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Research Note

Characterization of *Staphylococcus aureus* Strains Isolated from Raw Milk Utilized in Small-Scale Artisan Cheese Production

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ABSTRACT

Staphylococcus aureus is an important agent of bacterial mastitis in milking animals and of foodborne intoxication in humans. The purpose of this study was to examine the genetic and phenotypic diversity, enterotoxigenicity, and antimicrobial resistance of *S. aureus* strains isolated from raw milk used for the production of artisan cheese in Vermont. Cross-tabulations revealed that the 16 ribotypes identified among the 90 milk isolates examined were typically associated with a specific animal species and that more than half of these ribotypes were unique to individual farms. In general, specific *Eco*RI ribotypes were commonly associated with specific phenotypical characteristics, including staphylococcal enterotoxin production or the lack thereof. Limited antimicrobial resistance was observed among the isolates, with resistance to ampicillin (12.51%) or penicillin (17.04%) most common. Two isolates of the same ribotype obtained from the same farm were resistant to oxacillin with 2% NaCl. More than half (52.22%) of isolates produced toxin, and 31 of the 32 isolates solely produced staphylococcal enterotoxin type C. Although these data demonstrate that *S. aureus* strains found in raw milk intended for artisan cheese manufacture are capable of enterotoxin production, staphylococcal enterotoxin C is not typically linked to foodborne illness. Because *S. aureus* is a common contaminant of cheese, an understanding of the ecology of this pathogen and of the antimicrobial susceptibility and toxigenicity of various strains will ultimately contribute to the development of control practices needed to enhance the safety of artisan and farmstead cheese production.

Staphylococcus aureus is an important cause of bacterial mastitis in lactating animals and is frequently isolated from raw milk intended for direct consumption and/or the manufacture of raw milk products *(9, 10, 13)*. Staphylococcal mastitis also plays a key role in food safety because dairy animals with subclinical *S. aureus* mastitis may shed large numbers of the organism into milk. Numerous outbreaks of staphylococcal food poisoning linked to milk and milk products, including cheese, have been reported globally *(3, 11, 24)*. The proportion of dairy related illnesses from staphylococcal poisoning in the United States has decreased substantially in the past 40 years as a result of increased monitoring of mastitis in dairy cattle coupled with improved sanitation and the implementation of pasteurization *(24)*. Despite similar improvements, *S. aureus* has been reported as the leading etiologic agent of foodborne disease related to milk and milk products in France *(11)*.

Staphylococcal food poisoning occurs not as the result of the ingestion of the organism itself but through ingestion of preformed enterotoxins produced by some strains of *S. aureus (19)*. In addition to the classical staphylococcal enterotoxin (SE) types (SEA, SEB, SEC, SED, and SEE), extensive sequence data have led to the discovery of novel SEs and SE-like superantigens whose potential role in staphylococcal food poisoning has yet to be confirmed in many cases *(20)*. The behavior and enterotoxin production of *S. aureus* in cheese varies depending on the interaction of numerous factors, including cheese type, NaCl concentration, water activity, the activity, amount, and type of starter culture utilized, competition for nutrients, decreasing pH, and the production of undissociated weak acids and antistaphylococcal substances *(14, 19)*.

Data on the phenotypic and genotypic characteristics and antimicrobial resistance of *S. aureus* isolates recovered from milk intended for cheesemaking are scant, especially for the milk of sheep and goats. The objectives of this study were to identify the (i) genealogical relatedness, (ii) antimicrobial susceptibility, and (iii) enterotoxigenicity of *S. aureus* strains isolated from raw milk intended for small-scale artisan cheese manufacture in Vermont.

MATERIALS AND METHODS

Bacterial cultures. As part of surveillance work conducted in 2006 *(10)*, 2008 *(9)*, and 2009 (unpublished data), a total of 90 isolates were recovered from raw milk collected from 15 farms producing cheese from cow's ($n = 44$), goat's ($n = 19$), and/or sheep's ($n = 27$) milk. In almost all cases, the raw milk analyzed was used without prior heat treatment for the manufacture of raw milk cheese. The presence of *S. aureus* was detected and confirmed as previously described *(9, 10)*. Frozen stock cultures were

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TABLE 1. *Phenotypic variation among 90 isolates of* Staphylococcus aureus *identified by ribotype*

		Toxin production		Lipase activity		Hemolysis		Bound coagulase	
Ribotype	*n*	Negative	Positive	Negative	Positive	α	αβ[a]	Negative	Positive
DUP-14165	5	3	2	0	5[b]	4[b]	1	5[b]	0
DUP-14332	2	1	1	1	1	2[b]	0	2	0
DUP-14701	2	2	0	0	2[c]	1	1	2	0
DUP-19182	14	11[b]	3	4	10[b]	5	9	3	11
DUP-19182B	1	1	0	0	1	0	1	0	1
DUP-4003	3	2	1	3	0	0	3	0	3
DUP-4013	1	1	0	1	0	1	0	1	0
DUP-4017	1	0	1	1	0	1	0	1	0
DUP-4025	14	13[b]	1	14[b]	0	2	12	14[b]	0
DUP-4030	6	5[c]	1	6[b]	0	6[b]	0	5[b]	1
DUP-4045	8	0	8[b]	8[b]	0	0	8[c]	0	8[b]
DUP-4046	12	0	12[b]	3	9[b]	2	10	0	12[b]
DUP-4062	3	3[c]	0	2	1	2	1	3[b]	0
DUP-4063	2	0	2	2	0	0	2	0	2
DUP-4063B	8	0	8[b]	7	1	0	8[c]	0	8[b]
DUP-4063C	8	0	8[b]	4	4	0	8[c]	2	6
Total, no. (%)	90	42 (46.67)	48 (53.33)	56 (62.22)	34 (37.78)	26 (28.89)	64 (71.11)	38 (42.22)	52 (57.78)

[a] Double zone hemolysis.
[b] Significant correlation with ribotype at $P < 0.05$.
[c] Significant correlation with ribotype at $P < 0.1$.

regrown in brain heart infusion (BHI) broth incubated for 24 h at 35 ± 2°C. BHI cultures were then streaked for isolation on Trypticase soy agar plates with 5% sheep blood (TSA II; BD, Franklin Lakes, NJ), incubated at 37°C, and examined for hemolysis after 24 and 48 h. Purified colonies from TSA II were regrown on Baird Parker agar with egg yolk tellurite, incubated for 24 to 48 h at 35 ± 2°C, and examined for lipase activity as indicated by the presence of an iridescent film immediately surrounding colonies. Purified colonies from TSA II also were tested for bound coagulase (clumping factor) by the slide method. A single colony was mixed with 10 μl of reconstituted coagulase plasma with EDTA (BBL, BD, Sparks, MD) on a glass side and observed for clumping compared with the deionized water control.

Ribotyping. To examine subtype diversity, *S. aureus* isolates were characterized using the automated Riboprinter Microbial Characterization System (DuPont Qualicon, Wilmington, DE) according to the manufacturer's instructions with the restriction endonuclease *Eco*RI (Qualicon). Proprietary RiboExplorer software (version 2.0.3121.0) normalized the resulting fragment pattern data for band intensity and relative position and automatically assigned DuPont identifications (DUP-IDs). All patterns and DUP-ID assignments were confirmed by visual inspection. When patterns differed by a single weak band from an assigned DUP-ID, then each pattern was designated by the addition of an alphabetized letter (e.g., DUP-4063 and DUP-4063B).

Enterotoxin production, detection, and identification. In vitro enterotoxin production was carried out as recommended in the U.S. Food and Drug Administration's *Bacteriological Analytical Manual (6).* Supernatants obtained from this procedure were tested for the presence of SEA, SEB, SEC (C_1, C_2, and C_3), SED, and SEE with a visual immunoassay (SET-VIA, TECRA International, Frenchs Forest, New South Wales, Australia) as previously described *(7).* SE identification was carried out with the SET-ID (TECRA) assay, which was capable of identifying SEA through SEE.

Antimicrobial susceptibility. Antimicrobial susceptibility of isolates was evaluated using a commercial broth microdilution test (CMV1AMAF, Sensititre, TREK Diagnostics, Cleveland, OH). This method is widely used in veterinary diagnostic laboratories in the United States and adheres to the guidelines of the Clinical and Laboratory Standards Institute (CLSI). Antimicrobial resistance was based on the breakpoints established by the CLSI *(8),* and *S. aureus* ATCC 29213 was used as a quality control strain.

Data analysis. The association between phenotypic characteristics of isolates, ribotypes, and animal species was assessed statistically using Cross tabs with chi-square tests in SPSS for Windows (version 17.0.1, SPSS Inc., Chicago, IL). Results were considered significant at $P < 0.05$.

RESULTS

Ribotype diversity. The subtyping of one isolate per positive milk sample resulted in the identification of 16 ribotypes among the 90 isolates examined (Table 1). Cross-tabulations revealed that 13 of the 16 ribotypes were solely associated with isolates of a specific animal species, and nine of these associations were significant (Table 2). All but one of the individual isolates within the three remaining ribotypes were associated with a particular animal species. Half of the ribotypes identified were unique to individual farms. Further investigation of ribotype isolation on individual farms provided some insight into *S. aureus* epidemiology on small dairy farms that produce cheese. Two ribotypes were found among sheep's milk isolates collected from the bulk tank milk on farm B in 2006 (Table 2), whereas isolates from cow's milk on the same

TABLE 2. Staphylococcus aureus *ribotypes isolated from milk obtained from individual farms over 3 years*

Farm	Milk source	Ribotypes (*n*) isolated		
		2006	2008	2009
A	Cow	DUP-4025 (8), DUP-19182 (3)	DUP-4025 (2), DUP-19182 (3), DUP-4030 (3)	DUP-14332 (1)
B	Sheep, cow	DUP-4046 (8), DUP-4063C (1), DUP-4030 (2),[a] DUP-4017 (1)[a]	DUP-4046 (2)	
C	Sheep	DUP-4063B (2), DUP-4063C (7), DUP-4046 (1)	DUP-4063B (6)	
D	Cow	DUP-19182 (2)	DUP-4030 (1)	
E	Goat	DUP-4045 (5)	DUP-4045 (3)	
F	Cow		DUP-4025 (1), DUP-14332 (1)	DUP-14701 (2)
G	Goat	DUP-14165 (2)	DUP-14165 (3)	
H	Cow	DUP-19182 (4)		DUP-4062 (1)
I	Cow		DUP-19182 (1)	DUP-4025 (1)
J	Goat	DUP-19182 (1), DUP-4003 (2)	DUP-4063 (2)	
K	Cow		DUP-4046 (1)	DUP-4003 (1)
L	Cow		DUP-4062 (2)	
M	Cow		DUP-4025 (1)	
N	Cow			DUP-4025 (1), DUP-19182B (1)
O	Cow			DUP-4013 (1)

[a] DUP-IDs isolated from cow's milk.

farm collected later in 2006 were differentiated into two unique ribotypes not found among the sheep's milk isolates.

Phenotypic diversity. Several ribotypes were associated with individual phenotypic characteristics of isolates (Table 1). The proportion of isolates showing complete hemolysis on TSA II was significantly higher among isolates obtained from sheep's milk than among those obtained from cow's milk ($P = 0.002$). The proportion of isolates testing positive for bound coagulase was significantly higher among those obtained from either goat's or sheep's milk compared with isolates from cow's milk ($P = 0.003$ and <0.001, respectively). The proportion of isolates displaying αβ-hemolysis (double zone) was significantly higher in isolates positive for either bound coagulase ($P < 0.001$) or toxin production ($P = 0.006$) compared with isolates that were negative for these attributes. The association between isolates positive for bound coagulase and lipase was significant ($P = 0.018$) as was the association between toxigenic isolates and those positive for bound coagulase ($P < 0.001$).

SE production. Approximately half (52.22%) of isolates collected from 9 of the 15 farms and tested for SE production by the TECRA SET-VIA test were positive for SE. Isolates obtained from the remaining six farms were not positive for SE production using the methods described. A significantly higher proportion of isolates from both goat's milk ($P < 0.001$) and sheep's milk ($P < 0.001$) produced SE compared with isolates from cow's milk. The proportion of toxigenic isolates obtained from sheep's milk was also significantly higher than that from goat's milk ($P = 0.002$). Isolates of the same ribotype displayed similar enterotoxigenicity. Despite limitations in samples size, this association was significant for 6 of the 16 ribotypes (Table 1). Because ribotypes were common among particular farms, isolates from individual farms often had similar enterotoxigenicity, but this association could not be analyzed statistically due to limitations in sample size. Despite the isolation of several ribotypes, no isolate recovered from the raw cow's milk on farm A produced detectable SE. However, isolates of these same ribotypes recovered from other farms were positive for SE production.

Overall, 31 (96.88%) of the 32 isolates tested produced SEC alone. The remaining isolate (DUP-4025) produced SED alone; other isolates of this ribotype did not produce detectable SE. Although not included in the present analysis, two isolates cultured from anterior naris samples provided by a cheesemaker and a herdsman on farm A (ribotypes DUP-20365 and DUP-20365B) produced both SEA and SEC.

Antimicrobial susceptibility. Overall, 73 (82.95%) of the 88 isolates examined were susceptible to all the antimicrobials tested, and 15 (17.05%) were resistant to at least one antimicrobial. Ten (66.67%) of the 15 resistant isolates displayed coresistance to more than one drug. Nine of the 10 ampicillin-resistant isolates also were resistant to penicillin. Almost all (≥90%) of the isolates were inhibited by the lowest concentrations of oxacillin, pirlimycin, penicillin-novobiocin, tetracycline, and cephalothin, and approximately 80% of isolates were inhibited by the lowest concentrations of ampicillin, penicillin, and sulfadimethoxine (Table 3). Conversely, a minority of isolates were inhibited by the lowest concentration of ceftiofur. The MIC_{50} and MIC_{90} values for most antimicrobials tested were the same or within one doubling dilution of each other with the exception of ampicillin and penicillin. Resistance to penicillin was most common (17.04% of isolates) followed by ampicillin (12.51% of isolates) with a MIC_{90} of 0.5 μg/ml

TABLE 3. *MICs of various antimicrobials for* Staphylococcus aureus *isolates obtained from raw milk intended for artisan cheese manufacture*

Antimicrobial	% of isolates at a MIC (μg/ml) of[a]:												
	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	NI[b]
Ampicillin	*81.82*	5.68	**6.82** (R)	3.41 (R)	1.14 (R)	1.14 (R)	R	NT	NT	NT	NT	NT	NT
Penicillin	*82.95*	5.68 (R)	**5.68** (R)	2.27 (R)	2.27 (R)	1.14 (R)	R	NT	NT	NT	NT	NT	NT
Erythromycin	NT	*62.50*	**32.95**	I	I	I	NT	NT	NT	NT	NT	NT	4.55 (R)
Oxacillin + 2% NaCl	NT	NT	NT	NT	***97.73***	R	NT	NT	NT	NT	NT	NT	2.27 (R)
Pirlimycin	NT	NT	*89.77*	**5.68**	3.41	1.14 (R)	NT	NT	NT	NT	NT	NT	NT
Penicillin-novobiocin	NT	NT	NT	***100***	I	I	I	NT	NT	NT	NT	NT	NT
Tetracycline	NT	NT	NT	***93.18***	2.27	1.14	I	NT	NT	NT	NT	NT	3.41 (R)
Cephalothin	NT	NT	NT	NT	***98.86***	1.14	0	I	NT	NT	NT	NT	NT
Ceftiofur	NT	NT	22.73	***70.45***	4.55	2.27 (I)	NT	NT	NT	NT	NT	NT	NT
Sulfadimethoxine	NT	NT	NT	NT	NT	NT	NT	NT	*79.55*	**19.32**	1.14	0	NT

[a] Eighty-eight isolates were tested. The MICs that inhibit 50% (MIC_{50}) and 90% (MIC_{90}) of the isolates are shown in italic and bold, respectively. Values in bold italics represent both the MIC_{50} and the MIC_{90}. Some isolates were rated as intermediate (I) or resistant (R) according to CLSI guidelines *(9)*. NT, concentrations not tested for the indicated antimicrobial.

[b] NI, not inhibited. Growth occurred at highest concentration of antimicrobial tested.

for both drugs. In contrast, none of the isolates tested were resistant to penicillin-novobiocin, cephalothin, or sulfadimethoxine. Although most isolates were also susceptible to erythromycin, oxacillin, and tetracycline, 2.27, 4.55, and 3.41% of the isolates were not inhibited by these three drugs, respectively, at all concentrations tested.

Resistant phenotypes were observed in at least one isolate obtained from 6 of the 15 farms. In a few instances, isolates from an individual farm or of a particular ribotype had the same antimicrobial resistance patterns. Three ribotypes (DUP-14165, DUP-4003, and DUP-4062) were significantly associated with isolates resistant to at least one tested antimicrobial. Almost every isolate (ribotype DUP-14165) obtained from an organic goat dairy (farm G) was resistant to ampicillin, penicillin, and erythromycin. Both isolates from farm K, despite having different ribotypes (DUP-4046 and DUP-4003) and different sources (milk produced on the farm or purchased from an outside herd), were resistant to both ampicillin and penicillin. Although not significantly associated with the farm or ribotype, two of the six isolates of ribotype DUP-19182 from farm A were resistant to oxacillin.

DISCUSSION

This study examined the enterotoxigenicity, antimicrobial resistance, and ribotype diversity of *S. aureus* strains isolated from raw milk intended for small scale production of artisan cheeses in Vermont. Our observations support previous conclusions from investigations on the molecular epidemiology of *S. aureus* in cattle (reviewed by Anderson et al. *(4)*), which have suggested that a limited number of *S. aureus* strain types dominate in a given herd, with low strain variability, and that some strains may be widely distributed across a geographic region. It is unclear whether the association between specific ribotypes and animal species indicates a host preference or is simply a function of farm-specific subtypes, because farms typically kept only one species. In a comparison of *S. aureus* isolates from the mastitic milk of various ruminants, Mørk and colleagues *(21)* found that *S. aureus* genotypes were almost equally represented among ruminant species in Norway. Limited within-herd diversity observed in the present study is likely a result of the contagious nature of this pathogen. Similarly, the repeated isolation of specific ribotypes over a given season and over the course of years on individual farms suggests persistent infection, although the possibility of reinfection with the same subtype cannot be ruled out. The variation in strains detected over time on other farms suggests a change in the infecting strains but could also be the result of multiple strains infecting the various animals contributing to the bulk milk. The characterization of more than one isolate per positive milk sample would have provided more insight. In most cases, isolates of a particular ribotype had similar enterotoxigenicity, although this finding could be attributed to a broad distribution of closely related strains in this region *(26)*.

Numerous investigators have examined the toxigenicity of *S. aureus* isolates obtained from milk and milk products but have reported different results. Comparisons must be made cautiously because of regional differences in pathogen populations, bacteriologic culture methods, toxin production and detection methods, animal species, and the selection of animals for sample collection. In some cases, SEs are not produced, as was observed among strains isolated throughout a farm in Norway that produced raw milk cheese on a small scale *(17)*. In the present study, only half of the isolates tested produced detectable toxin, and 6 of the 15 farms did not yield a toxigenic isolate. The lack of SE production among isolates recovered from the raw cow's milk on farm A despite evidence that the same ribotypes recovered from other farms were positive for SE production certainly warrants further investigation.

Our results indicate that enterotoxigenic strains are relatively common among *S. aureus* isolates from raw milk intended for cheese manufacture in Vermont. In agreement with numerous other study findings, our findings identify SEC as a common SE produced by strains isolated from

milk of various species *(15, 26, 28).* In contrast, strains producing SEA and SED were implicated in the majority of staphylococcal food poisoning outbreaks and cases in the United Kingdom *(30)* and France *(18),* including those associated with the consumption of raw milk cheese.

In the present study, we observed a higher proportion of enterotoxigenic isolates in milk from small ruminants than in cow's milk. The lack of SE-producing isolates on farm A, a cow dairy, likely contributed to this finding because 20 of the 44 total cow's milk isolates tested were from farm A. Although SEC appears to be the predominant SE type produced among isolates of *S. aureus* obtained from milk, with a general lack of SEA and SEB production, contradictory reports do exist *(1, 2, 5).*

In general, limited antimicrobial resistance among strains was observed in the present study; antimicrobial resistant strains of *S. aureus* were identified among seven ribotypes isolated from six farms. The intraherd heterogeneity of antibiotic resistance observed in the present study supports the suggestion that universal immunization and treatment strategies may not prevent all *S. aureus* infections within a herd or flock *(23).* In general, MICs and proportions of resistant *S. aureus* isolates in the present study were low and comparable to those previously reported by other investigators for isolates obtained both domestically and internationally *(2, 4, 12, 22, 25, 29),* with few exceptions. For example, isolates in the present study were more susceptible to ampicillin, penicillin, and erythromycin than were isolates from both conventional and organic dairies in New York and Vermont *(27).* Resistance to oxacillin, an antimicrobial often used as a potential marker of methicillin resistance in *S. aureus,* has been reported for strains isolated from bovine mastitic milk *(12, 22, 29),* with notable variation in the reported MIC. According to Anderson and colleagues *(4),* resistance to penicillin appears to be the most commonly reported phenotype among mastitis-causing *S. aureus* isolates from cattle, with reported resistance rates generally ranging from 30 to 70%. Resistance to penicillin was the most common phenotype in the present study, but the resistance rate was at the low end of the ranges previously reported *(4, 25).* In cases of staphylococcal food poisoning, antibiotic resistant strains of *S. aureus* are not expected to be clinically more virulent or challenging *(16).* Because *S. aureus* is a common contaminant of cheese, an understanding of the ecology of this pathogen and the antimicrobial susceptibility and toxigenicity of various strains will ultimately assist the development of control practices to enhance the safety of artisan and farmstead cheese production.

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