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Diversity of *Staphylococcus* species and prevalence of enterotoxin genes isolated from milk of healthy cows and cows with subclinical mastitis

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ABSTRACT

The objectives of this study were to determine the occurrence and diversity of *Staphylococcus* spp. in milk from healthy cows and cows with subclinical mastitis in Brazil and to examine the profile of enterotoxin genes and some enterotoxins produced by *Staphylococcus* spp. A total of 280 individual mammary quarter milk samples from 70 healthy cows and 292 samples from 73 cows with subclinical mastitis were collected from 11 farms in the state of São Paulo, Brazil. *Staphylococcus* spp. were recovered from 63 (22.5%) samples from healthy cows and from 80 samples (27.4%) from cows with mastitis. The presence of *Staphylococcus aureus* was significantly different between these 2 groups and was more prevalent in the cows with mastitis. The presence of *Staphylococcus saprophyticus* was also significantly different between these 2 groups, but this organism was more prevalent in healthy cows. No statistically significant differences were observed in the numbers of other staphylococci in milk samples from the 2 groups. The *sea* gene was the most prevalent enterotoxin gene in both groups. Eight of 15 (53.3%) *Staph. aureus* carried this gene and all produced the SEA toxin. In the coagulase-negative staphylococci (CNS) group, 61 of 128 (47.5%) had the same gene and just 1 (1.6%) *Staphylococcus epidermidis* strain produced the enterotoxin in vitro. Because CNS were isolated from both groups of cows and most CNS contained enterotoxin genes but did not produce toxins, the role of CNS in mastitis should be carefully defined.

Key words: *Staphylococcus aureus*, coagulase-negative staphylococci, mastitis, enterotoxin

INTRODUCTION

Mastitis is one of the most common infectious diseases in dairy herds (Pol and Ruegg, 2007) and can be classified into clinical and subclinical forms. Clinical mastitis is defined based on clinical evidence of inflammation in at least one teat or the presence of lumps or flakes in the milk or an abnormal color or consistency of the milk from early jets of milk. In subclinical cases, animals are outwardly healthy and must be diagnosed using the California Mastitis Test (**CMT**) and SCC (Pantoja et al., 2009).

Staphylococcus are among the microorganisms most commonly isolated in mastitis cases (Pyörälä and Taponen, 2009), and *Staphylococcus aureus* is the most common cause of bovine mastitis and is responsible for the largest economic losses due to this condition (Melchior et al., 2006). Mastitis caused by this bacterial species may be subclinical or clinical (Pyörälä and Taponen, 2009).

Although *Staph. aureus* is considered the primary pathogen involved in intramammary infections, the role of CNS in this condition has recently been reexamined. These microorganisms, which were previously thought to be environmental contaminants, are now considered to be a frequent cause of mastitis, particularly subclinical mastitis (Taponen et al., 2007). The CNS seem to increase the number of somatic cells in milk, due to the presence of phagocytes combating local microorganisms, and to decrease milk production, and lead to intraalveolar fibrosis of the breast tissue and loss of secretory function of this tissue (Lüthje and Schwarz, 2006).

Because of the heterogeneity of this genus, which contains 47 species, mastitis caused by CNS is poorly understood and control of CNS mastitis is complicated (DSMZ, 2012). Infection by CNS is a persistent cause of intramammary inflammation, which may persist throughout the months of lactation in the absence of intervention (Gillespie et al., 2009). Park et al. (2011) isolated 263 CNS from milk samples from cows with intramammary infections, identifying 11 species, including *Staphylococcus chromogenes* (72.2%), *Staphylococcus xylosus* (9.1%), and *Staphylococcus haemolyticus* (6.1%), which were the most frequent species isolated.

Staphylococcus aureus produces many extracellular toxins (enterotoxins). These toxins are known to cause

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food poisoning in humans, and they may be involved in other types of infections such as mastitis. Classical staphylococcal enterotoxins (**SE**) were designated SEA through SEE (Bergdoll and Robbins, 1973). Later, new toxin genes were discovered, including *seg*, *seh*, *sei*, *selj*, *selk*, *sell*, *selm*, *seln*, *selo*, *selp*, *selq*, *selr*, *sels*, *selt*, *selu*, and *selv* (Betley et al., 1992; Ren et al., 1994; Munson et al., 1998; Jarraud et al., 2001; Kuroda et al., 2001; Orwin et al., 2001, 2003; Letertre et al., 2003; Omoe et al., 2003; Mempel et al., 2003; Thomas et al., 2006; Ono et al., 2008). Some of these enterotoxins are referred to as SE-like toxins, as their emetic properties have not yet been characterized (Lina et al., 2004).

Both coagulase-negative and coagulase-positive staphylococci carry genes for the production of these enterotoxins (Rall et al., 2010; Aydin et al., 2011; Park et al., 2011). However, although *Staph. aureus* is a strong enterotoxin producer, CNS seem to produce these enterotoxins at a low level, even under optimal conditions, or to not produce these toxins at all (Robbins et al., 1974; Rall et al., 2010; Aydin et al., 2011). Thus, this study aimed to examine the distribution of *Staphylococcus* species in milk samples from healthy cows and cows with subclinical mastitis and to determine the presence of genes for enterotoxins.

MATERIALS AND METHODS

Milk Samples

A total of 280 milk samples from 70 healthy cows (from the 4 mammary glands) and 292 milk samples from 73 cows with subclinical mastitis were collected over 12 mo from 10 farms in the state of São Paulo, Brazil. The samples were collected in sterile tubes after disinfection of the ostium with iodized alcohol (2.5%), and they were transported to the laboratory under refrigeration (4–8°C) in cool boxes with ice packs. All of the handlers used sterilized latex gloves.

The interpretation criteria used for diagnosis of subclinical mastitis were based on the CMT (Schalm and Noorlander, 1957) and SCC. Briefly, in the CMT, after excluding the first streams of milk, 3 mL of each milk sample was collected from a specific tray of the kit and an equivalent volume of the test reagent was added; the test reagent contained 10 mg of bromocresol purple, 1.5 g of sodium hydroxide, 15 mL of Teepol broth, and 1,000 mL of distilled water. The samples were scored from a range of negative to 5 plus signs (strongly positive), and samples with a score of at least 2 were suspected to be from cows with IMI and were subjected to somatic cell counting to confirm infection. This analysis was performed by flow cytometry using a Somacount 300 instrument (Bentley Instruments, Chaska, MN). Animals whose milk samples had >200,000 cells/mL and a positive CMT score of at least 2 and no clinical evidence of infection were classified as having subclinical mastitis (Pantoja et al., 2009).

Isolation and Identification of Staphylococcus spp.

All tests were performed with culture media from Oxoid (Basingstoke, UK) unless otherwise specified. The isolation of staphylococci was performed on Baird-Parker agar incubated at 35°C for 48 h. Characteristic colonies (black, with or without a halo) were tested for catalase, coagulase, and thermostable nuclease (TNase). Coagulase-positive species were subjected to testing with the Staphytect Dry Spot Test Kit (Oxoid). To separate the coagulase-positive species, we performed the Voges-Proskauer and β-galactosidase tests. A PCR for the *coa* and *nuc* genes was also performed using the protocol of Cremonesi et al. (2005).

The CNS strains were submitted to an antibiogram with bacitracin (0.04 U) and furazolidone (100 μg) to separate them from members of the genus *Kocuria*. Finally, the strains were identified using API Staph (BioMérieux, Marcy l'Etoile, France) and molecular methods, with *sod*A degenerate primers and the sequences were submitted to the European Molecular Biology Laboratory (EMBL) gene bank (Poyart et al., 2001).

PCR Testing for Genes Encoding Staphylococcal Enterotoxins

The Minispin Kit (GE Healthcare, Little Chalfont, UK) was used for DNA isolation according to the manufacturer's instructions. The primers used for the detection of SE genes are listed in Table 1. As positive controls, PCR reactions containing template DNA extracted from the standard *Staph. aureus* strains ATCC 13565 (*sea*), ATCC 14458 (*seb*), ATCC 19095 (*sec*), FRI 361 (*sed*, *seg*, *sei* and *sej*, *sel*, *sem*, *sen*, *seo*, *ser*), ATCC 27664 (see), FRI 137 (*seh*), FRI 326 (*seq*), and FRI 913 (*sek*) were carried out in parallel. Some additional PCR reactions received ultrapure water instead of template DNA to provide negative controls. One sample of each of *sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, *sei*, *sej*, *sell*, *selm*, *seln*, *selo*, *selp*, and *selr* amplicons was sequenced and the partial sequences were confirmed to correspond to GenBank accession numbers M18970, M11118, X05815, M28521, M21319, AY920261, U11702, AY920268, AB075606, AB679717.1, HE579071.1, HE579069.1, HE579073.1, FR714927.1, and GQ900416.1, respectively.