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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 *mec*A gene, biofilm (*ica*ADBC, *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 *mec*A gene. As for the biofilm genes, the *ica*ADBC 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 im-

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portant conclusions of a recent meta-analysis of existing literature by Reyher et al. (2012) is that challenge studies [experimental studies where the udder is challenged 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*, *sod*A, and *rpo*B 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