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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

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[1]Corresponding author: vlmores@ibb.unesp.br

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.

Table 1. Primers and their characteristics used for the detection of *Staphylococcus* spp. staphylococcal enteroxin genes

Gene	Sequence (5′–3′)	Product size, bp	Annealing temperature, °C	Reference
sea 1	TTGGAAACGGTTAAAACGAA			
sea 2	GAACCTTCCCATCAAAAACA	120	50	Johnson et al. (1991)
seb 1	TCGCATCAAACTGACAAACG			
seb 2	GCAGGTACTCTATAAGTGCC	478	50	Johnson et al. (1991)
sec 1	GACATAAAAGCTAGGAATTT			
sec 2	AAATCGGATTAACATTATCC	257	50	Johnson et al. (1991)
sed 1	CTAGTTTGGTAATATCTCCT			
sed 2	TAATGCTATATCTTATAGGG	317	50	Johnson et al. (1991)
see 1	AGGTTTTTTCACAGGTCATCC			
see 2	CTTTTTTTTCTTCGGTCAATC	209	50	Mehrotra et al. (2000)
seg 1	AAGTAGACATTTTTGGCGTTCC			
seg 2	AGAACCATCAAACTCGTATAGC	287	55	Omoe et al. (2002)
seh 1	GTCTATATGGAGGTACAACACT			
seh 2	GACCTTTACTTATTTCGCTGTC	213	46.4	Omoe et al. (2002)
sei 1	GGTGATATTGGTGTAGGTAAC			
sei 2	ATCCATATTCTTTGCCTTTACCAG	454	50	Omoe et al. (2002)
selj 1	CATCAGAACTGTTGTTCCGCTAG			
selj 2	CTGAATTTTACCATCAAAGGTAC	142	50	Nashev et al. (2004)
selk 1	TAGGTGTCTCTAATAATGCCA			
selk 2	TAGATATTCGTTAGTAGCTG	293	55	Omoe et al. (2005)
sell 1	TAACGGCGATGTAGGTCCAGG			
sell 2	CATCTATTTCTTGTGCGGTAAC	383	55	Omoe et al. (2005)
selm 1	GGATAATTCGACAGTAACAG			
selm 2	TCCTGCATTAAATCCAGAAC	379	55	Omoe et al. (2005)
seln 1	TATGTTAATGCTGAAGTAGAC			
seln 2	ATTTCCAAAATACAGTCCATA	282	55	Omoe et al. (2005)
selo 1	TGTGTAAGAAGTCAAGTGTAG			
selo 2	TCTTTAGAAATCGCTGATGA	214	55	Omoe et al. (2005)
selp 1	TGATTTATTAGTAGACCTTGG			
selp 2	ATAACCAACCGAATCACCAG	396	55	Omoe et al. (2005)
selq 1	AATCTCTGGGTCAATGGTAAGC			
selq 2	TTGTATTCGTTTTGTAGGTATTTTCG	122	55	Omoe et al. (2005)
selr 1	GGATAAAGCGGTAATAGCAG			
selr 2	GTATTCCAAACACATCTAAC	166	55	Omoe et al. (2005)
nuc 1	AGTTCAGCAAATGCATCACA			
nuc 2	TAGCCAAGCCTTGACGAACT	400	55	Cremonesi et al. (2005)
coa 1	CCGCTTCAACTTCAGCCTAC			
coa 2	TTAGGTGCTACAGGGGCAAT	204	57	Cremonesi et al. (2005)
RNAr 1	CCTATAAGACTGGGATAACTTCGGG			
RNAr 2	CTTTGAGTTTCAACCTTGCGGTCG	791	57	Mason et al. (2001)

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* Na_2HPO_4 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

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

Species	From healthy cows, no. (%)	From sick cows, no. (%)	Total, no. (%)
Staph. aureus	1 (1.6)	14 (17.5)	15 (10.5)
Staph. epidermidis	11 (17.5)	11 (13.8)	22 (15.4)
Staph. warneri	15 (23.8)	22 (27.5)	37 (25.9)
Staph. haemolyticus	5 (7.9)	7 (8.8)	12 (8.4)
Staph. saprophyticus	19 (30.2)	13 (16.3)	32 (22.4)
Staph. simulans	1 (1.6)	4 (5)	5 (3.5)
Staph. xylosus	7 (11.1)	6 (7.5)	13 (9.1)
Staph. cohnii ssp. *cohnii*	1 (1.6)	1 (1.3)	2 (1.4)
Staph. chromogenes	2 (3.2)	—	2 (1.4)
Staph. hominis	1 (1.6)	—	1 (0.7)
Staph. capitis	—	2 (2.5)	2 (1.4)
Total	63 (44.1)	80 (55.9)	143 (100)

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.

Staphylococcus saprophyticus was the most frequently isolated CNS in healthy animals (30.2%), followed by *Staph. warneri* (23.8%) and *Staph. epidermidis* (17.5%). 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 of the presence of genes encoding enterotoxins and the in vitro production of the classical enterotoxins are presented in Table 3. It should be noted that *sea* was the most frequently detected gene, occurring in 69 (48.3%) of the isolated staphylococci, including samples from both groups of cows, and 9 (13%) of the *sea*-positive staphylococci produced the SEA enterotoxin.

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.

Staphylococcus aureus is a major pathogen associated with bovine mastitis. This microorganism was detected in 4.8% of the 292 milk samples from cows with subclinical mastitis that were analyzed in this study. In the United States, Pol and Ruegg (2007) observed *Staph. aureus* in 2.9% of samples isolated from cows with subclinical mastitis. In a similar study, Karahan et al. (2009) observed that 28.6% of such samples were positive for *Staph. aureus* in Turkey. Out of the 80 strains of staphylococci isolated from cows with subclinical mastitis, *Staph. aureus* was the second most frequently isolated strain (17.5%) in the current study.

We isolated CNS from 66 (22.6%) of the 292 samples of milk from cows with mastitis. Pyörälä and Taponen (2009) concluded that these microorganisms can cause persistent infection, resulting in an increased number of somatic cells in milk, affecting milk quality, and reducing milk production from infected cows. However, our results showed that no CNS species were statistically associated with mastitis cases. In fact, *Staph. saprophyticus* was significantly ($P = 0.02$) 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.*

Table 3. Presence of enterotoxin genes and production of classical enterotoxins in *Staphyloccus* spp. isolated from milk of healthy and sick cows

	Staphylococcus species (no.)										
Gene/ Product	*aureus* (1)	*epidermidis* (11)	*warneri* (15)	*haemolyticus* (5)	*saprophyticus* (19)	*simulans* (1)	*xylosus* (7)	*cohnii* ssp. *cohnii* (1)	*chromogenes* (2)	*hominis* (1)	Total, no. (%)
Healthy cows (n = 63)											
sea/SEA	1/1	6/1	8	3	6		3				27 (42.9)
seb/SEB		1			1						2 (3.2)
sec/SEC											
sed/SED			1				1				2 (3.2)
see/SEE					1						1 (1.6)
seg		6	3	1	7		5	1	1		24 (38.1)
seh	1	2	2		2				1		8 (12.7)
sei		5	3	1	7		5		1		22 (35)
selj			1				1				2 (3.2)
sell											
selm		5	2	1	7		5		1		21 (33.3)
seln		4	2	1	6		5				18 (28.6)
selo		4	2	1	5		5				17 (27)
selp	1	4	5	2	3		2				17 (27)
selr		2			1		1		1		5 (7.9)
Sick cows (n = 80)	*aureus* (14)	*epidermidis* (11)	*warneri* (22)	*haemolyticus* (7)	*saprophyticus* (13)	*simulans* (4)	*xylosus* (6)	*cohnii* ssp. *cohnii* (1)	*capitis* (2)		Total, no. (%)
sea/SEA	7/7	6	11	4	7	1	4	1	1		42 (52.5)
seb/SEB		1									1 (1.3)
sec/SEC	2/2	2	3		1						8 (10)
sed/SED			1								1 (1.3)
see/SEE	1	1									2 (2.6)
seg	7	5	8	6	4	1	2				33 (41.3)
seh	3	2	4		2		1	1			13 (16.3)
sei	7	5	7	5	4	1	1				30 (37.5)
selj	2	1	1								4 (5)
sell	2	1	2		1						6 (7.5)
selm	7	3	5	5	3	0	1				24 (30)
seln	7	3	5	4	3	0	1				23 (28.8)
selo	7	3	4	4	3	0	1				22 (27.5)
selp	3	5	8	3	5	1	2		1		28 (35)
selr	1	1	2	1	1		1				73 (8.8)

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* ssp. *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* (φSa3mu) 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*, *sellj*, 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 *egc* cluster, which is localized in the type II pathogenicity island vSaß that can also carry the *seg-sei-selm-seln-selo* and, sometimes, *selu* 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 *senl*, 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 *seq* gene was also negative in all isolates and both of these genes can be found together in the pathogenicity island SaPI1, 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 *seq* 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 *selq* 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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