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

V. L. M. Rall,*1 E. S. Miranda,* I. G. Castilho,* C. H. Camargo,* H. Langoni,† F. F. Guimarães,† J. P. Araújo Júnior,* and A. Fernandes Júnior*

*Department of Microbiology and Immunology, and †Department of Hygiene Veterinary and Public Health, Universidade Estadual Paulista (UNESP), Botucatu, SP 18618-380, Brazil

**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
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, 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 the handlers used sterilized latex gloves.

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 (seg), 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, 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.
Classical Enterotoxin Production by Staphylococcus spp.

The *Staph. aureus* strains were cultivated in brain-heart infusion (BHI) broth and incubated overnight at 37°C. A volume of 0.1 mL was spread on sterilized cellophane over BHI agar with 1% yeast extract, and the Petri dishes were incubated overnight at 37°C. After this period, 2.5 mL of 0.01 M Na₂HPO₄ was added to the cellophane surface and, after homogenization with the colonies, the volume was transferred to a tube and submitted to centrifugation at 8,900 × g for 10 min at 4°C (Robbins et al., 1974).

For CNS, we used 30- to 40-cm dialysis sacs with 50 mL of double-concentrated BHI broth. The sacs were placed in 250-mL Erlenmeyer flasks and autoclaved at 121°C for 15 min. A loop of CNS (cultured in BHI at 37°C overnight) was mixed in 18 mL of 0.02 M phosphate buffer in 0.9% NaCl, pH 7.2, transferred to the Erlenmeyer flask, and incubated at 37°C for 24 h under agitation (130 rpm). After this period, the cultures were centrifuged at 8,000 × g for 10 min at 4°C (Donnelly et al., 1967).

The supernatants were tested for enterotoxins SEA, SEB, SEC, and SED using the reversed passive latex agglutination assay (RPLA) method (SET-RPLA, Oxoid) according to the manufacturer’s instructions. The same positive controls that were used for PCR were also used for the RPLA test.

**RESULTS**

We analyzed 280 samples of milk from 70 healthy cows and isolated *Staphylococcus* spp. from 63 (22.5%) samples. Of the 292 samples of milk from cows with...
subclinical mastitis, 80 (27.4%) were positive for these Staphylococcus spp.

Table 2 lists the staphylococcal species identified from milk from healthy cows and cows with mastitis. Staphylococcus aureus was the only species that was significantly more prevalent in the milk from cows with mastitis ($P = 0.001$). Staphylococcus saprophyticus was significantly more prevalent in milk from healthy cows ($P = 0.02$). The other staphylococci were present in similar numbers in milk samples from both groups, and no statistically significant differences between groups were seen for these other species.

Based on PCR analysis, sea was the most frequently observed enterotoxin gene, occurring in 27 of 63 (42.9%) isolates of staphylococci isolated from milk of healthy cows and in 42 of 80 (52.5%) isolates from animals with mastitis. In the cows with subclinical mastitis, the most frequently isolated Staphylococcus species was Staph. warneri (27.5%), followed by Staph. aureus (17.5%) and Staph. saprophyticus (16.3%).

Based on PCR analysis, sea was the most frequently observed enterotoxin gene, occurring in 27 of 63 (42.9%) isolates of staphylococci isolated from milk of healthy cows and in 42 of 80 (52.5%) isolates from animals with mastitis. The results of PCR analysis showed that no CNS species were statistically associated with mastitis cases. In fact, Staph. saprophyticus was significantly more prevalent in healthy cows than in cows with mastitis ($P = 0.02$) more prevalent in healthy cows. Because CNS are profusely spread on teat apices and other sites of a cow’s body as commensal organisms, they can cause mastitis once they are opportunistic pathogens (Taponen et al., 2008; Weese, 2012).

Some staphylococci are normally found on healthy skin of udders or on the hands of milkers. Staphylococcus epidermidis, Staph. simulans, and Staph. warneri are considered to be members of the bacterial microbiota in these regions, whereas Staph. xylosus and Staph. sciuri are considered to be environmental contaminants (Philpot and Nickerson, 2002). Data from this study demonstrate the isolation of several of these species in milk samples from healthy cows or cows with mastitis, with high prevalences of Staph. warneri (25.9%), Staph. aureus (17.5%), and Staph. warneri (27.5%).

**DISCUSSION**

Members of the genus Staphylococcus are major causative agents of bovine mastitis. In the current study, 292 samples of milk from cows with subclinical mastitis were tested and 27.4% were positive for these microorganisms.

**Table 2. Identification of Staphylococcus species isolated from milk of healthy cows or cows with subclinical mastitis**

<table>
<thead>
<tr>
<th>Species</th>
<th>From healthy cows, no. (%)</th>
<th>From sick cows, no. (%)</th>
<th>Total, no. (%)</th>
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<td>11 (13.8)</td>
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<td>5 (3.5)</td>
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<td>6 (7.5)</td>
<td>13 (9.1)</td>
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<td>80 (55.9)</td>
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Table 3. Presence of enterotoxin genes and production of classical enterotoxins in *Staphylococcus* spp. isolated from milk of healthy and sick cows

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STAPHYLOCCUS IN HEALTHY AND MASTITIC COWS
saprophyticus (22.4%), and Staph. epidermidis (15.4%) observed.

In the United States, Rajala-Schultz et al. (2004) found that CNS isolates were composed of 65.5% Staph. chromogenes, 10.1% Staph. simulans and 4.4% Staph. epidermidis. Lüthje and Schwarz (2006) isolated a total of 259 CNS strains from subclinical mastitis cases identified in Germany, including Staph. chromogenes (33.2%), Staph. simulans (23.2%), Staph. epidermidis (11.7%), Staph. xylosus, and Staph. haemolyticus (9.4%). In a study from Finland, Taponen et al. (2006) isolated CNS samples containing 36.4% Staph. simulans, 27.3% Staph. chromogenes, 3% Staph. haemolyticus, 3% Staph. warneri, and 5% Staph. epidermidis, and 1% Staph. hominis. In another study from the United States, Sawant et al. (2009) found Staph. chromogenes and Staph. epidermidis to be the most prevalent CNS species isolated, and Fessler et al. (2010) found similar results in cows from Germany, where the most frequent species were Staph. chromogenes and Staph. simulans.

Park et al. (2011) isolated 263 strains of CNS from IMI of cows in the United States and identified 11 species, including Staph. chromogenes (72.2%), Staph. xylosus (9.1%), Staph. haemolyticus (6.1%), Staph. sciuri spp. carnaticus (3%), Staph. hyicus (3%), Staph. simulans (2.7%), Staph. caprae (1.1%), Staph. epidermidis (0.8%), Staph. succinus (0.8%), Staph. capitis, and Staph. hominis (0.4%).

According to Supré et al. (2011), Staph. chromogenes, Staph. xylosus, Staph. cohnii, and Staph. simulans seem to affect udder health more so than other CNS species. In our study, Staph. chromogenes was not isolated from samples of milk from cows with mastitis and was only found in isolates from healthy cows at a low frequency of 3.2%. Staphylococcus simulans and Staph. cohnii were isolated from samples of milk from cows with mastitis, also at low frequencies (5 and 1.3%, respectively). We isolated Staph. xylosus in 13 of 143 (9.1%) tested samples, and Ruaro et al. (2013) observed this same value in their study.

From previous papers, the predominant CNS species in milk are Staph. chromogenes and Staph. simulans. All of these studies were conducted in the Northern Hemisphere, where animal handling procedures are very different from those used in Brazil. In previous Brazilian studies (Santos et al., 2010), the authors did not identify CNS at the species level, precluding a discussion of whether they observed increases in the predominant CNS species over what was observed in our study.

Staphylococcus spp. produce many virulence factors, several of which are involved in mastitis, particularly by producing enterotoxins. According to Table 3, sea was the most frequent enterotoxin gene based on our PCR test, occurring in 27 of 63 (42.9%) strains of staphylococci isolated from the milk of healthy cows and in 42 of 80 (52.5%) strains from cows with mastitis. Considering only the Staph. aureus strains, independent of the origin of milk, the frequency of sea isolation increased to 53.3% (8 of 15). Boynukara et al. (2008) also observed that this gene was the most frequent enterotoxin gene but found sea in only 23.6% of Staph. aureus strains isolated from subclinical mastitis cases. Findings similar to ours were obtained by Atanassova et al. (2001), who observed that the sea gene was present in 50% of Staph. aureus strains, and by Morandi et al. (2007), who observed that 48% of samples were positive for sea. Considering only CNS samples in the current study, the prevalence of sea isolated from healthy animals was 41.3%, whereas the prevalence in cows with mastitis was 43.8%. The phage φSa3 encodes the sea (φSa3nu) and selp (φSa3nu) genes, and selp was observed in 17 of 62 (27.4%) CNS strains isolated from healthy cows in the current study. Considering staphylococci from animals with mastitis, this phage was present in 3 (21.4%) Staph. aureus and 25 (37.9%) CNS isolates, or a total of 28 isolates (35%). In both groups of cows, we observed a significant difference considering the presence of these 2 genes (P = 0.002 for healthy cows and P < 0.001 for animals with mastitis).

We detected the seb gene in 2 CNS isolates (3.2%) from milk of healthy cows and 1 isolate (1.5%) from cows with mastitis; this gene seems to appear at low frequency in Staphylococcus spp. Boynukara et al. (2008) and Wang et al. (2009) found this gene at rates of 1.9 and 2.1%, respectively. In some studies, this gene was not observed (Cremonesi et al., 2005; Morandi et al., 2007; Ruaro et al., 2013).

The gene sec, which encodes SEC, was not found in milk samples from healthy cows but was present in 10% (8 of 80) of the staphylococci strains isolated from cows with mastitis. In an analysis of only Staph. aureus strains, the prevalence of this gene increased to 14.3%; similar frequencies of 15.5 and 16.1% were observed by Akineden et al. (2001) and Cremonesi et al. (2005), respectively. However, in other studies, this gene was found at low frequency or was not present, including studies by Boynukara et al. (2008) and Karahan et al. (2009).

According to Baba et al. (2002) and Holtfreter and Broker (2005), pathogenicity islands can carry the sec-sell-tst genes (SaPI2) or only the sec-sell genes (SaPI3). The sell gene was not observed in healthy animals (similar to sec) but was found in 6 (7.5%) strains from cows with mastitis: 2 (14.3%) Staph. aureus strains and 4 (6.1%) CNS strains.

The gene sed appeared at low prevalence and was present in 2 CNS strains (3.2%) from healthy cows.
and 1 (1.5%) from cows with mastitis. Boynukara et al. (2008) did not detect this gene in 106 Staph. aureus strains isolated from cows with subclinical mastitis.

The genes sed, selj, and selr are encoded together in plasmid pIB485 (Zhang et al., 1998; Omoe et al., 2003). In the healthy cows in this study, 2 (3.2%) CNS strains contained sed and selj (Staph. warneri and Staph. xylosus). In the cows with mastitis in this study, selj was more frequent than sed. The gene sed occurred in 1 (1.3%) CNS strain (Staph. warneri), and selj was present in 4 (5%): 2 (14.3%) Staph. aureus strains and 2 (3%) CNS strains (Staph. warneri and Staph. epidermidis). The gene selr occurred in 5 out of 62 (8.1%) CNS strains isolated from healthy cows and in 7 strains (8.8%) from cows with mastitis. This gene was present in 1 (7.1%) Staph. aureus strain and 6 (9.1%) CNS strains. Chiang et al. (2006) found this gene in 5.4% of 147 Staph. aureus strains in their study.

Only 1 (1.6%) CNS strain from healthy cows was positive for the presence of the see gene, and 2 (2.5%) strains from cows with mastitis were positive for this gene: 1 (7.1%) Staph. aureus strain and 1 (1.5%) CNS strain. Morandi et al. (2007), Chiang et al. (2006), and Wang et al. (2009) did not find the see gene in Staph. aureus strains. In an examination of CNS strains, Park et al. (2011) did not observe this gene in 263 strains.

The seg and sei genes are located together in the ecp cluster, which is localized in the type II pathogenicity island vSaB that can also carry the seg-sei-selm-seln-selo genes (Wang et al., 2009). In milk samples from healthy animals, seg was observed only in CNS strains and occurred in 24 (38.7%) strains. In samples from cows with mastitis, the frequency of this gene was higher but not statistically significant (P = 0.73) and the gene was also found in Staph. aureus, occurring in 33 (41.3%) strains: 7 (50%) Staph. aureus and 26 (39.4%) CNS. This gene was observed at similar frequencies in CNS strains from healthy cows (38.7%) and cows with mastitis (39.4%).

We were able to detect the sei gene in 22 of 62 (35.5%) CNS strains from healthy animals, and it was found together with seg at a frequency of 91.7%. In samples from cows with mastitis, the sei gene was observed in 30 (37.5%) strains, including 7 (50%) Staph. aureus strains that were positive for seg and 23 (34.8%) additional CNS strains in which the sei gene occurred with seg at a frequency of 88.5%. Despite the lower frequency of sei in relation to seg in both groups of animals, this difference in frequency was not statistically significant (P = 0.52 and P = 0.26, respectively). Although these genes are located together in a cluster, this difference can be explained by a mutation in the site of annealing of the primers or a cluster variation in these genes (Jarraud et al., 2001). Wang et al. (2009) obtained similar results and detected seg and sei in 31.4 and 31.8% of staphylococci strains, respectively.

The genes selm, seln, and selo were detected at frequencies of 33.8, 29, and 27.4%, respectively, of CNS strains from healthy animals. In samples from cows with mastitis, the rates of detection of these genes were higher: 36.4, 34.8, and 33.3%, but these numbers were not significantly different. Lower frequencies were found by Wang et al. (2009), with 26.9% positivity for selm, 17.3% for seln, and selo being absent. In another type of animal product used as food (pork), Hwang et al. (2007) observed that 37% of samples were positive for both the selm and seln genes and that selo was present in 27% of the strains.

The gene seh was detected in 8 (12.7%) isolates from healthy animals and was present in a single Staph. aureus strain and 7 out of 62 (11.3%) CNS strains. In cows with mastitis, the prevalence of this gene was higher (16.3%); it was detected in 3 (21.4%) Staph. aureus strains and in 10 (15.2%) CNS strains.

The selk gene was not observed in any strain. The seg gene was also negative in all isolates and both of these genes can be found together in the pathogenicity island SapPI, which also contains the seb gene. The seb gene also occurred at a low rate (3.2 and 1.5%) of CNS strains isolated from the milk of healthy cows and cows with mastitis, respectively, and the difference in the prevalence of this gene was not significant between the 2 groups (P = 0.58). Relatively few papers have described the detection of these genes. Wang et al. (2009) detected seb in 2.1% and selk in 3.2% of 283 Staph. aureus strains isolated from subclinical mastitis, whereas seg was not examined in this study. Omoe et al. (2005) found higher frequencies of these genes and observed frequencies of 30.1, 22.9, and 21.1% positivity for the seb, selk, and selg genes, respectively, although that study examined human clinical isolates and not those from livestock.

In our study, strains of Staphylococcus that possessed genes for the production of classical enterotoxins were tested for the production of these enterotoxins. We observed that 8 of 15 (53.3%) Staph. aureus strains from healthy cows and cows with mastitis contained the sea gene and all of these strains produced the toxin. In the CNS strains, only 1 strain (Staph. epidermidis) out of 128 (0.8%) containing the gene produced the SEA toxin. None of the CNS strains that contained the seb or sed genes produced these toxins. In our examination of sec, 2 Staph. aureus strains containing sec produced the SEC enterotoxin and 6 CNS strains did not. This lack of enterotoxin production by CNS strains despite the presence of the toxin genes has been reported previously (Rall et al., 2010; Aydin et al., 2011).
With the exception of *Staph. aureus* strains, which occurred predominantly in cows with mastitis, the other species of CNS were found indiscriminately in both groups of animals. Moreover, despite the presence of genes encoding classical enterotoxins in many CNS strains, only one strain produced these toxins. Further research is needed to demonstrate the true pathogenic potential of this group of organisms in mastitis.

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