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 mecA gene. The only gene relating to production of enterotoxins was sec. Among the genes relating to production of biofilm, icaD 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.
The aims of the present study were to identify the species of CNS in milk of mastitic ewes and investigate the presence of genes related to 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 Sáo Carlos, Sáo 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) >2.5x10⁵ 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, and were sent to a reference laboratory within the Brazilian Milk Quality Network, where counting procedures were performed using an electronic device (Somaccount 300; Bendley Instruments®).

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 after 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 “GAAATGATGAAAGG” 16S and L1 “CAAAGGATGCCGC” 23S (Couto et al. 2001).

The presence of genes meCA (Murakami et al. 1991), icaA, icaC, icaD, bap and bhp (Cucarella et al. 2004, Arciola et al. 2001, Qin et al. 2007), sec, seb, sec, sed and tst-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 nanostructured 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)
reported a CNS occurrence rate in milk samples from Santa Inês sheep of 64.3%. 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 (Luches 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 or 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 mecA 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 mecA expression may be present (Zafalon et al. 2012), but were not investigated in the present study. The presence of homologous mecA 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 icaA, icaC, icaD and bap among CNS species identified during weaning and the genes icaA, icaC and icaD after parturition are presented in the Tables 6 and 7, respectively. All the species of CNS studied were negative for the genes icaA 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 icaD 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 icaA, icaC, icaD, bap and bhp. The genes of the ica operon are responsible for synthesizing the production of biofilm. Strains of CNS carrying ica operon 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 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 icaC, icaD and bap among coagulase-negative Staphylococcus spp. identified in sheep with subclinical mastitis immediately before the drying-off period

| Species       | icaC                   | icaD                   | 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). icaC, icaD 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 icaC and icaD were found in a greater number of species before the drying-off period. There are reports that 60.0% of the CNS carried the icaD gene in milk samples from sheep with subclinical mastitis (Ergun et al. 2012). The presence of the icaA gene reported in the present study differed from the results found by other author (Martins 2013).
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 affected 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.

**Acknowledgements** - This study was funded by grants of the Research Support Foundation of the State of São Paulo (Fundação de Amparo à Pesquisa do Estado de São Paulo, FAPESP) (FAPESP procedural n°. 2012/23044-0). Our acknowledgements to Danilo Flávio Moraes Riboli by collaboration in laboratory activities for the identification of staphylococci.
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