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Molecular Types and Genetic Profiles of *Staphylococcus aureus* Strains Isolated from Bovine Intramammary Infections and Extramammary Sites[▽]

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Staphylococcus aureus **isolates collected from sites of intramammary infection during a 10-month period and from extramammary sites (dairy cow teat skin, teat canals, and skin lesions; milking liners; and hands and nostrils of milking personnel) at two separately managed Finnish dairy herd establishments were analyzed to study the sources and reservoirs of bovine *S. aureus* intramammary infection. Selected isolates were subjected to pulsed-field gel electrophoresis (PFGE) typing and PCR analysis for genes encoding hemolysins (*hla* to *hlg*), leukocidins (*lukED* and *lukM*), superantigens (*sea*, *sec*, *sed*, *seg* to *seo*, *seu*, and *tst*), adhesins (*fnbA* and *fnbB*), and penicillin and methicillin resistance (*blaZ* and *mecA*). *S. aureus* was found throughout the herds in 94% of the cows. Nine PFGE types were found, with the herds each having their own predominant type and sharing one type. The degree of diversity of PFGE types in herd II, which integrated foreign heifers, was higher than that in herd I. For both herds, the majority of the PFGE-typed isolates both from milk and from extramammary sites represented the predominant PFGE types. In isolates from herd I, the most prevalent genes were *hla-hlg*, *lukED*, and *fnbA*; in those from herd II, they were *hla*, *hld*, *hlg*, *lukED*, and *fnbA*. The other genes were pulsotype linked within the herds. The predominant PFGE types carried both *fnbA* and *fnbB*; only *fnbA* was detected in the other PFGE types. No connection between specific virulence genes and the origins of isolates was found. The results suggest that for the two herds, most *S. aureus* isolates from extramammary sites were indistinguishable from the isolates infecting the mammary gland and that those sites can thus act as origins and reservoirs of intramammary infections. However, contamination in the opposite direction cannot be excluded.**

Staphylococcus aureus is a major cause of contagious bovine mastitis. Depending on the country, prevalence values of 3.4 to 8.2% for quarter infections have been recorded previously in surveys in which samples have been taken from all quarters of all cows in randomly selected herds (30, 32, 43). Most dairy cows are probably exposed to *S. aureus*, as the organism is a frequent resident of the skin and mucous epithelia of dairy cows and other mammals, including humans, and is commonly found in the barn environment (35). The presence of the bacterium is likely to be of little consequence for the most part, but if the defense mechanisms of the host fail, *S. aureus* may become pathogenic.

S. aureus can produce more than 30 virulence factors that contribute to establishing and maintaining infection. The factors can be divided into two general groups, including surface-associated factors and degradative enzymes, together with exotoxins. *S. aureus* microbial surface components recognizing adhesive matrix molecules comprise surface proteins that promote colonization by being able to bind the host cellular matrix. This group includes fibrinogen-, fibronectin-, and collagen-binding proteins, which function during the initial stages of infection (31). Following successful colonization, *S. aureus* bacteria are able to secrete a plethora of other factors through which they obtain nutrients, invade, survive, and disseminate. These factors include enzymes and exotoxins, which are responsible for the pathological effects observed during the development of infection. Hemolysins (alpha, beta, delta, and gamma) and leukocidins are able to damage host cells by virtue of their cytolytic effects. In the event of the rapid progression of infection, the production of superantigens (enterotoxins, toxic shock syndrome toxin, and epidermolysins) can stimulate host defenses that can have severe, even lethal, consequences for the host (33).

Staphylococcal virulence factors have been identified for many *S. aureus* collections isolated in cases of bovine intramammary infection (IMI). In studies in which the production of staphylococcal virulence factors has been detected, most *S. aureus* isolates originating from bovine mastitis specimens have been shown to produce alpha- and beta-hemolysin and leukocidins or carry the respective genes (1, 34). Some superantigen determinants in particular, like *sec* and *tst*, have been common and typical for certain genetic lineages (9, 51). The genes *seg* to *seo*, which belong to the enterotoxigenic cluster *egc*, have been found frequently in *S. aureus* isolates from bovine mastitis specimens (9, 10, 40). Furthermore, the enterotoxin-encoding genes *sed* and *sej* have been detected in association with the penicillin resistance of certain strains (13). Such strains have been overrepresented in cases of persistent *S. aureus* mastitis (13).

Knowledge about the genetic variability within different *S. aureus* populations may help in the identification of the most likely source of an isolate. *S. aureus* has been found to be rather host specific (2, 14). Part of this specificity or host adaptation may be due to the acquisition or loss of mobile

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accessory genetic elements (7). It is not yet known whether specific virulence factors are associated with bovine mastitis. Genotypes of *S. aureus* strains residing in extramammary sites in dairy cows or in the barn environment have differed from those of strains isolated from sites of mastitis (50), which suggests that some bovine *S. aureus* strains may be specialized in infecting the bovine udder. This specialization may imply that strains predominantly causing IMI are more virulent than those present at extramammary sites. If specific virulence determinants could be identified, *S. aureus* IMI could be controlled by, for example, preventing the expression of these bacterial factors.

The aim of this study was to examine the molecular types and genetic profiles of *S. aureus* isolates originating from sites of IMI and to compare them with the molecular types and genetic profiles of *S. aureus* strains isolated from skin lesions and other body sites of dairy cows and from extramammary sites at two herd establishments.

MATERIALS AND METHODS

Herd and cow data. *S. aureus* isolates were collected from two commercial dairy herds (herds I and II) located in southern Finland and having a high incidence of *S. aureus* mastitis. The herds were selected based on the different management styles. On average, herd I had 30 lactating Holstein-Friesian cows and a mean annual milk production of 9,600 kg. The herd was closed; no animals were purchased from other herds. During the indoor season, the cows were housed in a free-stall barn with a concrete slatted floor and cubicles with peat as a bedding material; the cows grazed during the summer. The cows were milked twice a day in a 2-by-5 herringbone milking parlor. Udder health management included regular monitoring of the cows and milking machines, the maintenance of a milking order, forestripping before milking, the assessment of somatic cell counts in milk from all lactating quarters by the California Mastitis Test (CMT) every second week, the use of individual paper towels for udder cleaning, the use of gloves during milking, and postmilking teat disinfection using an iodine teat dip (0.75% active iodine). Animals with cases of clinical and subclinical mastitis were treated with antimicrobials, and treatment was always based on bacteriological diagnosis and susceptibility testing. Cows with a history of IMI in the preceding lactation received antimicrobial dry-cow therapy.

Herd II was an open herd that included replacement heifers from all over Finland. On average, the herd had 54 Ayrshire cows and a mean annual milk production of 9,185 kg. The cows were housed in a tied-stall barn with concrete floor cubicles containing peat bedding and were milked twice a day. Heifers were kept in a separate building for several months before transfer to the cow house, which usually occurred during early pregnancy. Mastitis control was similar to that for herd I, with the exception that quarters subclinically infected with *S. aureus* were usually not treated but prematurely dried off, especially if the isolate was penicillin resistant (produced β-lactamase).

Sample collection and microbiological procedures. Milk samples from all lactating cows with mastitis were collected by the herdsmen using an aseptic technique during a 10-month period from October 2002 to August 2003. The milk samples were submitted to the laboratories of the Finnish Food Safety Authority and Valio Ltd., where bacterial culturing was conducted according to routine methods (15). During the farm visits in March (herd I) and in May (herd II) of 2003, milk samples from all lactating quarters and samples from extramammary sites were collected as listed in Table 1 (this sampling is hereinafter referred to as the farm visit). Milk somatic cell counts were estimated by the CMT using the Nordic classification (18). In this method, scoring from 1 to 5 is used and a score of 3 (300,000 cells/ml of milk) is the threshold for mastitis. In herd II, three preparturient heifers close to parturition were included in the sampling. Milk samples were cultured according to routine methods, and bacterial species were preliminarily identified using conventional procedures described previously (15).

For the milk samples taken by the herdsmen before and after the farm visit, a quarter was considered to be infected with *S. aureus* when ≥500 CFU of *S. aureus*/ml was detected in the milk. For the milk samples taken at the farm visit, quarters were considered to have *S. aureus* IMI when ≥100 CFU of *S. aureus*/ml was detected in the milk if the CMT score was ≥3. Milk samples containing more than two bacterial species were considered to be contaminated. The IMI was classified as persistent if the same *S. aureus* pulsotype was repeatedly isolated from the same quarter during the same lactation. New infections were defined as those in quarters regarded as healthy before the farm visit but yielding samples in which *S. aureus* was detected later during the study period.

TABLE 1. Numbers and origins of samples collected from herds I and II during farm visits

Origin of samples	No. of samples from herd:		Total
	I	II	
Milk	108	197	305
Teat wall	108	204	312
Teat orifice	108	192	300
Teat canal	108	191	299
Skin lesion	8	33	41
Liner before milking	4	32	36
Liner after milking	108	186	294
Milkers' hands	2	2	4
Milkers' nostrils	2	4	6
Total	556	1,041	1,597

Samples from the teat walls (the skin of the teat barrels), the teat ends around the teat orifice, the teat canals, and all clearly visible skin lesions of the cows (mainly abrasions on the hind legs and a few lesions on teat or udder skin) and from the hands and nostrils of the milking personnel were collected by using sterile, singly packaged, cotton-tipped swabs (Technical Service Consultants Ltd., Heywood, United Kingdom). Swabs were moistened with Trypticase soy broth (TSB; Oxoid Ltd., Basingstoke, United Kingdom) before use. Teat canal samples were taken by rotating ultrafine, sterile, cotton-tipped swabs (Deltalab S. A., Barcelona, Spain) 360° in the canal. The teat was cleaned, disinfected with chlorhexidine solution, and dried with a paper towel before the insertion of the swab into the canal. Samples from the hands and nostrils of the staff were taken by rotating the swabs 360° between the bases of the fingers of both hands or in both nostrils. Four random swabs from liners used for herd I and swabs from all milking liners used for herd II were taken prior to milking to control for the cleanliness of the liners. Milking liners were also sampled after the milking of each cow. The swabs were rotated 360° at the point where the liner joined the short milking tube. Disposable latex gloves were used during the sampling.

After use, all swabs were immediately placed into sterile plastic containers filled with 5 ml of TSB. The containers were cooled and transported to the laboratory within a few hours. The isolation of *S. aureus* was carried out based on descriptions by Lancette and Tatini (19) with the following modifications. After the samples were kept for 30 min at room temperature, 5 ml of TSB containing 20% NaCl was added and the samples were subjected to a vortex and incubated for 24 h at 37°C. Subsequently, 0.1-ml aliquots of the cultures were transferred into duplicate plates of Baird-Parker agars and spread with a plastic triangle. The agars were incubated at 37°C for 24 h and checked and were then incubated another 24 h if no growth was observed. Black, dark gray, or shiny brown colonies with clear zones of hemolysis were selected for Gram staining and catalase and rabbit plasma coagulase tests. Other colonies suspected of being *S. aureus*, but without a clear zone, were transferred onto acriflavine agar and incubated at 37°C for 24 h. Colonies with yellow growth were tested as potential *S. aureus* colonies as described previously. The isolates were stored at −70°C in Protect bacterial preservers (Technical Service Consultants Ltd., Heywood, United Kingdom).

PFGE typing. All *S. aureus* isolates from mastitic milk, skin lesions, and human hands and nostrils were typed using pulsed-field gel electrophoresis (PFGE). At least one randomly selected isolate, if available, from the teat skin and/or the teat canal and/or the milking liner (after milking) per cow was typed to obtain insight into PFGE types in healthy and mastitic cows. Bacterial DNA for PFGE typing was digested with SmaI according to the method of Salmenlinna et al. (37). PFGE was conducted using the procedures of Murchan et al. (26). The relatedness of the fingerprints was assessed by using visual examination according to criteria described by Tenover et al. (44) and by using computer analysis with BioNumerics software (version 4.00; Applied Maths, Kortrijk, Belgium) with Dice coefficients and clustering by the unweighted-pair group method with arithmetic means; position tolerance was set at 1.0, and the cluster cutoff was set at an 80% similarity level. The different PFGE fingerprints (distinguished by seven or more band differences) were assigned different uppercase letters; patterns

TABLE 2. Numbers of cows and quarters with *S. aureus* IMI and pulsotypes infecting quarters[a]

Time point	Herd I		Herd II	
	No. of infected cows/ no. of infected quarters (no. of new infections)	Infecting pulsotype(s) (no. of infected quarters)	No. of infected cows/ no. of infected quarters (no. of new infections)	Infecting pulsotype(s) (no. of infected quarters)
Before farm visit	8/12 (ND[a])	A (8), B (4)	5/5 (ND)	A2 (1), D (2), E2 (1), F (1)
During farm visit	4/7 (1/3)	A (6), B (1)	6/7 (3/4)	D (6), F (1)
After farm visit	11/14[b] (8/11)	A (11), B (4)	3/3 (2/2)	D (3)

[a] ND, not determined.
[b] One quarter was infected with strains of pulsotypes A1 and B.

with one to six band shifts were accordingly represented by designations with numeric suffixes.

DNA isolation and PCR amplifications. All PFGE-typed *S. aureus* strains from herd I ($n = 102$) and 127 PFGE-typed strains from herd II were subjected to PCR. Bacterial DNA was isolated by using the DNeasy tissue kit (Qiagen, Hilden, Germany), with the modification that 11 U of lysostaphin (Sigma) was added at the cell lysis step. The DNA quantity for each isolation was measured using a GeneQuant II RNA/DNA calculator (Pharmacia) and diluted to a concentration of 100 ng/μl with Tris-EDTA buffer. The identification of *S. aureus* was confirmed according to the method of Brakstad et al. (3) by the amplification of the *nuc* gene. The β-lactamase gene *blaZ* was detected by primers designed by Vesterholm-Nielsen et al. (46) under the conditions described earlier by Haveri et al. (12). The genes *sea*, *sec*, *sed*, *tst*, and *mecA* were amplified by using primers and PCR conditions described by Mehrotra et al. (23); the primers were grouped into two multiplex sets, set 1 (for *sea*, *sec*, and *sed*) and set 2 (for *tst* and *mecA*). Primers for the genes *seg-sej*, *lukED*, *lukM*, *hla*, *hlb*, and *hld* were designed by Jarraud et al. (16), those for *sek-seo* were designed by Smyth et al. (40), those for *seu* were designed by Letertre et al. (20), those for *hlg* were designed by Lina et al. (21) and Jarraud et al. (16), and those for *fnbA* and *fnbB* were designed by Nashev et al. (28). One of the *hlg*-specific primers was modified as outlined by von Eiff et al. (47). Each 50-μl PCR mixture contained 2.5 U of DyNAzyme DNA polymerase (Finnzymes, Espoo, Finland), 200 μM deoxynucleoside triphosphate mix, 20 pmol of each primer, and 100 ng of DNA template. Multiplex PCRs were furthermore used for the detection of *seg*, *seh*, and *sei* (with set 3 primers); *hla* and *hlb* (with set 4 primers); *hld* and *hlg* (with set 5 primers); *sen* and *seo* (with set 6 primers); and *lukED* and *lukM* (with set 7 primers). Individual PCRs were conducted for *sej*, *sek*, *sel*, *sem*, *seu*, *fnbA*, and *fnbB*. Cycling conditions described by Mehrotra et al. (23) were used for the amplification of *seg* to *sej*, *lukED*, *lukM*, and *hla* to *hlg*. The conditions described by Smyth et al. (40) were used for *sek-seo* and *seu*. The conditions for the amplification of *fnbA* and *fnbB* included an initial denaturation step (5 min at 94°C), followed by 30 cycles of amplification (denaturation for 30 s at 94°C, annealing for 30 s at 50°C, and elongation for 1 min at 72°C) terminated with a 7-min incubation step at 72°C. The PCRs were carried out in a Peltier 200 thermal cycler (MJ Research, Waltham, MA), and each gene from each isolate was tested at least twice. The amplification products were analyzed by electrophoresis through 1% agarose gels. A positive control and a negative control (a reaction mixture without a DNA template) were included in each PCR run. The positive controls were strain FRI 913 (*sea sec sek sel tst lukED hlg*), ATCC 51811 (*seh*), ATCC 49775 (*seg sei sem sen seo*), ATCC 31890 (*lukM*), NCTC 8325 (*hla hld mecA*), CCUG 47326 (*fnbA fnbB*), and DO-204 (*sej*) provided by Justus-Liebig-Universität, Giessen, Germany, and strains EELA-7 (*sed seu*) and EELA-2285195 (*hlb*).

Statistical methods. A Pearson chi-square test was used to determine whether the presence of *S. aureus* at different sites of the teat (the teat canal, teat end, and teat wall) was associated with *S. aureus* IMI.

Nucleotide sequence accession number. The *fnbB* gene of a teat orifice isolate was sequenced at the Institute of Biotechnology (Helsinki, Finland), and the sequence is available from GenBank under accession number FJ178640.

RESULTS

***S. aureus* growth in milk and in other samples.** In herd I, *S. aureus* IMI was detected in 26 quarters of 17 cows during the study period. About half of these infections were persistent infections (Table 2). During the period before the farm visit, *S. aureus* mastitis was detected in 8 cows (27%) and in 12 quarters (10%). At the time of the farm visit, *S. aureus* IMI was detected in 4 cows (15% of 27 cows) and 7 quarters (7% of 108 quarters). Twenty-eight percent of the samples from the other sites harbored *S. aureus* (Table 3); 48% of the quarters and 85% of the cows had *S. aureus* on the teat walls and teat orifices and/or in the teat canals. The presence of *S. aureus* in the teat canal ($P < 0.01$) and in the teat end ($P < 0.05$) was associated with *S. aureus* IMI. Eighty percent of the quarters that became infected with *S. aureus* during the study also had the bacterium on the teat skin and/or in the teat canals, whereas for those sites on the unaffected quarters, the respective figure was only 42%. Skin lesion specimens from seven cows (26%) supported the growth of *S. aureus*, and two of the cows had concomitant *S. aureus* mastitis. Two of the four randomly sampled milking liners were contaminated with *S. aureus* before milking. During the follow-up period after the farm visit, *S. aureus* mastitis was detected in 11 cows (37%) and in 14 quarters (12%).

In herd II, *S. aureus* was isolated from 10 cows and 11 quarters during the study period. Approximately half of the IMIs were new infections (Table 2). Five cows (9%) and five quarters (2%) had *S. aureus* mastitis before the farm visit. During the farm visit, 6 of the 51 cows (12%) had *S. aureus*

TABLE 3. *S. aureus* isolates from herd I and their PFGE types grouped according to sample origin

Origin of samples	No. of samples with *S. aureus*/ total no. of samples (%)	No. (%) of PFGE-typed *S. aureus* isolates	PFGE type(s) (no. of isolates)
Milk	37[a] (ND[b])	38 (100)	A1 (27), B (10), A1 and B (1)
Teat wall	27/108 (25)	9 (33)	A1 (7), B (1), A1 and B (1)
Teat orifice	27/108 (25)	13 (48)	A1 (10), B (3)
Teat canal	32/108 (30)	14 (44)	A1 (11), B (3)
Skin lesion	7/8 (88)	7	A1 (1), B (5), A1 and B (1)
Liner before milking	2/4 (50)	2	A1 (2)
Liner after milking	28/108 (26)	12 (43)	A1 (11), B (1)
Milkers' hands	2/2 (100)	2	A1 (2)
Milkers' nostrils	2/2 (100)	2	A1 (1), C (1)
Total	165[a] (ND)	99 (60)	A1 (72), B (23), A1 and B (3), C (1)

[a] Number of samples with *S. aureus*; includes follow-up samples taken after treatments.
[b] ND, not determined.

TABLE 4. *S. aureus* isolates from herd II and their PFGE types grouped according to sample origin

Origin of samples	No. of samples with *S. aureus*/ total no. of samples (%)	No. (%) of PFGE-typed isolates	PFGE type(s) (no. of isolates)
Milk	21[a] (ND[b])	21 (100)	D (17), F (2), A2 (1), E2 (1)
Teat wall	131/204 (64)	43 (34)	D (38), E1 (2), A2 (1), B (1), F (1)
Teat orifice	130/192 (68)	37 (28)	D (33), F (2), A2 (1), B (1)
Teat canal	35/191 (18)	20 (57)	D (16), B (2), A2 (1), F (1)
Skin lesion	26/33 (79)	26 (100)	D (24), E1 (1), F (1)
Liner before milking	0/32 (0)	0	
Liner after milking	48/186 (26)	40 (83)	D (38), B (1), E1 (1)
Milkers' hands	0/2 (0)	0	
Milkers' nostrils	3/4 (75)	3	D (2), G (1)
Total	390[a](ND)	192 (49)	D (168), F (7), B (5), A2 (4), E1 (4), E2 (1), G (1)

[a] Number of samples with *S. aureus*; includes follow-up samples taken after treatments.
[b] ND, not determined.

IMIs, with 7 infected quarters (3% of 204). *S. aureus* was detected in 44% of samples from the other sites (Table 4). Ninety-six percent of the cows and 81% of the quarters had the bacterium on the teat skin or in the teat canals. The presence of *S. aureus* in the teat canal was associated with the presence of *S. aureus* in the milk ($P < 0.01$). Twenty-one cows had *S. aureus* in the skin lesion specimens, four cows having concomitant *S. aureus* IMIs. *S. aureus* mastitis was detected in three cows (6%) and in three quarters (1%) during the follow-up period after the farm visit.

Comparison of *S. aureus* pulsotypes between and within herds. PFGE typing produced nine fingerprints, which were classified as pulsotypes A1 to G (Fig. 1). Pulsotypes A1 (from herd I) and A2 (from herd II) and pulsotypes E1 and E2 (from herd II) were considered to be possibly related, with four band differences. Pulsotype B was detected in both herds.

In herd I, *S. aureus* pulsotypes A1 and B were isolated from milk samples throughout the study (Table 2). Pulsotype A1 caused mastitis in 13 cows (19 quarters). In six of those cows (eight quarters), the same pulsotype was detected several times in the same quarter, indicating persistent IMI. Pulsotype A1 predominated in samples from the extramammary sites (Table 3) and was detected in 90% of *S. aureus*-positive cows. Pulsotype A was present on the teat skin and/or in the teat canals of six cows without *S. aureus* IMI. Pulsotype B caused mastitis in eight cows (eight quarters). At the time of the farm visit, pulsotype B was isolated from extramammary sites in three healthy cows. Pulsotype B was common in the skin lesion samples. Indistinguishable pulsotypes were isolated from quarter milk and teat skin samples and/or teat canal samples from the same cows. The same pulsotype was found in skin lesion samples and milk samples from the same cows. For two cows, pulsotypes A1 and B were isolated from milk samples obtained from different quarters at the same time. Milk from a single quarter of one cow harbored pulsotypes A1 and B.

In herd II, pulsotype D was continuously isolated from milk during the study period (Table 2). Pulsotype D caused mastitis in seven cows (eight quarters) and was isolated several times from the milk of three cows. At the farm visit, pulsotype D was present in the extramammary sites in 41 cows. Two prepartum heifers sampled had pulsotype D in the colostrum and on the teat skin, and all three heifers had it on the skin lesions. Pulsotype F was found together with pulsotype D in the extramammary sites in four cows, and pulsotype E1 was found in the extramammary sites in three. For one cow, pulsotype F was repeatedly isolated from the same mastitis quarter. Pulsotypes A2, B, and E2 were sporadic; each of them was isolated from a single cow. Six cows had *S. aureus* strains of unrelated pulsotypes in the milk samples and the teat skin or teat canal samples from the same quarter, but the pulsotype found in milk was also present on the skin of another quarter. The same pulsotype, D, was found in skin lesion specimens and milk samples from the same cows. Two cows had pulsotype D in the teat canals and on the teat skin, and these quarters developed mastitis caused by that pulsotype after the sampling day.

In 25 samples, variations in colony morphology, color, or the hemolysis phenotype were visually detected. To examine whether the colony variants represented different pulsotypes, two colonies from each of these samples were PFGE typed. Except for three samples from herd I (milk, teat skin, and skin lesion samples) (Table 3), each sample had only one pulsotype.

***S. aureus* virulence genes.** Variations in the gene profiles among the most common pulsotypes, A1, B, and D, were observed. The individual genes or the different gene profiles of multiple *S. aureus* isolates were not linked with the origins of the isolates.

Pulsotype A1 isolates from the cows and the milking liners of herd I ($n = 72$) had *hla-hlg*, *lukED*, *fnbA*, and *fnbB* and, with one exception, *blaZ*, *sed*, and *sej* but seldom had *lukM* ($n = 8$), and only one pulsotype A1 isolate had *sem* and *seo*. Pulsotype A1 isolates from milkers' hands ($n = 2$) and nostrils ($n = 1$) were positive for *hla-hlg*, *lukED*, *fnbA*, *fnbB*, *blaZ*, *sed*, and *sej*, as were pulsotype A1 isolates from the cows. Pulsotype B isolates from herd I ($n = 26$) had *hla-hlg*, *lukED*, *lukM*, *fnbA*, *seg*, and *sei*, and the majority ($n = 19$) had *sem-seo*; *seu* was detected in nine strains, and *blaZ* was detected in two (from

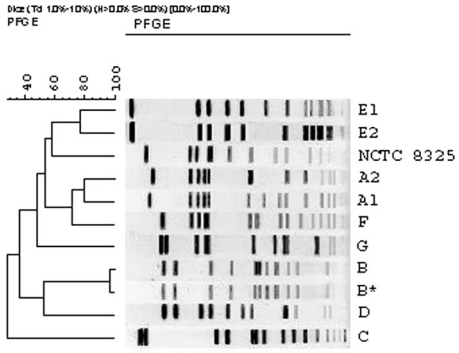

FIG. 1. Dendrogram showing the genetic relatedness of *S. aureus* pulsotypes A1 to G observed in herds I and II during the study period. Numbers at the upper left indicate percent similarity.

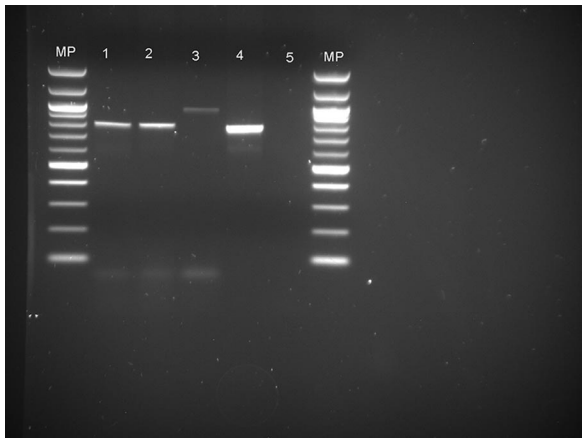

FIG. 2. Agarose gel electrophoresis analysis showing PCR amplification products for the *fnbB* gene of *S. aureus*. Lanes: MP, DNA molecular size markers; 1 and 2, *fnbB*-positive isolates 774 and 196; 3, *fnbB* variant (teat orifice isolate); 4, *fnbB*-positive control CCUG 47326; and 5, negative control.

milk and skin lesion samples), but *sed* or *sej* was detected in none. A pulsotype C isolate from a human nostril had a unique gene profile (*hla hld hlg lukED fnbA sec sel seg sei tst*).

In herd II, pulsotype A2 isolates ($n = 4$) carried only *hla*, *hld*, *hlg*, *lukED*, and *fnbA*. Pulsotype B isolates from herd II ($n = 5$) possessed *hla-hlg*, *lukED*, *lukM*, *fnbA*, *seg*, *sei*, *sem-seo*, *seu*, *sec*, *sel*, and *tst.* All of the 105 PCR-screened pulsotype D strains harbored *hla*, *hld*, *hlg*, *fnbA*, and *fnbB*, and 103 had *lukED*, but only a few were positive for *lukM* ($n = 4$), *blaZ* ($n = 3$), *seg* and *sei* ($n = 2$), *sed* ($n = 1$), *sem* ($n = 1$), or *seo* ($n = 1$). Pulsotype D isolates found in the nostrils of two milkers were positive for *hla*, *hld*, *hlg*, *lukED*, *lukM*, *fnbA*, and *fnbB*. Pulsotype E1 strains ($n = 4$) were positive for *hla*, *hld*, *hlg*, *lukED*, *fnbA*, *blaZ*, *seg*, *sei*, *sem-seo*, and *seu*. The pulsotype E2 isolate carried *hla*, *hld*, *hlg*, *lukED*, *fnbA*, *seg*, and *sei*. The genes *hla*, *hld*, *hlg*, *lukED*, *fnbA*, *blaZ*, *sea*, *seh*, and *sek* were detected in pulsotype F strains ($n = 7$). Pulsotype G from the nostrils of the third milker harbored *hla*, *hld*, *hlg*, *lukED*, *lukM*, *fnbA*, *seg*, *sei*, *sem-seo*, and *seu*.

No strains with *mecA* were detected.

For one *S. aureus* isolate of pulsotype E1 originating from mastitic milk and one isolate of pulsotype D from a teat orifice, the PCR amplification products for *fnbB* were heavier than the corresponding fragment of the positive control (Fig. 2). The amplification products of the teat orifice isolate were submitted to the Institute of Biotechnology (Helsinki, Finland) for sequencing. The sequence was exported into Chromas Lite version 2.01 (Technelysium Pty. Ltd., Australia). The sequence was sent to GenBank (accession no. FJ178640) and is 90% identical to the corresponding GenBank sequences of fibronectin-binding protein genes in *S. aureus* strains Mu3 (accession no. NC_009782) and Mu50 (accession no. NC_002758).

DISCUSSION

The presence of *S. aureus* in sites of IMI and at extramammary sites in two herds in which the pathogen continuously caused mastitis was investigated. The proportion of animals harboring *S. aureus* on their udder skin was very high in both herds. The contamination of milking equipment by *S. aureus* was common, and the bacterium was also isolated from the hands and nostrils of milkers. In both herds, the majority of *S. aureus* isolates comprised predominant genotypes, which were unique to the herds. The number of pulsotypes in herd II (an open herd), which included heifers raised off site, was higher ($n = 7$) than that in herd I (a closed herd; $n = 3$). Our results agree with those of Middleton et al. (24), who also found more heterogeneity among *S. aureus* isolates from an open herd than among those from a closed herd. Nearly all strains subjected to PCR analysis carried genes for alpha-, beta-, and gamma-hemolysin and the genes *lukED* and *fnbA*. The other genes were less frequent and pulsotype linked within the herd, but no connection between specific virulence genes and the origin of the isolate was found. In both herds, strains of the predominant pulsotypes typically possessed *fnbA* and *fnbB*, whereas only *fnbA* was present in the other types. The results from both PFGE typing and PCR showed that strains with genotypes indistinguishable from those of strains in sites of mastitis also dominated extramammary sites within both herds.

The high prevalence of *S. aureus* on teat skin and in canals indicates that virtually all cows in these herds could have exposure to this pathogen via their own skin. Postmilking teat dipping, practiced for both herds, did not appear to prevent the growth of *S. aureus* on the teats. The efficacy of the teat dips may not be as good under field conditions as under experimental conditions, as recently shown in a Norwegian field study (49). In herd II, extramammary sites may be more likely routes of transmission than milk from infected quarters. It was easier to maintain a milking order for this stanchion-barn herd than for herd I, with its loose-housing system. This circumstance may indicate that postmilking liner contamination by *S. aureus*, seen after the milking of most of the cows, originated from the teat skin and teat canals of healthy cows.

We were able to detect very small numbers of *S. aureus* CFU by using a selective enrichment method. This strategy was possibly one reason for the high level of recovery of *S. aureus* from the extramammary sites, a phenomenon that was reported by Matos et al. (22). Although only a small percentage of these cows developed *S. aureus* IMI during the study period, IMI may be more likely if bacteria are present around the teat orifice than if the teats are free from *S. aureus*. This trend was directly demonstrated in two of the cows, for which the same pulsotype that infected the udder quarter was also isolated from the teat skin prior to IMI. Cows with teat canals colonized by *S. aureus* are at particular risk of developing mastitis; one-third of the mammary quarters in herd I had *S. aureus* in the teat canals, which may be one reason for the high number of new infections in this herd. Furthermore, tissues affected by teat abrasions and teat traumas in particular are likely to contain high numbers of *S. aureus* (27), and teat lesions are common in many herds. In our study herds, the skin lesions harbored the same *S. aureus* strains as the milk from the same cows with IMIs.

Zadoks et al. (50) typed *S. aureus* isolates collected from sites of IMIs, teat skin, teat canals, milking equipment, and milking personnel for 43 herds by using PFGE and binary typing. The number of isolates varied from 1 to 20 per herd.

Based on the complete data, the majority of teat skin isolates were different from those infecting the udders. The authors concluded that specific udder-pathogenic *S. aureus* strains exist and are mostly different from those present on the udder skin. In our study, the results largely contradict their findings but agree with those of Jørgensen et al. (17), who reported that the genotypes of strains from IMI sites and teat skin were indistinguishable by PFGE typing. In our herds, genotypes that probably were best adapted to multiplying on the teat skin, in the teat canals, and in skin lesions also caused most IMIs. Occasional exceptions were one sporadic genotype (E2) in herd II that was isolated only from milk and two other genotypes (B and E1) that were detected only on the teat skin and were not isolated from sites of IMI.

To the best of our knowledge, only one previous study comparing virulence characteristics of *S. aureus* strains isolated from IMI sites and extramammary sites is available (8). The authors found milk-associated *S. aureus* genotypes to be more likely to produce biofilm than genotypes associated with extramammary sources (teat skin and milking liners). In our study, no association between the sample origin and virulence genes was established. However, in both herds, the genes *fnbA* and *fnbB*, encoding fibronectin-binding proteins (FnBPs), coexisted in the most common strains, those of pulsotypes A1 and D. In other pulsotypes, which were less frequent, only *fnbA* was detected. In the work of Salasia et al. (36), *fnbA* was more common than *fnbB* in *S. aureus* isolates originating from cows with bovine mastitis in Indonesian and German dairy herds. The proteins FnBPA and FnBPB enable the attachment of *S. aureus* to host tissues, facilitate the invasion of the organism into epithelial cells (4, 48), and are involved in the colonization of other surfaces like medical devices (11). Strains not expressing FnBPA and FnBPB were demonstrated previously to have highly reduced abilities to invade bovine mammary epithelial cells in vitro (5). It is speculated that *S. aureus* genotypes predominant in dairy herds can exhibit high colonization and survival rates in bovine hosts, which favors a benign relationship between the microbe and the host animal. In herd II in particular, pulsotype D may have benefited from its possibly high potential for adherence and may have therefore become predominant. Other explanations for the finding that a strain with a small number of virulence genes could resist competition with other strains are hard to find.

The genes for superantigens were ubiquitous in isolates from herd I. The predominant genotype, A1, typically isolated from cows with persistent infections, carried enterotoxin-encoding genes *sed* and *sej* and the gene for penicillin resistance, *blaZ*. The prognosis for mastitis caused by penicillin-resistant *S. aureus* strains is poor (38, 42), and wider spreading of these strains may be expected if they are present also at the extramammary sites. Furthermore, the presence of the *blaZ* gene has been thought to indicate the coexistence of some other virulence factor(s), which together with *blaZ* may contribute to the persistence of *S. aureus* in the mammary gland (13). In our previous study, the responses of IMIs caused by *blaZ*-positive strains to treatment tended to vary among different pulsotypes, but strains that carried *sed*, *sej*, and *blaZ* were overrepresented in chronic IMIs (13). Superantigens such as enterotoxins have been suggested to enhance the persistence of bovine IMI due to their immunosuppressive effect on the host animal (6). The use of antimicrobials in the herds may also have an impact; antimicrobials like β-lactams and fluoroquinolones have been shown in vitro to stimulate HLA production by *S. aureus* (29), and this effect may be possible for enterotoxins as well.

Many factors contribute to the selection and diversity of *S. aureus* strains in herds. The number and diversity of *S. aureus* strains is likely to increase if new strains are introduced via cattle import, as shown here for herd II, in agreement with the results of Middleton et al. (24). Sommerhäuser et al. (39) proposed that the successful control of contagious mastitis may increase the diversity but decrease the spread of strains within the herd and that failure in control may lead to the dissemination of only one or a few dominant strains throughout the herd. Preparturient heifers can be an important reservoir of *S. aureus*, and strains originating from their skin have been shown to cause IMI in cows of the same herd (24). However, heifers can also harbor different strains from those in lactating cows (50). In our study, two precalving heifers in herd II harbored *S. aureus* in the colostrum, and the strains were similar to those isolated from lactating cows.

Milking personnel may also carry the same *S. aureus* types as the cows, as noted here for both herds, but the origin of the colonization remains unknown. However, the findings from recent epidemiological studies of human and animal *S. aureus* strains, including bovine mastitis isolates, suggest host specificity, although a high degree of variation within and between *S. aureus* clonal lineages originating from different host animals was found (2, 14, 41, 45). A recent study by Herron-Olson et al. (14) showed that strains from cattle with *S. aureus* mastitis carried several unique genes, which may be suggested to play a role in the adaptation of the strains to the bovine udder. Some other genes, such as *bap*, involved in biofilm formation, and gene complexes like the immune evasion cluster may be less important for IMI, as these genes are rare in bovine isolates, in contrast to human isolates (25, 41).

In conclusion, the *S. aureus* strains causing bovine IMI were not different from those isolated from extramammary sites. Teat skin and especially the teat canals were important potential reservoirs of *S. aureus* causing IMI. This information may be useful in planning mastitis control strategies for herds suffering from *S. aureus* mastitis. However, identical pulsotypes from different herds may harbor different virulence and resistance genes, and placing these types in the same virulence class may be an oversimplification. The relative significance of different genes is poorly understood and should be further studied in larger populations.

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