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Detection of classical and newly described staphylococcal superantigen genes in coagulase-negative staphylococci isolated from bovine intramammary infections

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Abstract

The coagulase negative staphylococci (CNS) are the most prevalent mastitis pathogen group yet their virulence characteristics have not been well described. We investigated the presence of 19 classical and newly described staphylococcal superantigen (SAg) genes in CNS isolates from bovine intramammary infections (IMI). A total of 263 CNS representing 11 different *Staphylococcus* spp. were examined, and 31.2% ($n = 82$) of CNS isolates had one or more SAg genes; there were 21 different SAg gene combinations. The most prevalent combination of SAg genes (*seb, seln*, and *selq*; $n = 45$) was found in *S. chromogenes*, *S. xylosus*, *S. haemolyticus*, *S. sciuri* subsp. *carnaticus*, *S. simulans* and *S. succinus*. The genes for SAgs appear to be widely distributed amongst CNS isolated from bovine IMI.

Keywords

coagulase-negative staphylococci; staphylococcal superantigens; multiplex PCR

1. INTRODUCTION

Staphylococcal superantigens (SAgs) including staphylococcal enterotoxins (SEs) and toxic shock syndrome toxin-1 (TSST-1) were originally identified in *S. aureus*. Staphylococcal enterotoxins were named according to their emetic activities following oral administration in

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a primate model. Several SEs were designated as SE-like (SEl) since they either lack emetic properties or their emetic activities have not been tested in this model (Lina et al., 2004). There are 5 different types of classical SEs, SEA through SEE, which are antigenically distinct (Bergdoll, 1989). Recently, new types of SEs (SEG, SEH, SEI, SElJ, SElK, SElL, SElM, SElN, SElO, SElP, SElQ, SElR, SES, SET, SElU, and SElV) have been described in *S. aureus* (Ono et al., 2008; Seo and Bohach, 2007; Thomas et al., 2006).

Compared to *S. aureus*, a relatively limited numbers of studies have examined the prevalence of SAg genes in Coagulase negative staphylococci (CNS) isolates. Crass and Bergdoll (1986) first identified CNS that produced TSST-1, SEA, or both from patients with toxic shock syndrome. Some studies also demonstrated that classical SEs and TSST-1 were produced by CNS species isolated from human clinical samples and intramammary infections of cattle and other ruminants (Bautista et al., 1988; Kuroishi et al., 2003; Valle et al., 1991; Cunha et al., 2007). Conversely, several larger surveys failed to detect genes encoding SAgs in CNS from human and veterinary specimens (Becker et al., 2001a; Becker et al., 2001b; Jaulhac et al., 1991; Kreiswirth et al., 1987; Nemati et al., 2008).

These observations collectively suggest that CNS isolates from bovine IMI could be a possible source of SAgs. Survey studies examining genetic constructs for SAg in CNS heretofore have been limited in scope, with either a limited number of samples collected or a limited number of SAg genes probed. To build upon these previous investigations we collected milk from more than 1000 cows on 6 dairy farms to gain a better insight into the prevalence of CNS with genes for SAgs. The focus of the current study was to investigate the prevalence and distribution of CNS bovine IMI isolates that possess the genes for classical and/or newly described SAgs.

2. MATERIALS AND METHODS

2.1. Bacterial strains

Milk samples were aseptically collected in duplicate from functional mammary quarters on six dairies in Idaho and Washington. A mammary quarter was considered infected when a single type of CNS was presumptively identified on sheep blood agar after 48 h incubation at 37 °C and 5% CO_2, from duplicate milk samples, and the number of colony-forming unit/ml of milk was greater than 200 (National Mastitis Council, 1987). A total of 263 CNS isolates which caused IMI were identified and speciation was determined by partial 16S rRNA and/or *rpoB* gene sequence analyses as previously described (Park et al., 2009). The species of CNS from bovine IMI used in the present study included: *S. chromogenes* (*n* = 190), *S. xylosus* (*n* = 24), *S. haemolyticus* (*n* = 16), *S. sciuri* subsp. *carnaticus* (*n* = 9), *S. hyicus* (*n* = 8), *S. simulans* (*n* = 7), *S. caprae* (*n* = 3), *S. epidermidis* (*n* = 2), *S. succinus* (*n* = 2), *S. capitis* (*n* = 1), and *S. hominis* (*n* = 1). Six *S. aureus* reference strains (Table 1) were used to establish multiplex PCR for 19 different SAg genes. All bacterial strains were stored in 20% glycerol solution at −80 °C.

2.2. Genomic DNA purification

Genomic DNA was purified as previously described (Pitcher et al., 1989). Briefly, bacteria were cultured in 5 ml of Todd Hewitt broth (THB, Becton Dickinson Diagnostic Systems, Sparks, MD) at 37 °C for 18 h and then harvested by centrifugation at 12,000 g for 5 min. The cell pellet was resuspended in TE buffer (50 mM Tris-HCl pH 7.0, 10 mM EDTA), digested by treatment with 10 μl of lysostaphin (1 mg/ml, Sigma-Aldrich Co., St. Louis, MO) and 100 μl of lysozyme (100 mg/ml, Sigma-Aldrich Co.) at 37 °C for 1 h. Cell lysates were treated with 500 μl of lysis buffer (5 M guanidine thiocyanate, 5 mM EDTA, 0.5% v/v sarkosyl) followed by 250 μl of ammonium acetate solution (7.5 M, Sigma-Aldrich Co.).

After mixing with an equal volume of chloroform:isoamyl alcohol (24:1), the sample was centrifuged at 18,000 g for 10 min. The upper aqueous phase containing DNA was collected into a fresh microtube and precipitated with isopropanol, followed by washing with 70% ethanol. The DNA concentration was adjusted to100 ng/μl by the addition of deionized water to achieve an OD = 2.0 at 260 nm (Nanodrop Technologies, Wilmington, DE).

2.3. Multiplex PCR for SAg genes

A multiplex PCR method described by Omoe et al. (2005) was used to amplify 19 different SAg genes (18 SE and TSST-1 genes). It appeared that primers for *sea, seb, seln*, and *selq* genes used in a previous study generated non-specific amplification in some species of CNS isolates, particularly *S. chromogenes*, the most prevalent species in bovine IMI. Thus primers for *sea, seb, seln* and *selq* genes were replaced with newly designed ones. Additionally, one more primer for the *selu* gene was included in this study. Primers and the expected size of PCR products for each SAg gene are described in Table 1. Multiplex PCR was performed with 4 different sets of primer mixtures (Set 1: *sea, seb, sec, sed, see;* Set 2: *seg, seh, sei, selj, selp;* Set 3: *selk, selm, selo, tst1;* Set 4: *sell, seln, selq, selr, selu)*. Each reaction mixture (50 μl) consisted of 10×PCR buffer (Applied Biosystems Inc., Foster City, CA), 1U *Taq* DNA polymerase (Applied Biosystems Inc.), 0.4mM of dNTP mix (Applied Biosystems Inc.), 0.3 μM of each primer, 100 ng of template DNA and sterile deionized water. Multiplex PCR for SAg genes was carried out with the following thermal cycling conditions: an initial denaturation of DNA at 95°C for 10 min was followed by 35 cycles of amplification (95 °C for 30 s, 53 °C for 45 s, and 72 °C for 90 s), ending with a final extension at 72 °C for 10 min. The mixture of genomic DNA from *S. aureus* reference strains were used for multiplex PCR of each set as a positive control. All PCR products were analyzed by 1% agarose gel electrophoresis in 0.5× Tris-borate-EDTA buffer. Amplification of non-template controls was attempted with each reaction to determine if DNA contamination occurred.

3. RESULTS

3.1. Modification of multiplex PCR for SAg genes

The modified multiplex PCR method in this study successfully amplified 19 different SAg genes from *S. aureus* reference strains (Fig. 1A and 1B). The sizes of the PCR products obtained from the positive control reference strains corresponded to their predicted sizes (Fig.1 and Table 1). The SAg genes of all reference strains determined by a modified multiplex PCR were exactly identical to those previously described in other studies (Table 2). Non-specific reactions were not observed. An amplicon was not observed in the negative control, *S. aureus* RN4220. In addition, this modified multiplex PCR specifically amplified SAg genes from *S. chromogenes* as well as other CNS species of isolates without non-specific amplifications as shown in Fig. 2A and 2B, demonstrating representative multiplex PCR results observed in various CNS species isolated from bovine IMI.

3.2. Prevalence and distribution of SAg genes

Of the 263 CNS IMI isolates tested, 82 CNS isolates (31.2%) were found to have one or more SAg genes (Table 3). Isolates with the *selq* gene (22.4%, 59/263) were most prevalent followed by those with the *seb* (20.9%, 55/263) or *seln* gene (20.9%, 55/263) (Table 3). The *see*, *sell*, *selm*, *selp*, or *tst1* gene was not detected in any of isolates in this study (Table 3). The SAg genes were widely distributed among 9 different CNS species including *S. chromogenes*, *S. xylosus*, *S. hyicus*, *S. haemolyticus*, *S. sciuri* subsp. *carnaticus*, *S. simulans, S. capitis, S. epidermidis*, and *S. succinus* (Table 3). Of the *S. chromogenes*, 22.1 % (42/190) of the isolates were SAg gene-positive (Table 3). *Staphylococcus xylosus* was the second most prevalent CNS species in bovine IMI and 45.8% (11/24) of the isolates

were SAg gene-positive (Table 3). All *S. hyicus* ($n = 8$) and *S. capitis* ($n = 1$) were found to have SAg genes. No SAg genes were detected amongst *S. caprae* and *S. hominis* isolates (Table 3).

A total of 21 different SAg gene combinations were observed among the 82 SAg gene-positive isolates (Table 4). The most common SAg gene combination was *seb*, *seln*, and *selq*, which was present in 54.9% (45/82) of SAg gene-positive CNS isolates (Table 4). Most of SAg gene-positive isolates had both classical and newly described SAg genes (73.2%, 60/82) (Table 4). Nineteen isolates had only newly described SAg genes whereas 3 isolates had classical SAg genes only (Table 4). Of the 42 SAg gene-positive *S. chromogenes* isolates, the combination of *seb*, *seln*, and *selq* genes was most prevalent (69.0%, 29/42) and the same combination also was most commonly observed in SAg gene-positive *S. xylosus* isolates (Table 4).

4. DISCUSSION

Staphylococcal SAgs are typically associated with *S. aureus* as virulence factors and have profound effects on the host immune system through the subversion of immune responses and delays in the establishment of pathogen-specific immunity (McCormick et al., 2001; Seo et al., 2007). Several previous studies (Bautista et al., 1988; Kuroishi et al., 2003; Valle et al., 1991) have suggested the possibility that various CNS species isolated from ruminant mastitis possess the genes to produce some of the SAgs. We found that one or more classical and/or newly described SAg genes were observed in 31.2% of CNS isolates and these SAg genes were widely distributed in 9 different CNS species. In this study, a group of *seb*, *seln*, and *selq* genes was the most prevalent SAg gene combination ($n = 45$) in CNS IMI isolates and was observed in various CNS species (Table 4). The current study demonstrated that the *seb* gene was the most common classical SAg while only 3 isolates harbored the *sec* gene. This was not consistent with other findings (Cunha et al., 2007; Kuroishi et al., 2003; Valle et al., 1991) that SEC was the most prevalent classical SAg in CNS isolates regardless of the origin of isolates.

Still other investigators reported that no SAg genes were detected in human and veterinary CNS isolates and thus, SAgs, were not associated with CNS isolates (Becker et al., 2001a; Becker et al., 2001b; Nemati et al., 2008). Nemati et al. (2008) investigated the presence of 18 different SAg genes in CNS isolated from clinical or subclinical bovine IMI using PCR as similar to our study and showed that none of the CNS isolates were positive for SAg genes. They raised a possibility that the failure of SAg gene detection could be due to the low sequence similarity of SAg genes between CNS and *S. aureus*. However, we note in their study genomic DNA was prepared by a boiling method routinely used in PCR technique for Gram-negative microorganism. In our study we based our DNA extraction on cell lysis using lysostaphin and lysozyme rather than boiling cells. We conjecture that our use of enzymes to lyse cells may result in a better yield and quality of DNA, leading to fewer false-negative detection results, than might be found with boiling cells which could have denatured the DNA before extraction.

In this study, we noticed a particular phenomenon that SAg genes detected in CNS IMI isolates seemed to be unstable. Generally, the amplification intensity of SAg genes from CNS IMI isolates was much weaker than those from *S. aureus* reference strains (the positive control) (Fig. 2A and 2B) although an equal amount of genomic DNA was used for multiplex PCR. Furthermore, the amplicon intensity became weaker or even disappeared when genomic DNA was extracted from the culture following several passages of the organisms under laboratory conditions. Thus it could be hypothesized that genetic elements harboring SAg genes in CNS from bovine IMI are unstable, with stability losses with

passing generations. Evidence in support of this hypothesis can be found in a recent report (Ubeda et al., 2007) where SAg genes in some *S. aureus*, chromosomally integrated in a staphylococcal pathogenic island, could be replicated as multimeric plasmids and transferred to other staphylococci, both *S. aureus* and CNS. However, replication of multimeric plasmids was unstable such that some daughter cells in the passage of culture lost multimeric plasmid harboring SAg genes. Clearly further study is necessary to define the genetic elements harboring SAg genes in CNS and to identify culture conditions which are needed to stabilize the maintenance of genetic elements.

To the best of our knowledge, this is the first study that investigated extensively and showed the presence of classical and/or newly described staphylococcal SAg genes in various species of CNS isolates from bovine IMI. We acknowledge the need to determine under what conditions those detected SAg genes in CNS isolates are maintained and expressed at the protein level. Our findings support the conjecture that CNS isolates from bovine IMI could serve as a possible reservoir of classical and newly described SAgs typically associated with the *S. aureus* pathogen.

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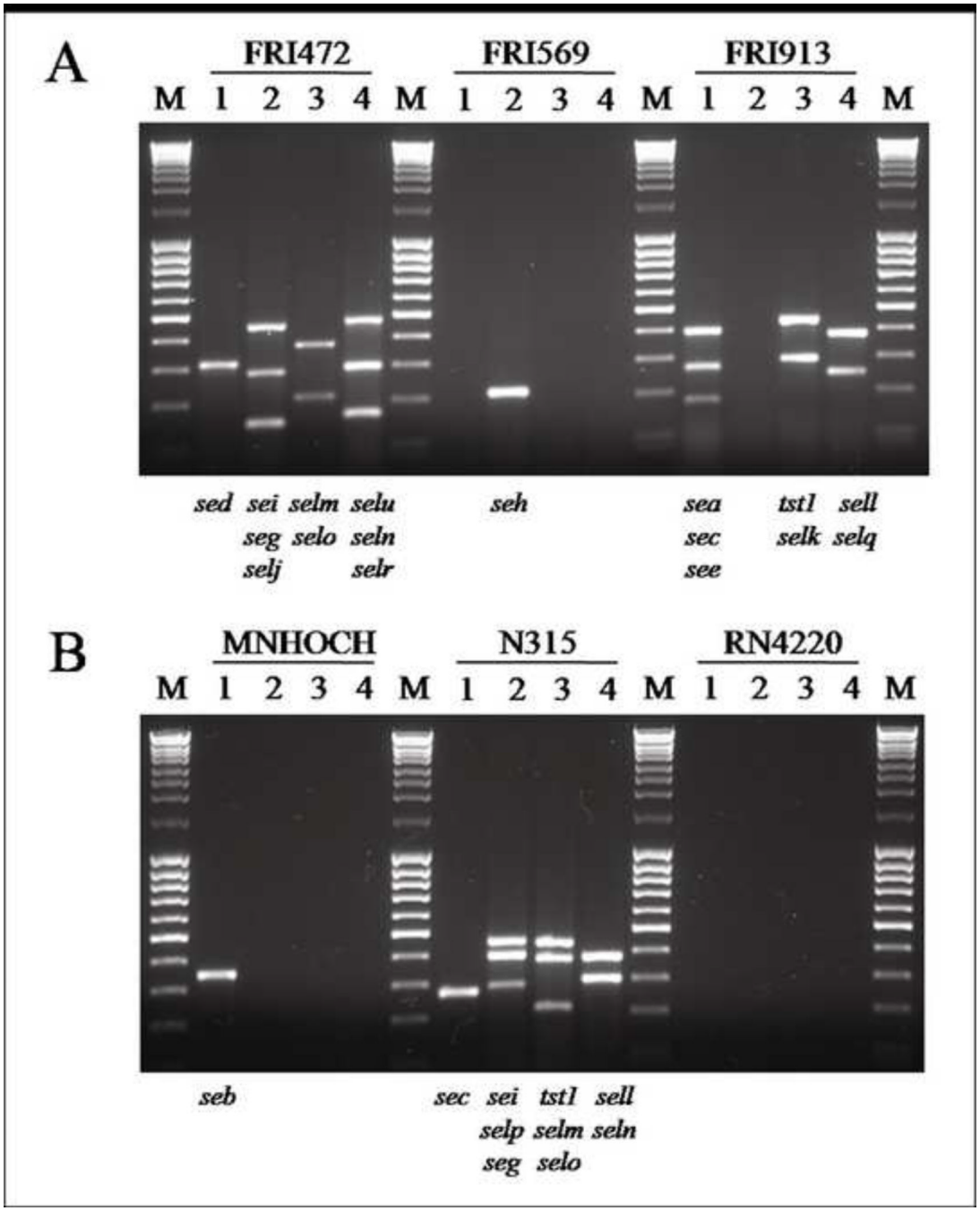

FIG. 1.

Detection of staphylococcal superantigen genes from *S. aureus* reference strains by multiplex PCR. The SAg genes detected from *S. aureus* reference strains by multiplex PCR of each primer set were indicated at the bottom of each lane. Lanes; M,100 bp DNA ladder; 1,.primer set 1; 2, primer set 2; 3, primer set 3; 4, primer set 4.

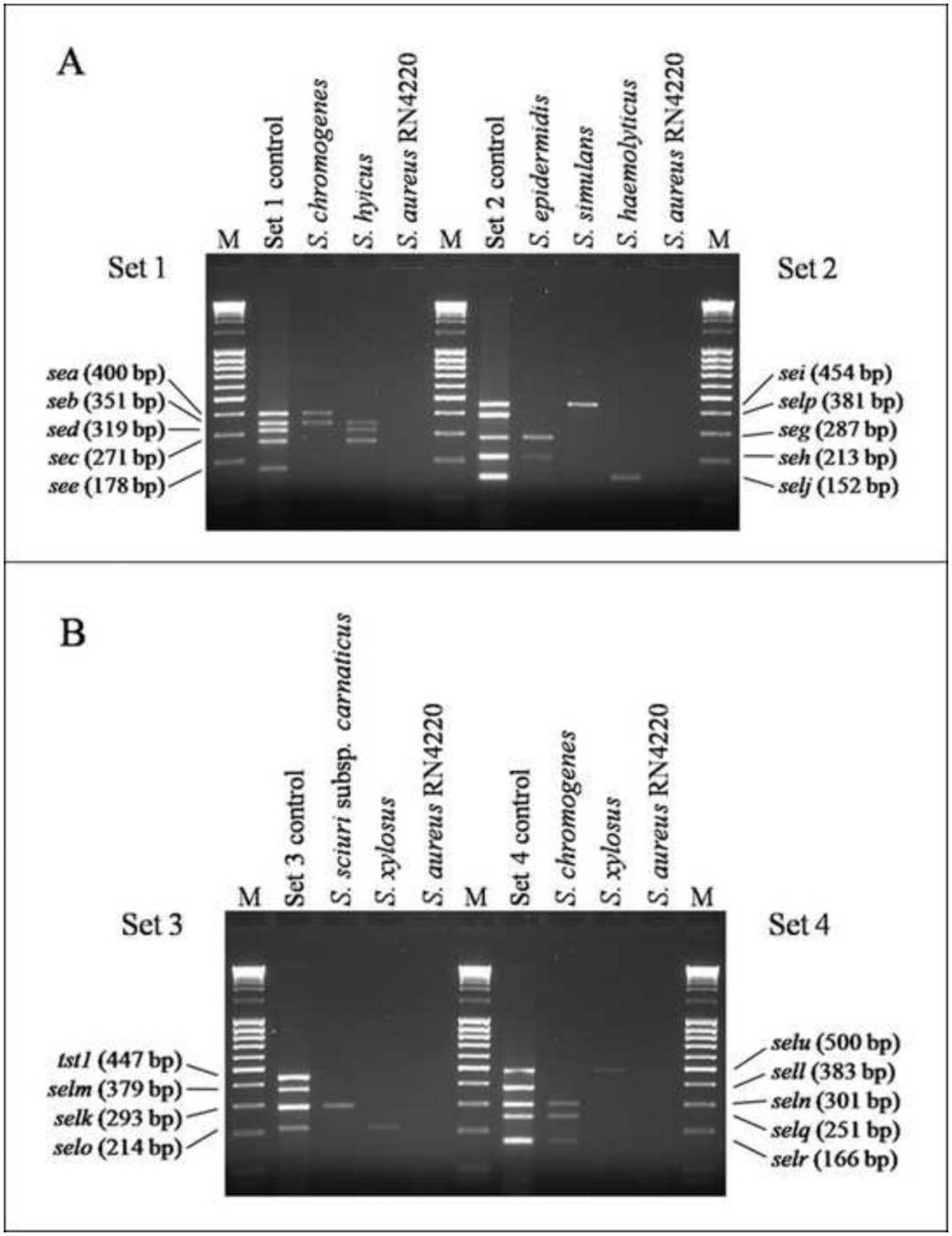

FIG. 2.

Detection of staphylococcal superantigen genes from CNS isolates from bovine intramammary infections by multiplex PCR. The mixtures of genomic DNAs from 5 *S. aureus* reference strains were used for multiplex PCR as a positive control for each primer sets. Genomic DNA extracted from *S. aureus* RN4220 was used as a negative control for multiplex PCR. Results shown are representative data obtained from various species of bovine IMI CNS isolates harboring SAg genes. (A) primer set 1 and 2, (B) primer set 3 and 4; Lane M: 100 bp DNA ladder.

Table 1

Nucleotide sequences of primers and expected size of PCR products (bp) of staphylococcal superantigenic toxins used in this study

Gene	Primer	Oligonucleotides sequence (5′to 3′)	PCR product (bp)	PCR set	References
sea	SEA-F	CAGCATACTATATTGTTTAAAGGC	400	1	This study
	SEA-R	CCTCTGAACCTTCCCATC			
seb	SEB-F	GTATGGTGGTGTAACTGAGCA	351	1	This study
	SEB-R	TCAATCTTCACATCTTTAGAATCA			
sec	SEC-F	CTCAAGAACTAGACATAAAAGCTAGG	271	1	(Becker et al., 1998)
	SEC-R	TCAAAATCGGATTAACATTATCC			
sed	SED-F	CTAGTTTGGTAATATCTCCTTTAAACG	319	1	(Becker et al., 1998)
	SED-R	TTAATGCTATATCTTATAGGGTAAACATC			
see	SEE-F	CAGTACCTATAGATAAAGTTAAAACAAGC	178	1	(Becker et al., 1998)
	SEE-R	TAACTTACCGTGGACCCTTC			
seg	SEG-F	AAGTAGACATTTTTGGCGTTCC	287	2	(Omoe et al., 2002)
	SEG-R	AGAACCATCAAACTCGTATAGC			
seh	SEH-F	GTCTATATGGAGGTACAACACT	213	2	(Omoe et al., 2002)
	SEH-R	GACCTTTACTTATTTCGCTGTC			
sei	SEI-F	GGTGATATTGGTGTAGGTAAC	454	2	(Omoe et al., 2002)
	SEI-R	ATCCATATTCTTTGCCTTTACCAG			
selj	SEJ-F	ATAGCATCAGAACTGTTGTTCCG	152	2	(Omoe et al., 2005)
	SEJ-R	CTTTCTGAATTTTACCACCAAAGG			
selk	SEK-F	TAGGTGTCTCTAATAATGCCA	293	3	(Omoe et al., 2005)
	SEK-R	TAGATATTCGTTAGTAGCTG			
sell	SEL-F	TAACGGCGATGTAGGTCCAGG	383	4	(Omoe et al., 2005)
	SEL-R	CATCTATTTCTTGTGCGGTAAC			
selm	SEM-F	GGATAATTCGACAGTAACAG	379	3	(Omoe et al., 2005)
	SEM-R	TCCTGCATTAAATCCAGAAC			
seln	SEN-F	CATCATGCTTATACGGAGGAG	301	4	This study

Gene	Primer	Oligonucleotides sequence (5′ to 3′)	PCR product (bp)	PCR set	References
	SEN-R	CCCACTGAACCTTTTACGTT			
selo	SEO-F	TGTGTAAGAAGTCAAGTGTAG	214	3	(Omoe et al., 2005)
	SEO-R	TCTTTAGAAATCGCTGATGA			
selp	SEP-F	TGATTTATTAGTAGACCTTGG	381	2	(Omoe et al., 2005)
	SEP-R	ATAACCAACCGAATCACCAG			
selq	SEQ-F	TCAAGGAGTTAGTTCTGGAAATT	251	4	This study
	SEQ-R	GCTTACCATTGACCCAGAGA			
selr	SER-F	GGATAAAGCGGTAATAGCAG	166	4	(Omoe et al., 2005)
	SER-R	GTATTCCAAACACATCTAAC			
selu	SEU-F	ATCAGAAACAAACATTAAAGCCCA	500	4	This study
	SEU-R	TGACCATTTCCTTCGATAAACTTTAT			
tst1	TST-F	AAGCCCTTTGTTGCTTGCG	447	3	(Becker et al., 1998)
	TST-R	ATCGAACTTTGGCCCATACTTT			

Table 2

S. aureus reference strains used in this study

Strains	Superantigen gene(s)	Reference
FRI 472	*sed, seg, sei, selj, selm, seln, selo, selr, selu*	(Monday and Bohach, 1999)
FRI 569	*seh*	(Su and Wong, 1995)
FRI 913	*sea, sec, see, selk, sell, selq, tst1*	(Bania et al., 2006)
MNHOCH	*seb*	(Monday and Bohach, 1999)
N315	*sec, seg, sei, sell, selm, seln, selo, selp, tst1*	(Kuroda et al., 2001)
RN4220	None	(Monday and Bohach, 1999)

Table 3

Staphylococcal superantigen (SAg) genes observed in coagulase-negative staphylococcal isolates from bovine intramammary infections

Species[a]	No. of isolates	No. of SAg gene-positive isolates																			
		SAg genes[b]	sea	seb	sec	sed	see	seg	seh	sei	selj	selk	sell	selm	seln	selo	selp	selq	selr	selu	tst1
S. chromogenes	190	42	4	33	1	1	0	0	0	0	2	4	0	0	33	0	0	37	1	3	0
S. xylosus	24	11	0	9	0	0	0	1	0	0	0	0	0	0	9	1	0	9	0	1	0
S. haemolyticus	16	6	0	3	0	1	0	0	0	0	3	0	0	0	3	0	0	3	0	0	0
S. sciuri subsp. *carnaticus*	9	7	0	2	0	0	0	0	0	4	0	1	0	0	2	0	0	2	0	0	0
S. hyicus	8	8	0	4	2	3	0	0	0	0	0	7	0	0	4	0	0	4	0	0	0
S. simulans	7	5	0	3	0	1	0	0	0	1	0	0	0	0	3	0	0	3	0	0	0
S. caprae	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
S. epidermidis	2	1	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0
S. succinus	2	1	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0
S. capitis	1	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
S. hominis	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Total	263	82	4	55	3	6	0	2	2	5	5	12	0	0	55	1	0	59	1	4	0

[a]The species was determined by partial 16S rRNA gene sequence analysis except *S. caprae* and *S. capitis* which were determined by partial *rpoB* gene sequence analysis.

[b]No. of isolates that were positive for at least one SAg gene.

Table 4

Combinations of staphylococcal superantigen (SAg) genes observed in 82 SAg gene-positive coagulase-negative staphylococcal isolates from bovine intramammary infections

Species[a]	Combinations of SAg genes	No. of isolates
S. chromogenes (*n* = 42)	*sea, seb, selj, selk, seln, selq*	1
	sea, seb, selj, selk, seln, selq, selr	1
	sea, seb, selk, seln, selq	1
	sea, selk	1
	seb, seln, selq	29
	seb, seln, selq, selu	1
	sec	1
	sed	1
	selq	4
	selu	2
S. xylosus (*n* = 11)	*seb, seln, selq*	8
	seb, seln, selq, selo	1
	seg	1
	selu	1
S. hyicus (*n* = 8)	*seb, sec, sed, selk, seln, selq*	1
	seb, sed, seln, selq	1
	seb, selk, seln, selq	2
	sec, sed, selk	1
	selk	3
S. sciuri subsp. *carnaticus* (*n* = 7)	*seb, seln, selq*	2
	sei	4
	selk	1
S. haemolyticus (*n* = 6)	*seb, sed, seln, selq*	1
	seb, seln, selq	2
	selj	3
S. simulans (*n* = 5)	*seb, seln, selq*	3
	sed	1
	sei	1
S. capitis (*n* = 1)	*seh*	1
S. epidermidis (*n* = 1)	*seh, seg*	1
S. succinus (*n* = 1)	*seb, seln, selq*	1

[a]The species was determined by partial 16S rRNA gene sequence analysis except *S. caprae* and *S. capitis* which were determined by partial *rpoB* gene sequence analysis.