Enterotoxin production, enterotoxin gene distribution, and genetic diversity of *Staphylococcus aureus* recovered from milk of cows with subclinical mastitis

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Objective—To evaluate enterotoxin production, enterotoxin gene distribution, and genetic diversity of *Staphylococcus aureus* in milk obtained from cows with subclinical mastitis.

Sample—Milk samples obtained from 350 cows (1,354 mammary glands) on 11 Wisconsin dairy farms.

Procedures—Of 252 *S aureus* isolates obtained from 146 cows, 83 isolates (from 66 cows with subclinical mastitis) were compared genotypically by use of pulsed-field gel electrophoresis and via PCR identification of toxic shock syndrome toxin 1 (*TSST-1*) and classical *S aureus* enterotoxin genes (*sea*, *seb, sec*, *sed*, and *see*).

Results—Among the 83 *S aureus* isolates, ≥ 1 enterotoxin genes were identified in 8 (9.6%). Enterotoxin gene distribution was as follows: *TSST-1*, 7 isolates (8.4%); *sec*, 5 isolates (6.0%); and *sed*, 2 isolates (2.4%). Enterotoxin genes *sea*, *seb*, and *see* were not identified. Twelve pulsotypes and 5 subtypes were identified among the 83 isolates; 5 of the 12 pulsotypes were represented by only 1 isolate. In cows of 1 herd, only a single *S aureus* pulsotype was detected; in cows on most other farms, a variety of pulsotypes were identified. One pulsotype was recovered from 4 farms (n = 23 cows) and another from 5 other farms (16). Isolates with an enterotoxin gene were represented by 6 pulsotypes.

Conclusions and Clinical Relevance—*S aureus* classical enterotoxins and *TSST-1* were rarely recovered from milk samples obtained from cows with subclinical mastitis in Wisconsin. Diverse pulsotypes of *S aureus* were detected within and among farms, indicating that different strains of *S aureus* cause subclinical mastitis in dairy cows. (*Am J Vet Res* 2011;72:1361–1368)

Staphylococcus aureus is a frequent cause of clinical and subclinical mastitis in dairy cows,[1–3] and the reported prevalence of infected cows in affected herds ranges widely.[4–6] In addition to causing mastitis in cows, *S aureus* can produce toxins responsible for food poisoning of humans.[7] The ingestion of foods contaminated with *S aureus* enterotoxins can cause fever, vomiting, nausea, abdominal pain, and diarrhea.[8,9] Depending on geographic location, staphylococcal food poisoning has been reported to be responsible for 14% to 40% of all microbial foodborne illness among humans.[10,11] The pathogenicity of *S aureus* is related to the production of extracellular protein toxins and virulence factors such as exotoxins, extracellular enzymes, surface proteins, and capsule polysaccharides.[7] These factors are responsible for a variety of diseases caused by *S aureus* in humans and other animals.

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Abbreviations

PFGE	Pulsed-field gel electrophoresis
SCC	Somatic cell count

Enterotoxigenic strains of *S aureus* produce at least 19 variants of enterotoxins, including the classical enterotoxins (*sea* through *see*), toxic shock syndrome toxin 1 gene (*TSST-1*), and newly discovered enterotoxins (*seg* through *ser* and *seu*). These enterotoxins are known as superantigens because of their ability to polyclonally activate T cells. This activation leads to excessive production of proinflammatory cytokines and T-cell proliferation, causing massive systemic release of proinflammatory cytokines that can lead to clinical signs that include fever, hypotension, and shock. The superantigens might facilitate immunosuppression in cattle and contribute to chronic intramammary infection. Milk and dairy products are considered to be a primary source of human exposure to *S aureus* enterotoxins.[12] Several researchers have reported variability in the prevalence of *S aureus* enterotoxin genotypes among farms and countries.[13,14] In studies[7,14–16] conducted in Germany, Brazil, Japan, and the United States, the prevalence of enterotoxin genes in *S aureus* isolates has been reported to range from 10% to 70%. This variability may be related to differences in severity of mastitis, geographic location of herds, laboratory methods, and the identification of different enterotoxins among studies.

In dairy herds, control of mastitis caused by *S aureus* is based on rapid identification of infected individuals and adoption of strategies that decrease the opportunity for spread among cows. The use of molecular methods for bacterial typing has proven helpful in human and veterinary epidemiological investigations to establish virulence factors, to identify bacterial strains for vaccine production, and for targeting antibacterial drugs for more effective disease control.[17,18]

Several researchers have suggested that a few specialized clones of *S aureus* are responsible for most intramammary infections,[17,19–21] whereas other researchers have suggested that strains are herd specific.[22,23] In addition, the presence of predominant strains within herds has been a consistent finding.[19,20,22] At least 1 group has suggested that mastitis control programs should be focused on specific strains within herds.[24] Further information about the association between virulence factors and strain may therefore improve mastitis control programs on dairy farms.

The purpose of the study reported herein was to evaluate the production of classical enterotoxins and *TSST-1*, distribution of enterotoxin genes, and genetic diversity of *S aureus* recovered from milk obtained from cows with subclinical mastitis.

Materials and Methods

Herd and cow selection criteria—Wisconsin dairy herds (n = 11) were recruited through practicing veterinarians and extension agents. Enrollment criteria required herds to have mastitis problems caused by *S aureus*, and most herds based this information on results of previous bacteriologic cultures of bulk tank milk. Study personnel collected milk samples from eligible cows during a single visit to each farm. Cows were selected for sample collection on the basis of the following criteria: 4 to 305 days in lactation, SCC > 200,000 cells/mL (determined at the most recent Dairy Herd Information Association monthly test), and normal appearance of milk on the day of sample collection. Milk samples were obtained from cows with apparently new subclinical infections (SCC > 200,000 cells/mL only in the month of collection) and from cows with apparently chronic infections (SCC > 200,000 cells/mL for ≥ 2 consecutive months previous to the visit).

Collection of milk samples—Two milk samples for microbiological culture and an additional sample for SCC assessment were collected from all mammary glands of cows included in the study by use of aseptic techniques.[25] Milk samples were cooled rapidly, transported to the University of Wisconsin-Madison Milk Quality Laboratory, and plated onto bacterial culture medium on the day of collection. Determination of SCC was performed via flow cytometry at a commercial Dairy Herd Information Association laboratory.[a] For analysis, SCCs were logarithmically transformed (base 10) to achieve homogenous variance.

Bacteriologic culture—For the primary culture, 0.10 mL of each milk sample collected from each mammary gland was streaked on half of a blood agar plate and one-quarter of a MacConkey agar plate. Plates were incubated at 37°C for 24 hours. Except for use of a greater inoculum volume, pathogens were identified to species level by use of laboratory procedures as defined by National Mastitis Council guidelines.[25] The increased inoculum volume was used to enhance recovery of *S aureus* organisms that were being shed at a low level. Furthermore, to improve recovery of *S aureus*, an enhancement method was used for some samples that initially yielded negative culture results.[26] Briefly, if no microbial colonies were detected following primary culture (after 24 hours of incubation), the same refrigerated milk samples were incubated for 6 hours at 37°C, after which 1 mL of milk was plated on a commercial culture medium[b] developed to identify *S aureus*. By use of this system, *S aureus* was initially identified following the manufacturer's instructions.[b] *Staphylococcus aureus* was then confirmed to be present by replating 2 representative colonies on blood agar and differentiation from other staphylococci by means of a mannitol test and coagulase tube test. Final confirmation was provided by use of a commercial biochemical identification system.[c] Suspected *Streptococcus* spp were identified as gram-positive cocci that had a negative catalase reaction and by use of the Christie, Atkins, Munch-Petersen test and esculin reaction. Gram-negative bacteria were identified on the basis of results of culture on MacConkey agar, assessment of motility and indole and ornithine reactions, and growth on triple sugar iron agar. In addition, identification of most aerobic gram-positive bacteria was determined by use of the appropriate commercial identification system[c,d] with a confidence level > 70%. All milk samples were screened for *Mycoplasma* spp by use of comingled milk (32 gland samples from 8 cows/plate) inoculated on mycoplasma culture medium[e] and incubated under microaerophilic conditions. Milk samples and isolates were frozen and stored until used for further analysis. Milk samples were considered contaminated if ≥ 3 dissimilar colony types were found in the same sample. Except for *S aureus*, results of cultures were considered negative when < 3 CFUs of the same colony type were seen on the plate. To increase the sensitivity of recovering *S aureus*, the presence of ≥ 1 CFU was considered a positive result. This alternative detection limit was chosen to minimize false-negative results caused by intermittent shedding of *S aureus*. A mammary gland was considered infected when the same mastitis pathogen was isolated from the duplicate milk samples collected from that gland. Each isolate used in further analysis was obtained from a different mammary gland.

Detection of *S aureus* enterotoxin genes—Selected *S aureus* isolates (n = 83) were used to perform PCR analysis to detect genes of the classic enterotoxins and *TSST-1*. Isolates were selected from among all farms by use of nonprobability sampling. Genomic DNA was extracted by use of a commercial kit.[f] Primers for sets described elsewhere[27] were used for the amplification of the genes encoding *sea*, *seb*, *sec*, *sed*, *see*, and *TSST-1*. All primers were synthesized at a commercial laboratory.[g] All *S aureus* strains used as positive controls were obtained from the University of Wisconsin Food Research Institute.

For PCR amplification, the reaction mixture (25 µL) contained 5X reaction buffer,[h] 2.5mM $MgCl_2$, 200µM of each deoxyribonucleotide triphosphate, 5mM forward primers, 5mM reverse primers, 1.25 U of *Taq* DNA polymerase, and nuclease-free water. All PCR reactions were performed by use of a DNA thermal cycler.[i]

The cycling conditions for the amplification reaction were as follows: initial denaturation at 94°C for 4 minutes followed by 37 cycles of denaturation at 94°C for 2 minutes; annealing of primers for *sea*, *sec*, *sed*, and *TSST-1* at 51°C, for *seb* at 48°C, and for *see* at 52°C for 1 minute 30 seconds; and extension at 72°C for 1 minute 30 seconds, followed by a final elongation at 72°C for 2 minutes. One negative control was also included in each series of amplifications. Following amplification reactions, the PCR products were analyzed via electrophoresis in a 1.5% agarose gel at 105 V for 45 minutes in 0.5X Tris-borate-EDTA buffer (45mM Tris-HCL, 45mM boric acid, and 1mM EDTA). A 100-bp ladder[h] was loaded after every 10 wells loaded with a PCR product. Following electrophoresis, the gels were stained with ethidium bromide solution (0.05 mg/L) and examined under UV florescence. The presence of a band at the expected product size was considered a positive result.

Detection of *S aureus* enterotoxins—Samples (n = 83) used for detection of enterotoxin genes were also assessed for the presence of selected enterotoxins by use of a commercial ELISA kit.[j] The enterotoxins of interest were *sea*, *seb*, *sec*, *sed*, and *see*. A plate contained 96-wells; 83 wells were used for assessment of enterotoxin protein of isolates, 11 wells were used for positive controls, and 2 wells were used for negative controls.

PFGE of DNA macrorestriction fragments—The same isolates (n = 83) were analyzed via PFGE of DNA macrorestriction fragments with the restriction endonuclease *Sma*I[k] following a modified procedure that has been previously described.[28] *Staphylococcus aureus* isolates placed in agarose plugs were digested overnight with *SmaI* to improve the digestion quality. Pulsed-field gel electrophoresis of DNA digests was performed with a variable field angle system.[l] All restriction bands were normalized to *S aureus* (National Collection of Type Cultures [NCTC] 8325), which was included in the first, eighth, and final lanes of each gel. Gels were stained with ethidium bromide for 1 hour, destained in distilled water, and photographed.[m] Macrorestriction patterns were analyzed visually and by use of a computer program.[n] Visual interpretation guidelines[29] and the results were based on agreement between the same 2 observers (LO and CH). Isolates with identical restriction profiles were assigned the same type and identified with a capital letter. Isolates that differed from main types by 1 to 3 band shifts consistently for a limited number of genetic events were assigned subtypes and indicated with a numeric suffix. Isolates with > 3 such differences were considered different types.

Results

Duplicate milk samples were obtained from each of 1,354 mammary glands of 350 cows located on 11 commercial dairy farms that were visited between January and June 2007. Among the 350 cows, 40 had < 4 functional mammary glands.

The median herd size was 170 milking cows (range, 50 to 900). Farms milked cows in parlors (n = 6) or stallbarns (5), and 4 herds were milked 3 times daily. Overall, recommended practices such as wearing gloves during milking, forestripping, premilking and postmilking teat disinfection, and comprehensive intramammary nonlactating cow treatment were widely used.

Of 1,354 mammary gland samples, the most common microbiological result was no growth of bacteria (616 [45.5%] mammary gland samples). The distribution of additional microbiological culture results was as follows: *S aureus*, 252 (18.6%) mammary gland samples; coagulase-negative staphylococci, 203 (15.0%) mammary gland samples; *Streptococcus* spp, 52 (3.8%) mammary gland samples; gram-negative bacteria, 34 (2.5%)

Table 1—Proportion, distribution, and pulsotype characterization of *Staphylococcus aureus* isolates in mammary gland milk samples collected from 350 cows with subclinical mastitis on 11 farms.

			Proportion of mammary gland samples positive for *S aureus*		Proportion of mammary gland samples selected for analysis		
Farm	Total No. of cows	Total No. of mammary gland samples	No. of cows	No. of mammary glands (%)	No. of cows	No. of mammary glands (%)	Pulsotype* (No. of isolates)
1	29	113	8	13 (11.5)	6	9 (10.8)	A (6), B (2), and C (1)
2	29	115	13	16 (14.0)	5	5 (6.0)	F (3) and G (2)
3	38	143	34	73 (51.0)	9	11 (13.2)	A (3), D (1), E (1), and G (6)
4	34	133	6	10 (7.5)	7	10 (12.0)	I (9) and J (1)
5	32	125	20	44 (35.2)	10	12 (14.5)	A (12)
6	29	116	8	9 (7.7)	4	5 (6.0)	F (1) and H (4)
7	30	114	17	28 (24.5)	4	7 (8.4)	A (2), B (4), and L (1)
8	30	116	7	8 (6.9)	2	2 (2.4)	F (1) and G (1)
9	40	155	17	29 (18.7)	11	12 (14.5)	B (1) and K (11)
10	29	111	11	15 (13.5)	6	7 (8.4)	F (2) and G (5)
11	30	113	5	7 (6.2)	2	3 (3.6)	F (1) and G (2)
Total	350	1,354	146	252† (18.0)	66	83† (33)	

*Different pulsotypes are denoted by letters A to L. †Each isolate was obtained from a different mammary gland.

mammary gland samples; other pathogens including *Corynebacterium* spp, *Arcanobacterium pyogenes*, *Bacillus* spp, *Proteus* spp, and yeast, 59 (4.4%) mammary gland samples; and contaminated, 138 (10.2%) mammary gland samples. *Staphylococcus aureus* were recovered from 252 mammary gland samples obtained from 146 cows. Of these isolates, 209 were isolated on blood agar, and 43 were recovered by use of secondary inoculation of incubated samples as described. On the basis of SCC history, *S aureus* infections among 252 mammary glands were categorized as chronic infection (n = 149 [59%]), defined as SCC > 200,000 cells/mL for ≥ 2 consecutive months prior to the visit; new infection (36 [14%]), defined as SCC > 200,000 cells/mL only in the month of collection; or undefined status (67 [27%]), when SCC records were unavailable. Of 252 *S aureus* isolates obtained from mammary glands, few were nonhemolytic (6 [2.4%] mammary gland samples), mannitol negative (5 [2%] mammary gland samples), or coagulase negative (12 [4.8%] mammary gland samples), and all had a confidence level ≥ 70% according to the commercial identification system.[c] Proportion of mammary glands with positive results for *S aureus* among farms ranged from 6.2% to 51.0% **(Table 1)**. The $\log_{10}$SCC values among *S aureus*–containing mammary gland samples ranged from 4.4 to 7.0 (mean, 6.0).

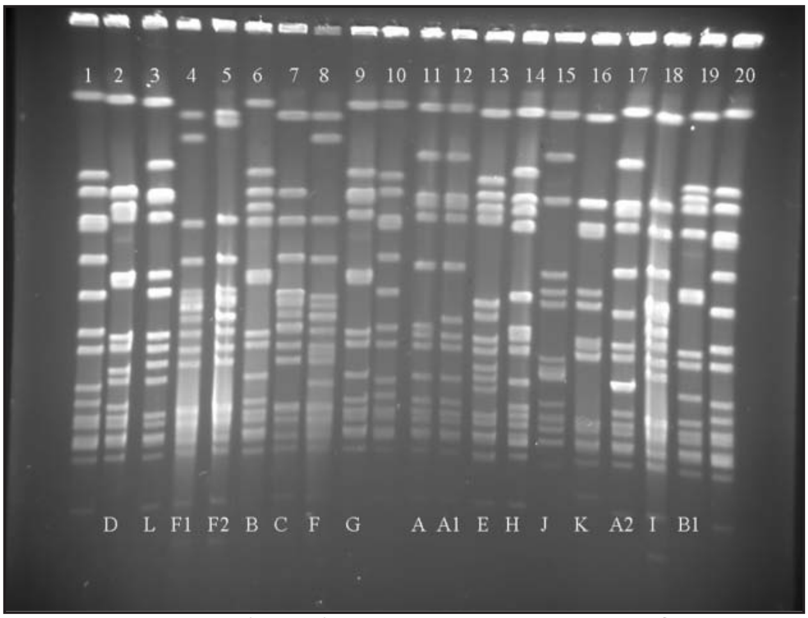

Figure 1—Distribution of DNA fingerprint patterns obtained via *Sma*I digestion and PFGE analysis of genomic DNA from 83 *Staphylococcus aureus* isolates recovered from mammary gland milk samples collected from 66 cows with subclinical mastitis on 11 farms. The PFGE analysis revealed 12 pulsotypes (A to L) and 5 subtypes (A.1, A.2, B.1, F.1, and F.2). All restriction bands were normalized to *S aureus* (National Collection of Type Cultures [NCTC] 8325), which was included in lanes 1, 10, and 20.

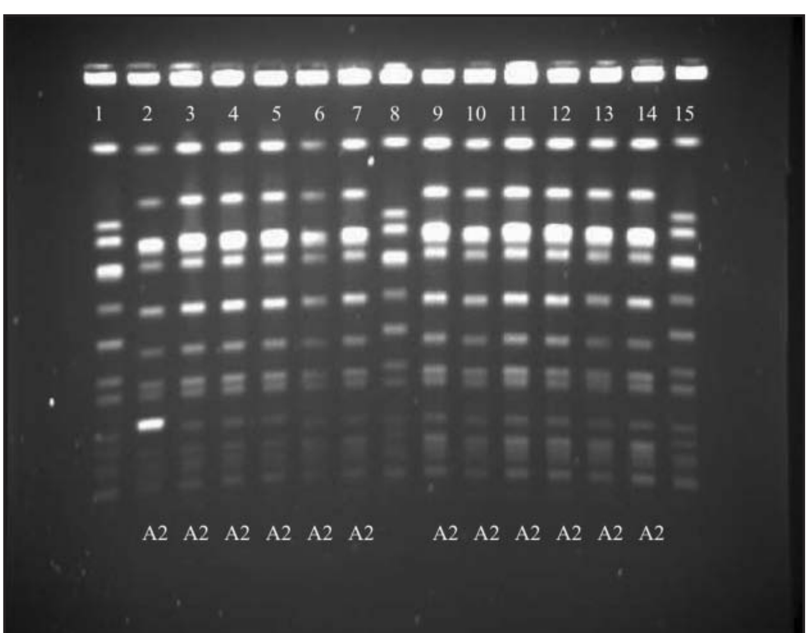

Figure 2—Comparison of representative DNA fingerprint patterns obtained via *Sma*I digestion and PFGE analysis of genomic DNA from *S aureus* isolates recovered from 12 mammary gland milk samples collected from cows with subclinical mastitis on 1 farm (lanes 2 through 7 and 9 through 14). All restriction bands were normalized to *S aureus* (NCTC 8325), which was included in lanes 1, 8, and 15. Notice that the *S aureus* isolates in samples collected from cows in this herd were homogeneous for a single pulsotype.

Of the 252 *S aureus* isolates obtained from 146 cows, 83 isolates were selected from 66 (45%) cows for assessment of enterotoxin genes and enterotoxin protein and for molecular characterization by use of PFGE. A single 96-well plate allowed for assessment of enterotoxin protein of 83 isolates; 11 wells were used for positive controls, and 2 wells were used for negative controls. The selected isolates represented all 11 farms and met specific identification requirements, such as growth in pure culture by use of blood agar, positive mannitol and coagulase reactions, and ≥ 70% confidence level reported by use of a commercial identification system.[c] The median number of isolates selected per farm was 7 (range, 2 [2.5%] to 12 [14.5%]; Table 1). The $\log_{10}$SCC values for the milk samples from which the selected *S aureus* isolates were obtained ranged from 4.2 to 7.0 (mean, 6.3).

Identification of *S aureus* enterotoxins—Among the 83 selected isolates, 8 (9.6%) were positive for ≥ 1 enterotoxin gene. For these 8 isolates, the distribution of genes encoding enterotoxins was as follows: *TSST-1*, 7 (8.4%) isolates; *sec*, 5 (6.0%) isolates; and *sed*, 2 (2.4%) isolates. Enterotoxin genes *sea*, *seb*, and *see* were not recovered from any mammary gland samples. Samples that encoded enterotoxin genes were obtained from cows on 7 of the 11 farms. Isolates with positive test results for *sec* were obtained from milk samples from 4 farms,

Table 2—Distribution of *S aureus* pulsotypes A through L by identification of enterotoxin gene and detection of enterotoxin in mammary gland milk samples collected from 66 cows with subclinical mastitis on 11 farms.

		Enterotoxin genes detected		No enterotoxin genes detected	
Farm	No. of isolates	Enterotoxin detected (No. of isolates)	No enterotoxin detected (No. of isolates)	Enterotoxin detected (No. of isolates)	No enterotoxin detected (No. of isolates)
1	9	—	C (1)	A (1)	A (5) and B (2)
2	5	—	G (1)	—	F (3) and G (1)
3	11	—	—	D (1)	A (3), E (1), and G (6)
4	10	—	—	—	I (9) and J (1)
5	12	—	—	—	A (12)
6	5	—	—	—	F (1) and H (4)
7	7	—	A (1)	—	A (1), B (4), and L (1)
8	2	F (1)	—	—	G (1)
9	12	—	K (1)	—	B (1) and K (10)
10	7	—	F (2)	G (1)	G (4)
11	3	—	G (1)	—	F (1) and G (1)

— = Not applicable.
See Table 1 for remainder of key.

isolates with positive test results for *sed* were obtained from milk samples from 2 farms, isolates with positive test results for *TSST-1* were obtained from milk samples from 6 farms, and isolates with positive test results for the 3 enterotoxins were obtained from milk samples from 2 farms. The $\log_{10}$SCC values for the milk samples from which the *S aureus* isolates that were positive for enterotoxin genes were obtained ranged from 6.0 to 7.0 (mean, 6.6).

Samples (n = 83) used for detection of enterotoxin genes were also assessed for the presence of selected enterotoxins proteins by use of an ELISA to detect these proteins in milk. Only 4 (4.8%) samples produced enterotoxin A to E. Curiously, 3 samples lacked the presence of any gene encoding the enterotoxins assessed in this study. Moreover, 2 had minimal concentrations of enterotoxin (0.206 and 0.244 μg/mL).

All isolates were typeable by use of PFGE. Digestion of genomic DNA from *S aureus* isolates with *Sma*I produced 9 to 14 fragments that ranged in size from < 60 to 650 kbp. Twelve pulsotypes (A to L) and 5 subtypes (A.1, A.2, B.1, F.1, and F.2) were identified by use of PFGE among samples from the 11 farms. The most predominant pulsotype and subtype, respectively, were A (n = 23) and A.2 (12; **Figure 1**). Five of 12 pulsotypes were represented by a single isolate, and 8 of the pulsotypes were found only on a single farm. The most predominant pulsotypes were A (n = 23) and G (16), recovered from 4 and 5 different farms, respectively. The $\log_{10}$SCC values for the milk samples from which those pulsotypes were obtained ranged from 6.1 to 7.0 (mean, 4.4).

Isolates from only 1 herd (containing 80 lactating cows) were homogeneous for a single pulsotype (A.2; **Figure 2**). On that farm, 44 of 133 (35%) collected mammary gland samples were infected with *S aureus*. More than 2 pulsotypes were identified in mammary gland samples from approximately a third of herds. Two pulsotypes were identified in mammary gland samples from 7 herds. Eight pulsotypes were unique for an individual herd, but 5 were not predominant in the specific herd from which they were recovered (Table 1). Four pulsotypes were identified on the farm that had the greatest proportion of isolates with positive test results for *S aureus*.

Isolates that carried an enterotoxin gene were represented by 5 pulsotypes, predominantly pulsotype F (n = 3; **Table 2**). The same combinations of enterotoxins were recovered from multiple pulsotypes. For example, *TSST-1* and enterotoxin gene *sec* were found together in 3 different pulsotypes (including C, F, and G). Specific pulsotypes had different characteristics among herds. For example, pulsotype A was present in mammary gland samples from 4 herds but encoded enterotoxin genes only in samples from 1 herd. Isolates expressing enterotoxin were represented by 4 pulsotypes. Of 6 mammary gland samples from the same farm, enterotoxin was found in only 1, although all strains were indistinguishable by use of PFGE.

Discussion

Each herd enrolled in the present study was selected because its history suggested that *S aureus* would be recovered from mammary gland samples collected from cows with subclinical mastitis, and *S aureus* had been previously isolated from bulk tank milk. Unfortunately, the project budget did not allow the characterization of enterotoxin from isolates obtained from all subclinically infected cows (n = 146); nevertheless, isolates from 66 cows were assessed. In the present study, the number of *S aureus* isolates evaluated for enterotoxin was comparable to that evaluated in similar studies[7,16,30]; furthermore, sample collection was performed on a greater number of farms, compared with previous studies conducted in Brazil, Germany, and the United States. Previous studies have included 72 isolates obtained from cows[30] with clinical and subclinical mastitis in Brazil and 78 isolates obtained from cows[16] with clinical mastitis in the United States. German researchers identified several toxin genes recovered from 103 *S aureus* strains originating from 66 cows in 8 herds.[7] The selection criteria used in the study reported here were intended to minimize clustering of characterized isolates within herds and apply consistent microbiological diagnostic criteria. The selected 83 isolates were contributed from each of the 11 herds, and no herd contributed > 15% of the isolates that were further characterized. However, results of this study should not be used to estimate statewide prevalence of enterotoxin or enterotoxin genes because such estimates would require investigation of a random selection of herds and isolates.

The presence of classical enterotoxin genes in *S aureus* isolates recovered from cows with mastitis has been investigated, although none of the previous studies[7,16,31,32] evaluated milk samples obtained from cows with subclinical mastitis on a variety of farms in the United States. The inclusion of cows with subclinical mastitis in the present study is potentially important because this milk can be legally sold for human consumption.

Previously, researchers have reported variability in the prevalence of enterotoxin genotypes among farms and countries.[7,32,33] The recovery of enterotoxin genes in the present study was in agreement with results of other studies.[31,32] In 1 study[32] conducted in Mexico, none of the isolates (n = 41) yielded the expected amplification products for the *sea*, *seb*, and *sec* genes; however, *sed*, *see*, and *TSST-1* genes were not assessed. The authors concluded that these genes do not participate in the pathogenesis of *S aureus* mastitis in cows in that country. In contrast, other researchers[16] found that 73 of 78 (93.6%) *S aureus* isolates recovered from cows with clinical mastitis on 2 US farms carried some enterotoxin genes. Genes for *sea* and *see* were not identified, but the gene for *sed* was frequently detected (52.6% of isolates). Those researchers reported greater prevalence of newly identified enterotoxin genes (*seg* to *seq*), compared with classical enterotoxin genes (*sea* to *see*). The results of the present study may differ from previous investigations because of different clinical characteristics of mastitis and inclusion of a broader population of farms or because more recently identified enterotoxins were not investigated. It is also possible that there is variability in the prevalence of enterotoxin genes among geographic regions.

Coexistence of *sec* and *TSST-1* was detected in 4 mammary gland samples in the present study. The combination of *sec* and *TSST-1* in *S aureus* recovered from cows with subclinical mastitis on 8 farms in Germany[7] and from cows with peracute mastitis on Japanese farms[34] has been previously reported. Interestingly, the Japanese researchers reported that all isolates recovered from cows with peracute clinical mastitis produced *TSST-1* and *sec*, whereas the cows from which isolates were obtained that produced both *TSST-1* and *sec* in the present study had subclinical mastitis. The production of this combination of enterotoxins and its role in mastitis are unknown, and there is little homology between these 2 toxins. It has been reported that a virulence factor is considered important when the gene encoding it as well as its expression is present in the isolated strains.[13] The present study involved only cows with subclinical mastitis, for which the causative organisms were expected to be less virulent than those associated with clinical cases reported in other studies.

Consistent with results of other studies,[15,31] none of the isolates recovered in the present study had *sea*, *seb*, or *see* genes. Researchers[31] have previously failed to identify *sea*, *sed*, and *see* in 84 *S aureus* isolates recovered from milk from 84 dairy cows with subclinical mastitis in Spain. In contrast, *see* and *seb* were found in greater prevalence in milk from cows with clinical and subclinical mastitis in Brazil.[30] In that study, 72 *S aureus* isolates were analyzed for production of *sea*, *seb*, *sec*, and *sed* enterotoxins and *TSST-1* by use of an optimum-sensitivity plate method and 52.8% of these strains produced *sea* and *seb* enterotoxins.

Similar to results of past research,[35] production of enterotoxin was evident in only a few milk samples in the present study. In another Brazilian study,[35] 209 milk samples from cows with clinical and subclinical mastitis caused by *S aureus* were analyzed; approximately 4% (n = 9) of the isolates produced enterotoxins A to D as determined by use of a reverse passive latex agglutination method. The present study used an ELISA for detection of enterotoxin because it is an official Association of Official Analytical Chemists test. Using PCR techniques, we were unable to identify genes that encoded for enterotoxin in 3 mammary gland samples in which enterotoxin was detected. It is known that *sed* is encoded by the plasmid pIB485[36] that also encodes *sej*, and they are separated from each other by an intergenic region[37]; hence, it is possible that the positive results of the ELISA could have been caused by a cross-reaction. A previous study[38] that used isolates recovered from cows with subclinical mastitis revealed an association of 98.6% between *sed* and *sej*. Another possible reason for cross-reaction is that *S aureus* may be able to produce unidentified enterotoxins.[39] In addition, the difference between ELISA results and amplification of enterotoxin genes could be an indication of the existence of sequence variations in *se* genes.

Macrorestriction analysis with *Sma*I and PFGE was performed to evaluate genetic relatedness among the *S aureus* isolates in the present study. Among all isolates, 8 of the 12 pulsotypes were unique to individual herds. Similarly, a prior study[22] revealed that two-thirds of *S aureus* pulsotypes (n = 34) isolated from 181 cows with subclinical mastitis in Korea were unique to 1 herd. In another investigation,[7] 103 *S aureus* isolates were obtained from 60 cows with clinical mastitis on 8 farms in Germany; it was concluded that a limited number of specialized clones of *S aureus* were responsible for mastitis in cows. The results of the present study were similar because 3 predominant pulsotypes (A, G, and K) represented 50 (60%) isolates obtained among 8 farms. Another study[20] compared PFGE and binary typing for the differentiation of 38 *S aureus* isolates obtained from dairy cows on farms in The Netherlands. Those researchers reported a limited number of predominant types among herds, suggesting that certain variants may have a predilection for causing intramammary infection. In the present study, homogeneous pulsotypes were identified in mammary gland samples from only 1 herd; this small farm contained 80 lactating cows (Holstein, Jersey, and crossbred) that were housed in a tie stall barn and milked twice each day. Farm personnel used only minimal control procedures for contagious mastitis. The findings of the present study suggest that a herd will generally have a predominant strain of *S aureus* but that both heterogeneity and homogeneity among herds exist. For example, in contrast to the homogeneous herd, pulsotypes from herd 4 were heterogeneous (4 pulsotypes) but 1 pulsotype (G) was predominant in this herd.

Although the present study did not include sufficient enterotoxin-producing strains of *S aureus* to test the association between pulsotype and enterotoxin gene, the diversity of pulsotypes that carried the same enterotoxin genes suggested that this association may be lacking. Future studies that include a larger number of isolates obtained from many farms, including a variety of pulsotypes and subtypes, should be performed to determine potential associations. Other researchers have reported a lack of association between pulsotype and enterotoxin genes.[16,40] However, some studies[31,41,42] have identified certain similarities between the pulsotype and enterotoxin biotypes. In those 3 studies, isolates obtained from different sources (human, animal, or food origin) were analyzed and a possible variation based on host was detected. One study[42] involved analysis of 91 strains of enterotoxigenic *S aureus* and 20 nonenterotoxigenic strains isolated from raw cow's milk, raw cow's milk cheeses, and the dairy environment. The investigators concluded that 1 pulsotype was exclusively responsible for the production of enterotoxin D, but for the other enterotoxins, no association was evident.

It is important to emphasize that most outbreaks of staphylococcal food poisoning among humans have involved enterotoxin A and that none of the isolates recovered from the cows on the study farms had *sea*, *seb*, or *see* genes. Further studies are needed to examine the prevalence of newly identified enterotoxins and their association with outbreaks of human disease.

Classical enterotoxins and *TSST-1* were rarely identified in *S aureus* recovered from mammary gland milk samples obtained from cows with subclinical mastitis in Wisconsin. Most herds had heterogeneity of *S aureus* pulsotypes; however, 1 herd had pulsotype homogeneity. Results of the present study indicated that there are diverse *S aureus* pulsotypes that cause mastitis. Future research should be directed toward studying the presence of newly discovered enterotoxins and risk factors related with the diversity of *S aureus* causing mastitis in dairy cows.

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a. Agsource CRI, Verona, Wis.
b. Petrifilm Staph Express plates, 3M, Saint Paul, Minn.
c. API Staph, bioMerieux, Durham, NC.
d. API 20 Strep, bioMerieux, Durham, NC.
e. Biological Media Services, School of Veterinary Medicine, University of California-Davis, Davis, Calif.
f. DNeasy kit, Qiagen, Valencia, Calif.
g. DNA Synthesis Facility, Biotechnology Center, University of Wisconsin, Madison, Wis.
h. Flexi buffer, Promega Corp, Madison, Wis.
i. MyCycler, 96 wells, Bio-Rad Laboratories Inc, Hercules, Calif.
j. Tecra Staphylococcal Enterotoxins A-E ELISA kit, 3M, Saint Paul, Minn.
k. New England Bio Labs, Beverly, Mass.
l. CHEF-DR III, Bio-Rad Laboratories, Hercules, Calif.
m. FOTO/Analyst Investigator Eclipse, 8-bit standard dual light workstation, FOTODYNE Inc, Hartland, Wis.
n. Quantity One analysis software, version 4.6.3, Bio-Rad Laboratories Inc, Hercules, Calif.

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