Effect of Abrasion of Teat Orifice Epithelium on Development of Bovine Staphylococcal Mastitis

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ABSTRACT

The predisposing effect of teat damage on mastitis caused by staphylococci and the pathogenicity of *Staphylococcus aureus*, *Staphylococcus hyicus*, and *Staphylococcus epidermidis* were investigated with an experimental model. The study included three experiments in which the teat canal orifice of 5 cows was slightly abraded. Experimental and control quarters were challenged with a staphylococcal suspension, and the status of the quarters was monitored. Virulence of the staphylococcal strains was studied using a protein-binding test with ^{125}I-labeled proteins (fibronectin, fibrinogen, vitronectin, collagen type I and II, and IgG). Abrasion on the teat orifice epithelium was a predisposing factor for staphylococcal infections. Teat canal infection or colonization developed in 93% of experimental quarters and in 53% of control quarters; IMI developed in 73% of experimental quarters, but in none of the control quarters. Quarter IMI developed more consistently when the contaminating agent was *S. aureus*. *Staphylococcus hyicus* was very effective in causing teat canal infections, but *S. epidermidis* appeared to be less infectious. The *S. aureus* strain had strong binding sites for most of the proteins tested. The *S. hyicus* and *S. epidermidis* strains showed no binding, or only very weak binding, which correlated with lower infection rates.

(**Key words**: bovine, staphylococcal mastitis, teat orifice epithelium)

Abbreviation key: **CNS** = coagulase-negative staphylococci.

INTRODUCTION

Results of a Finnish mastitis survey (17) indicated that 17% of 16,970 quarter milk samples examined microbiologically contained mastitis pathogens. The largest groups of bacteria were coagulase-negative staphylococci (**CNS**) and *Staphylococcus aureus*. The CNS group included mainly *Staphylococcus hyicus* and *Staphylococcus epidermidis* (11). The pathogenicity of *S. aureus* has been studied extensively, but CNS strains have received little attention until recently. Most CNS mastitis cases are subclinical or teat duct colonizations that slightly increase milk SCC (9), but CNS also can provoke a clinical disease that may result in persistent IMI that are difficult to cure (12).

To establish an IMI, the causative organism has to overcome two major defenses: the first barrier against invading bacteria is the teat canal and associated tissues; the second barrier is formed by leukocytes in the milk. The teat canal acts as a physical barrier, and its three

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principal defense mechanisms are adsorption of bacteria onto keratin, desquamation of bacteria-coated keratin by the shearing forces associated with milk flow through the canal, and desiccation of the canal lumen, mainly by evaporation from the external teat orifice, allowing the resealing of keratinized surfaces (21, 28). A lesion at the teat end, or in the teat canal, is assumed to be a predisposing factor to staphylococcal IMI. In practice, the most frequent causes of such lesions are faulty milking machines, poor milking technique, and perturbations in the environment.

Staphylococci are able to colonize teat end epithelium and to adhere to tissue proteins, including fibronectin, fibrinogen, fibrin, vitronectin, and collagen (8, 15, 16, 20). These proteins may become exposed to the surface after damage to the epithelium (27) and may then promote direct adherence of staphylococci, predominantly to the sites of the lesions. Bacteria passing the teat canal barrier encounter humoral and cellular defense mechanisms in the milk, most importantly, neutrophilic leukocytes that phagocytose bacteria. However, the killing capacity (phagocytosis, oxidative burst) is suppressed in milk (6, 19). Furthermore, inflammation of the udder increases vascular permeability, which leads to the presence of plasma proteins in the milk (5, 23). Bacteria that specifically accumulate plasma proteins on the surface may appear more hostlike. This surface structure might interfere with defense mechanisms of the host (4, 29, 30).

The purpose of this study was to investigate the effect of experimentally induced teat canal lesions on the development of mastitis caused by staphylococci and to compare the pathogenicity of the CNS, *S. hyicus* and *S. epidermidis*, with that of *S. aureus*.

MATERIALS AND METHODS

Bacteria

The bacterial species used in the three experiments were *S. aureus* [M60 (2)], *S. hyicus*, and *S. epidermidis*. The CNS, *S. hyicus* 51/90 and *S. epidermidis* 67/90, were isolated from clinical mastitis IMI and were identified with Staph Trace® (Bio Merieux, Marcy-l'Etoile, France) and were supplied by the National Veterinary and Food Research Institute, Helsinki, Finland.

Bacterial suspensions were prepared by inoculation of 1 cfu from an overnight culture on blood agar into tubes containing 10 ml of meat extract broth (Oxoid, Basingstoke, Hampshire, England). After 20 h of incubation at 37°C, the tube contents were pooled and diluted to correspond to McFarland's standard .5 (10). Additionally, the colony-forming units of the bacterial suspensions were determined by culture of .1 ml of serial dilutions on blood agar and counts of the colonies that developed.

For evaluation of in vitro binding of plasma proteins to the bacteria, the strains were grown overnight at 37°C in 100 ml of brain-heart infusion broth (Difco, Detroit, MI) and centrifuged (15,000 × *g*, 30 min, 8°C). The bacterial sediments were suspended in 5 ml of PBS, pH 7.4, containing 6 *M* guanidinium hydrochloride and were stirred for 1 h at room temperature (20°C). Subsequently, the cells were washed three times with PBS and resuspended in 5 ml of PBS and kept frozen at −20°C until further use.

For the binding assay, the bacterial suspensions were homogenized for 10 s in an ultrasonic water bath (Branson, Danbury, CT) and then adjusted to measure absorbance at 600 nm (1 cm) = 1.40. This homogenate corresponded to the value of a Cowan 1 (ATCC 12598) suspension of *S. aureus*, gravimetrically adjusted to 1 mg/ml. Bacterial aliquots (300 μl) were mixed with 100 μl of PBS containing .05% Tween 20 and .1% BSA fraction V (Sigma Chemical Co., St. Louis, MO) and 100 μl of the ^{125}I-labeled ligand in Eppendorf tubes. The ligands contained .5 ng of N-terminal 24-kDa fragment of bovine fibronectin, 150,000 cpm; 1 ng of human IgG, 68,000 cpm; 1.0 ng of bovine fibrinogen, 18,000 cpm; 2 ng of vitronectin, 11,000 cpm; and 2 ng of collagen, 13,000 cpm, respectively. All proteins were labeled by the chloramine T method (13). The bacteria were allowed to react for 2 h at room temperature with the ligands. Subsequently, the cells were centrifuged for 10 min at maximum speed in an Eppendorf centrifuge (Eppendorf, Hamburg, Germany). The supernatant was discarded, the pellets were washed twice with 1 ml of PBS-Tween, and the radioactivity associated with the bacteria was determined in a gamma counter (LKB-Wallac, Turku, Finland). Binding of the ligands to the bacteria was expressed as a percentage of the

total radioactivity added to the samples. For comparison, the reference strains *S. aureus* Cowan 1 ATCC 12598, the protein A negative strain *S. aureus* Wood 46 ATCC 10832, and *S. hyicus* NCTC 10350 (a porcine isolate) were included (from the collection of reference strains of H.-P. Müller, Uppsala, Sweden).

Cows

Seven lactating Finnish Ayrshire dairy cows in midlactation were used. The cows had calved one to three times and had mean daily milk production of 19.1 kg. Cows were kept in a tie stall and were fed hay for ad libitum intake and grass silage and concentrate according to milk production. The cows were milked twice daily. Udder health was monitored before each experiment by bacteriological culture and measurement of NAGase concentrations in milk.

In each experiment, the same five cows were used. One quarter was experimentally exposed, one quarter served as a control, and the remaining two quarters were unused. In Experiment 1, the right fore quarter was exposed, and the right hind quarter was the control. In Experiment 2, the left fore quarter was exposed, and the left hind quarter was the control. In Experiment 3, the right hind quarter was exposed, and the left hind quarter was the control. The experiments were designed such that different quarters were exposed to damage in different experiments. The time between experiments was 1 mo. For Experiment 3, two cows had to be replaced because of continuous IMI.

Experiments

The cows were sedated with xylazin (.05 mg/kg; Bayer, Leverkusen, Germany), teat ends were cleaned with 70% ethanol, and the ends of teat canals of exposed quarters were abraded with a teat slitter (Swiss model number 35880; Hauptner, Solingen, Germany). The instrument was inserted 2 mm into the teat canal and rotated 360°C to abrade the epithelium epidermal tissue. The keratinized layer is thus damaged, leaving the dermis partly visible. Subsequently, the exposed and control quarters were contaminated with the same staphylococcal suspension. Bacterial suspension (2 ml of 10^8 cfu/ml) was pipetted onto cotton pledgets placed in large rubber finger cots. These partially unrolled cots were then placed over each teat end and kept in place for 30 min.

Monitoring

Clinical signs and other parameters were monitored according to a prescheduled protocol on d −14, −7, 0 (the day of the experiment), .5, 1, 2, 3, 5, 7, 10, 14, and 21). Bacteriological samples were taken aseptically from teat canals and from normal premilking milk after the first few streams were discarded. Milk was also sampled directly through the teat wall with a 23-gauge needle to confirm the presence of IMI when the previous milk sample yielded bacteria. Teat canal samples were taken with a calcium alginate swab (Calgiswab Type 1®; Spectrum Laboratories Inc., Houston, TX) by gentle rotation of the swab in the teat canal at a depth of 3 mm. The swabs were cultured by rotation on blood agar. After 18 to 20 h of incubation at 37°C, bacterial colonies were counted semiquantitatively (as colony-forming units per swab), and bacterial types were identified by routine bacteriological methods. *Staphylococcus* spp. were classified using the Staph Trac® system. Milk samples were streaked out with calibrated (.03 ml) plastic loops on blood agar. After 18 to 20 h of incubation at 37°C, bacterial colonies were counted (colony-forming units/.03 ml). Bacteriological identification continued according to the same protocol as with swab samples. Concentrations of NAGase in milk were measured fluorometrically (18) from frozen samples. Clinical symptoms (temperature and changes in the udder such as pain, swelling, and abnormal milk appearance) were monitored throughout the experiment.

The criterion for teat canal infection (i.e., colonization) was that ≥10 colonies of the same bacterial species were isolated from a swab sample. An intramammary quarter was considered to have IMI when ≥10 colonies of bacteria were isolated from a .03-ml milk sample and when NAGase was ≥10. The bacterial species isolated in the follow-up samples after challenge were confirmed to be the same as in contamination using colony morphology and the Staph Trac® system.

Differences among the groups were analyzed by using the chi-square test (25). The Mann-Whitney U statistic was used to test whether maximum NAGase in the different groups differed significantly (25). Generally, the small number of quarters used in the experiments limited the value of statistical tests.

RESULTS

Teat Canal Infections

Quarters with abraded teat canals (14 out of 15) and one-half of the control quarters were infected ($P < .5$) (Table 1). Infections in exposed quarters were more severe than in control quarters by semiquantitive bacterial counts on blood agar; growth of *S. aureus* from exposed quarters was thicker (confluent) than growth of *S. hyicus* and *S. epidermidis* (about 200 cfu per swab). Growth of all bacterial strains isolated from control quarters was sparse (<50 cfu per swab). Average duration of infections was 7 d in exposed quarters and 3 d in control quarters. The infection was present in all cows within .5 d with *S. aureus*, .5 to 2 d with *S. hyicus*, and .5 to 3 d with *S. epidermidis*.

IMI

Based on the laboratory findings (bacteriology and NAGase), 11 of 15 exposed quarters became infected, but all of the control quarters remained uninfected ($P < .1$). With *S. aureus* IMI, inflammation parameters were higher than for IMI caused by CNS (Table 2), but differences were not significant between *S. aureus* and *S. hyicus* ($P = .28$). The NAGase increased between d 3 and 5 and peaked on d 6. *Staphylococcus aureus* IMI lasted the longest; on d 7, *S. aureus* IMI persisted in all 5 exposed quarters, *S. hyicus* IMI in 3 quarters, and *S. epidermidis* IMI in 2 quarters. After 3 wk, *S. aureus* and *S. epidermidis* IMI remained in 2 quarters, but *S. hyicus* was no longer detected.

The *S. aureus* IMI developed into clinical mastitis in four of the five cows (Table 2). Symptoms included high temperature, pain and swelling in the infected quarter, and changes in milk appearance (clots, change in color, and viscosity). *Staphylococcus hyicus* IMI developed into clinical mastitis in two cows, and symptoms were similar to those caused by *S. aureus*.

Virulence of Staphylococcal Strains

The staphylococcal strains used in this study were examined for their ability to bind specifically to several plasma and extracellular matrix proteins. The *S. aureus* strain M60 showed strong and specific binding to the N-terminal fragment of bovine fibronectin, fibrinogen, vitronectin, and human IgG and, in these features, was comparable with the reference strains Cowan 1 and *S. hyicus* NCTC 10350. In contrast, the *S. hyicus* strain 42, a bovine isolate, and the *S. epidermidis* strain 67 did not bind to fibrinogen or IgG and showed only very low binding activity to the other proteins, which is comparable with the reference strain *S. aureus* Wood 46. The binding profile of *S. aureus* M60 is shown in Figure 1.

TABLE 1. Number of quarters that developed teat canal infections and IMI in experimental and control quarters.[1]

	Teat canal infection[2]		IMI[2]	
Bacteria	Experimental[3]	Control[4]	Experimental	Control
S. aureus	5	2	5	0
S. hyicus	5	5	4	0
S. epidermidis	4	1	2	0

[1]*Staphylococcus aureus* was used in Experiment 1, *Staphylococcus hyicus* in Experiment 2, and *Staphylococcus epidermidis* in Experiment 3.

[2]n = 5.

[3]Ends of teat canals were abraded and contaminated with staphylococcal suspension.

[4]Ends of teat canals were contaminated with staphylococcal suspension.

TABLE 2. Clinical symptoms and maximum NAGase in experimental quarters.

Cow	Staphylococcal strain	Clinical symptoms[1]		NAGase[2]		
		General	Local	Maximum	Average	SE[3]
(no.)						
1	*S. aureus*	++	++	123.2		
2	*S. aureus*	–	+	80.9		
3	*S. aureus*	++	++	18.6		
4	*S. aureus*	–	–	21.5		
5	*S. aureus*	+	++	33.8	55.6	20.3
1	*S. hyicus*	–	–	18.1		
2	*S. hyicus*	–	–	21.3		
3	*S. hyicus*	–	+	43.8		
4	*S. hyicus*	+	+	22.1		
5	*S. hyicus*	–	–	41.8	29.3	5.4
1	*S. epidermidis*	–	–	18.0		
2	*S. epidermidis*	–	–	10.0		
3	*S. epidermidis*	–	–	10.0		
4	*S. epidermidis*	–	–	2.0		
5	*S. epidermidis*	–	–	8.0	9.6	2.6

[1]– = No clinical symptoms; + = body temperature <39.5°C, mild soreness, swelling of the udder, abnormal milk appearance; and ++ = body temperature >39.5°C, moderate soreness, swelling of the udder, abnormal milk appearance.

[2]NAGase units (18).

[3]NAGase of *S. aureus* and *S. hyicus* differs from that of *S. epidermidis* ($P < .01$). *Staphylococcus aureus* differs from coagulase-negative staphylococci ($P < .05$).

DISCUSSION

Teat canal infections probably often precede quarter IMI. Teat canal lesions are important in mastitis development. Jackson (14) reported that a teat canal lesion resulted from a malfunctioning milking machine. Clinical mastitis developed in 55.6% of those cows, but only in 5.9% of control cows. However, in that study (14), lesions did not precede IMI. Bright et al. (3) reported that removal of the teat canal keratin layer predisposed the udder to mastitis when the teats were challenged by being dipped in solution containing *Streptococcus agalactiae*. When quarters of cows in the control group were dipped without removal of the keratin layer, the rate of IMI was lower. In an experimental contamination test with *Actinomyces pyogenes*, Seinhorst et al. (24) showed that the nonlactating quarters did not automatically become infected. However, damage to the epithelium at the teat end markedly increased IMI after topical application of *A. pyogenes*.

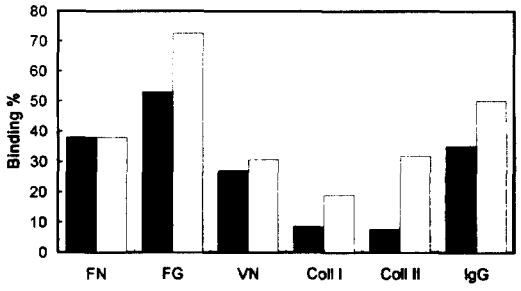

Figure 1. Binding of ^{125}I-labeled proteins to *Staphylococcus aureus* strain M60 (solid bars) and to *S. aureus* Cowan 1 (open bars). Binding is expressed as a percentage of the total protein added. FN = 24-kDa N-terminal fragment of bovine fibronectin, FG = bovine fibrinogen, VN = vitronectin, Coll I = collagen type I, Coll II = collagen type II, IgG = human IgG.

Teat lesion and subclinical mastitis are significantly correlated. Subclinical mastitis occurs 1.75 times more frequently in damaged quarters than in healthy quarters. If a teat is damaged, the risk of the cow acquiring mastitis during a 10-mo milking period increases by 50%. If the lesion reaches the teat canal, the risk is even higher (1). If teat canal infections could be prevented, mastitis might be better controlled (7).

In our experiment, *S. aureus* caused mastitis in quarters that had teat lesions in four out of five cows, and *S. hyicus* caused mastitis in two cows. Subclinical IMI appeared in one cow challenged with *S. aureus* and in two cows challenged with both *S. hyicus* and *S. epidermidis*. Control quarters exhibited no signs of subclinical or clinical IMI. However, teat canal infections appeared also in control quarters. Apparently, a lesion on the epithelium of the teat canal is a predisposing factor to teat canal infections, IMI, and subsequent clinical mastitis.

Staphylococcal species have traditionally been divided into coagulase-positive and CNS groups. Coagulase-positive strains are considered to be contagious, major pathogens, but CNS are minor pathogens and have been classified as skin flora opportunists (9). However, CNS can produce clinical mastitis, but clinical signs are generally less intense than in mastitis caused by *S. aureus*. The degree of inflammation of the udder is also less than that associated with mastitis caused by other pathogens (22). Honkanen-Buzalski (11) reported that, for subclinical mastitis, mean SCC was 776×10^3 in quarters infected with CNS and 2373×10^3 in quarters infected with *S. aureus*. In our experiment, the difference was also marked in the severity and duration of symptoms between these two groups (based on NAGase and bacteriological findings). The two CNS strains also differed. *Staphylococcus hyicus* appeared to be more infective than *S. epidermidis*. *Staphylococcus hyicus*, which was the most common CNS isolate in the Finnish mastitis survey (17), causes clinical mastitis (11). In our experiment, *S. hyicus* caused teat canal infections in all exposed and control quarters, indicating that it may be an important natural teat canal colonizer.

The protein-binding assays showed differences between the staphylococcal strains. The most virulent staphylococcal strain in this study, *S. aureus* M60, strongly bound all six proteins tested. Three of the strongly bound proteins, vitronectin, fibrinogen, and fibronectin, occur in an immobilized form in wounded tissues. These interactions between bacteria and host-proteins might be assumed to be important in adhesion of the *S. aureus* strain M60 to the sites of experimental lesions in the teats. This hypothesis was suggested by Thomas et al. (26), who reported a tropism of *S. aureus* and *Streptococcus dysgalactiae* for exposed connective tissue, but not for healthy epithelium. The results of our study corroborate this hypothesis in that exposure of wounded teats to strain M60 in all cases led to development of quarter IMI, but exposure of the healthy teats to strain M60 did not. Interactions between bacteria and host proteins may not, however, be restricted to adhesion mechanisms. After the onset of mastitis, many plasma proteins leak into milk. The possibility of *S. aureus* binding host-derived proteins might affect bacterial survival within the mammary gland. Bacterial cells covered with a dense layer of plasma proteins may appear more hostlike, possibly providing an alternative explanation for reduced clearance of various bacteria by the leukocytes.

CONCLUSIONS

Despite the small number of quarters used in the experiments, the results indicate that a lesion on the teat orifice epithelium is a predisposing factor to staphylococcal mastitis. Furthermore, classifying staphylococci as coagulase-positive and CNS does not seem to provide sufficient information about the character and prognosis of the disease produced by them, especially in the case of CNS. *Staphylococcus aureus* is the most virulent of the staphylococci, but exposure of teats to CNS can lead to quarter IMI, and the probability of subclinical mastitis development is high. Because CNS lack some of the virulence factors of *S. aureus*, better characterization of these properties is necessary to understand the pathogenicity of CNS. Economically subclinical mastitis is important, and CNS merit further research.

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