobial (50mg of cloxacillin-benzathine). Values with different letters in the same line: p<0.05.

Table 3. Coagulase-negative *Staphylococcus* spp. isolated before weaning of sheep with subclinical mastitis

Species	Experimental groups					
	G1		G2		G3	
	N	%	N	%	N	%
S. warneri	8	38.1	2	12.5	5	25.0
S. simulans	5	23.8	5	31.3	4	20.0
S. xylosus	4	19.0	2	12.5	2	10.0
S. epidermidis	2	9.5	3	18.8	4	20.0
S. lentus	1	4.8	-	-	-	-
S. caprae	1	4.8	-	-	-	-
S. haemolyticus	-	-	2	12.5	-	-
S. hominis	-	-	-	-	1	5.0
S. cohnii	-	-	-	-	1	5.0
S. capitis	-	-	-	-	1	5.0
S. chromogenes	-	-	2	12.5	1	5.0
S. auricularis	-	-	-	-	1	5.0
TOTAL	21	100.0	16	100.0	20	100.0

G1 = Control group, composed of sheep that did not receive intramammary antimicrobials, G2 = sheep that were treated with conventional antimicrobials (100mg of cloxacillin–benzathine), and G3 = sheep that were treated with nanoencapsulated antimicrobials (50mg of cloxacillin-benzathine).

reported a CNS occurrence rate in milk samples from Santa Inês sheep of 64.3%. A similar rate was also reported by other authors (Pereira et al. 2014, Santana et al. 2013). CNS can also be isolated from animals with clinical mastitis (Lucheis et al. 2010). These microorganisms are considered to be colonizers of the skin of the teat. Thus, any deficiency in managing these animals can facilitate entry of these microorganisms into the mammary gland (Laffranchi et al. 2001). Before weaning, *S. warneri* was the most prevalent species in G1 and G3. In G2, *Staphylococcus simulans* presented greater occurrence, followed by *S. epidermidis.*

Table 4 shows the species of CNS identified in the sheep 15 and 30 days after parturition. *S. warneri* remained as the species with greatest occurrence in G1 and G3, and in the latter, even after the treatment at the end of the previous lactation. In G2, 15 days after parturition, *S. simulans* was the species with greatest occurrence, as it also was before treatment.

Table 5 presents the distribution of the *sec* gene in CNS species before treatment, and also 15 and 30 days after parturition. Among the enterotoxins genes investigated, this was the only one identified. There are no reports in the literature correlating the failure in the sheep mastitis treatment with CNS strains and genes responsible for production of enterotoxins or the toxin responsible for toxic shock syndrome. On the other hand, a correlation has been made with public health risks due to consumption of milk contaminated with microorganisms that produce enterotoxins and the toxin responsible for toxic shock syndrome (Mariano et al. 2007, Nader Filho et al. 2007, Ferreira et al. 2014).

None of the microorganisms isolated presented the *mec*A gene, which relates to resistance to oxacillin, and this was in agreement with the findings of other authors (França et al. 2012, Silva 2012, Martins 2013). Other oxacillin resistance mechanisms that are unrelated to *mec*A expression may be present (Zafalon et al. 2012), but were not investigated in the present study. The presence of homologous *mec*A genes or other classes of penicillin-binding proteins may be related to oxacillin resistance mechanisms (Mendonça et al. 2012). In goats, microorganisms isolated from animals carrying clinical mastitis presented greater virulence capacity than microorganisms isolated from subclinical mastitis (Bezek & Hull 1995). So, the lack of identification of the remaining genes relating to production of enterotoxins may have been because the samples were from sheep with subclinical and not clinical mastitis.

The distributions of the genes *ica*C, *ica*D and *bap* among CNS species identified during weaning and the genes *ica*A, *ica*C and *ica*D after parturition are presented in the Tables 6 and 7, respectively. All the species of CNS studied were negative for the genes *ica*A and *bhp* immediately before the drying-off period. However, the gene icaC was present in CNS specimens isolated from sheep from G1 and G3, while the genes *ica*D and *bap* were found in CNS isolated from sheep distributed in all three groups. CNS identified 15 and 30 days after the parturition of the subsequent lactation did not present the *bap* gene.

Biofilm production can contribute towards maintaining CNS within the mammary gland. It can be encoded by the genes *ica*A, *ica*C, *ica*D, *bap* and *bhp*. The genes of the *ica* operon are responsible for synthesizing the production of biofilm. Strains of CNS carrying *ica* genes can produce biofilms, which may be related to the difficulty of attaining a cure for the mammary gland after treatment (Arciola et al. 2001). Even with inactivation or absence of the *ica* operon,

Table 4. Coagulase-negative *Staphylococcus* spp. isolated from treated and untreated sheep, 15 and 30 days after parturition and lactation subsequent to treatment

Species	Experimental groups											
	G1				G2				G3			
	15 days post-partum		30 days post-partum		15 days post-partum		30 days post-partum		15 days post-partum		30 days post-partum	
	N	%	N	%	N	%	N	%	N	%	N	%
S. warneri	4	25.0	6	40.0	1	7.7	2	18.2	2	28.6	4	50.0
S. xylosus	3	18.8	1	6.7	-	-	1	9.1	1	14.3	-	-
S. epidermidis	2	12.5	1	6.7	2	15.4	-	-	1	14.3	-	-
S. cohnii	1	6.3	1	6.7	1	7.7	2	18.2	-	-	1	12.5
S. haemolyticus	2	12.5	1	6.7	-	-	-	-	-	-	-	-
S. simulans	1	6.3	2	13.0	4	30.8	1	9.1	-	-	1	12.5
S. hominis	1	6.3	2	13.0	-	-	-	-	1	14.3	-	-
S. saprophyticus	1	6.3	-	-	-	-	-	-	1	14.3	-	-
S. capitis	1	6.3	1	6.7	1	7.7	2	18.2	-	-	-	-
S. lentus	-	-	-	-	1	7.7	1	9.1	-	-	-	-
S. caprae	-	-	-	-	1	7.7	1	9.1	-	-	-	-
S. schleiferi schleiferi	-	-	-	-	1	7.7	-	-	1	14.3	-	-
S. sciuri sciuri	-	-	-	-	1	7.7	-	-	-	-	1	12.5
S. chromogenes	-	-	-	-	-	-	1	9.1	-	-	1	12.5
TOTAL	16	100	15	100	13	100	11	100	7	100	8	100

G1 = Control group, composed of sheep that did not receive intramammary antimicrobials, G2 = sheep that were treated with conventional antimicrobials (100mg of cloxacillin–benzathine), and G3 = sheep that were treated with nanoencapsulated antimicrobials (50mg of cloxacillin-benzathine).

Table 5. Distribution of the *sec* gene in coagulase-negative *Staphylococcus* identified during weaning and after treatment at 15 and 30 days after parturition and subsequent lactation

Species	Experimental groups					
	G1		G2		G3	
	N	%	N	%	N	%
			Pretreatment			
S. warneri	6	60.0	-	-	1	33.3
S. simulans	3	30.0	1	50.0	-	-
S. xylosus	1	10.0	-	-	1	33.3
S. epidermidis	-	-	1	50.0	-	-
S. chromogenes	-	-	-	-	1	33.3
			15 days post-partum			
S. warneri	2	100.0	-	-	1	50.0
S. simulans	-	-	1	100.0	-	-
S. epidermidis	-	-	-	-	1	50.0
			30 days post-partum			
S. warneri	2	50.0	1	25.0	4	57.1
S. epidermidis	1	25.0	-	-	1	14.3
S. simulans	1	25.0	-	-	1	14.3
S. cohnii	-	-	1	25.0	-	-
S. lentus	-	-	1	25.0	-	-
S. xylosus	-	-	1	25.0	-	-
S. sciuri sciuri	-	-	-	-	1	14.3

G1 = Control group, composed of sheep that did not receive intramammary antimicrobials, G2 = sheep that were treated with conventional antimicrobials (100mg of cloxacillin–benzathine), and G3 = sheep that were treated with nanoencapsulated antimicrobials (50mg of cloxacillin-benzathine).

Table 6. Distributions of the genes *ica*C, *ica*D and *bap* among coagulase-negative *Staphylococcus* spp. identified in sheep with subclinical mastitis immediately before the drying-off period

Species	*ica*C		*ica*D		*bap*	
	N	%	N	%	N	%
			G1			
S. epidermidis	1	33.3	2	33.3	-	-
S. xylosus	1	33.3	-	-	1	100.0
S. simulans	1	33.3	1	17.0	-	-
S. warneri	-	-	3	50.0	-	-
			G2			
S. epidermidis	-	-	-	-	-	-
S. xylosus	-	-	-	-	1	100.0
S. simulans	-	-	1	50.0	-	-
S. warneri	-	-	1	50.0	-	-
			G3			
S. epidermidis	1	33.3	-	-	1	100.0
S. xylosus	-	-	-	-	-	-
S. simulans	2	67.0	3	37.5	-	-
S. warneri	-	-	4	50.0	-	-
S. chromogenes	-	-	1	12.5	-	-

G1 = Control group, composed of sheep that did not receive intramammary antimicrobials, G2 = sheep that were treated with conventional antimicrobials (100mg of cloxacillin–benzathine), and G3 = sheep that were treated with nanoencapsulated antimicrobials (50mg of cloxacillin-benzathine). *ica*C, *ica*D and *bap* = genes responsible for biofilm formation.

strains of *bap*-positive *S. aureus* continued to present *in vitro* biofilm synthesis (Cucarella et al. 2004), thereby becoming 10 to 1,000 times more resistant to antimicrobials than free cells (Amorena et al. 1999). Genes *ica*C and *ica*D were found in a greater number of species before the drying-off period. There are reports that 60.0% of the CNS carried the *ica*D gene in milk samples from sheep with subclinical mastitis (Ergun et al. 2012). The presence of the *ica*A gene reported in the present study differed from the results found by other author (Martins 2013).

Table 7. Distribution of the genes *ica*A, *ica*C and *ica*D in species of coagulase-negative *Staphylococcus* identified 15 and 30 days after parturition in sheep belonging to the three experimental groups

Species	*icaA*				*icaC*				*icaD*			
	15 days post-partum		30 days post-partum		15 days post-partum		30 days post-partum		15 days post-partum		30 days post-partum	
	N	%	N	%	N	%	N	%	N	%	N	%
						G1						
S. haemolyticus	1	50.0	1	50.0	2	28.6	1	11.1	2	25.0	1	7.7
S. xylosus	1	50.0	-	-	3	42.9	1	11.1	3	38.0	1	7.7
S. hominis	-	-	-	-	1	14.3			1	13.0	1	7.7
S. warneri	-	-	-	-	1	14.3	4	44.4	1	13.0	6	46.0
S. epidermidis	-	-	-	-	-	-	1	11.1	1	13.0		
S. simulans	-	-	-	-	-	-	1	11.1	-	-	2	15.0
S. capitis	-	-	1	50.0	-	-	-	-	-	-	1	7.7
S. cohnii	-	-	-	-	-	-	1	11.1	-	-	1	7.7
						G2						
S. xylosus	-	-	-	-	-	-	1	20.0	-	-	1	17.0
S. hominis	-	-	-	-	-	-	-	-	-	-	-	-
S. warneri	-	-	-	-	1	25.0	1	20.0	-	-	1	17.0
S. epidermidis	-	-	-	-	2	50.0	-	-	2	67.0	-	-
S. simulans	-	-	-	-	1	25.0	1	20.0	1	33.0	1	17.0
S. capitis	-	-	-	-	-	-	-	-	-	-	2	33.3
S. cohnii	-	-	-	-	-	-	1	20.0	-	-	1	17.0
S. lentus	-	-	-	-	-	-	1	20.0	-	-	-	-
						G3						
S. warneri	-	-	-	-	1	50.0	3	75.0	1	50.0	3	60.0
S. epidermidis	-	-	-	-	1	50.0	-	-	1	50.0	-	-
S. simulans	-	-	-	-	-	-	1	25.0	-	-	-	-
S. chromogenes	-	-	-	-	-	-	-	-	-	-	1	20.0
S. sciuri sciuri	-	-	-	-	-	-	-	-	-	-	1	20.0

G1 = Control group, composed of sheep that did not receive intramammary antimicrobials, G2 = sheep that were treated with conventional antimicrobials (100mg of cloxacillin–benzathine), and G3 = sheep that were treated with nanoencapsulated antimicrobials (50mg of cloxacillin-benzathine). *ica*A, *ica*C and *ica*D = genes responsible for biofilm formation.

Six untreated sheep presented *S. warneri* with virulence factor genes before weaning. All of them continued to present subclinical mastitis during the subsequent lactation. The multiple correspondence analysis among the effects of treatment before drying the sheep and the presence of microorganisms and genes demonstrated that this species of staphylococci when presented the *sec* and *icaD* genes was related to the absence of spontaneous recovery of sheep, i.e. with the permanence of cases of the disease when not effected the treatment. In G2, three sheep presented reinfection 30 days after parturition, after being treated with 100mg of cloxacillin-benzathine. It was established *S. xylosus* carrying the gene *bap* was related with the reinfection. Bacteria inside biofilms are subject to less action by neutrophils, which facilitates bacterial growth for long periods (Rasmussen & Givskov 2006). Consequently, the antimicrobial may be inefficient. Then, sheep that presented CNS strains carrying genes responsible for formation of biofilm during the pre-partum period can continue to present subclinical mastitis during the drying off period and over the next lactation. In the sheep treated with nanoparticulate antimicrobials, the cases of lack of cure by 15 and 30 days after parturition occurred in mammary halves infected by *S. epidermidis, S. warneri* and *S. simulans*. The absence of cure mastitis in this group of animals was not related to the bacterial species or their genes, probably by this type of treatment being more effective than the use of conventional cloxacillin and the absence of treatment, in the 15 days prepartum.

CONCLUSIONS

The identification of several CNS species in the milk of sheep with mastitis is consistent with the widespread occurrence of these microorganisms in the etiology of subclinical mastitis in ewes. These bacteria have genes for virulence factors that can negatively interfere with disease control methods.

The knowledge about the ability of CNS remaining in the mammary halves of sheep, even after intramammary treatment, provides information regarding the epidemiology of the disease and contributes towards adoption of measures for future control.

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