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Collagen Binding, Elastase Production, and Slime Production Associated with Coagulase-Negative Staphylococci Isolated from Bovine Intramammary Infections†

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Collagen binding, elastase production, slime production, and associated somatic cell counts were determined with 160 strains of coagulase-negative staphylococci isolated from bovine intramammary infections. Mean binding values for type I and II collagen with *Staphylococcus epidermidis*, *S. chromogenes*, and *S. hyicus* strains were 5.8, 6.6, and 7.4 and 4.3, 4.2, and 4.9%, respectively. Eleven of 28 (39.3%) *S. epidermidis*, 1 of 38 (2.6%) *S. chromogenes*, and 1 of 94 (1.1%) *S. hyicus* strains were elastase positive. Slime production was noted with 12 (42.9%) *S. epidermidis*, 1 (2.6%) *S. chromogenes*, and 11 (11.7%) *S. hyicus* strains. No differences in somatic cell counts were observed with type I or type II collagen binding, elastase production, or slime production with *S. epidermidis* or *S. chromogenes*. However, somatic cell counts associated with *S. hyicus* strains with collagen type I binding affinities of >5 and type II binding affinities of >3 were 320.7 × 10³ compared with 163.9 × 10³ for strains with lower binding affinities.

Historically, virulence of mastitis pathogens has been determined by the magnitude of specific cellular immune responses (14, 19). Polymorphonuclear leukocytes preferentially migrate into the udder in response to bacterial infection (18), increasing the somatic cell counts (SCC), which are highly predictive of infection and inversely correlated with milk production (14, 19). Past recommendations (20) considered SCC of normal milk to be 500 × 10³ or less. A recent study (14) indicated that normal SCC may be as low as 50 × 10³ and that significant milk losses occur at SCC above this level.

Coagulase-negative staphylococci are the organisms most frequently isolated from bovine intramammary infections (IMI) (1, 5, 6). Associated SCC levels have been placed at 462 × 10³ per ml (1, 3, 5). Since these SCC levels were within the 500 × 10³ level previously considered normal, coagulase-negative staphylococci were classified as nonpathogenic or minor pathogens (3). The realization that normal milk SCC were much lower than realized (14) has resulted in reclassification of the coagulase-negative staphylococci as mammary gland pathogens.

Delineation of virulent coagulase-negative staphylococci from nonvirulent resident flora has been difficult because virulence factors have not been well defined (8, 9). Gemmell and Schumacher-Perdreau (9) determined that coagulase-negative staphylococci produce as many as eight toxins and enzymes that could contribute to virulence. Of these, an enzyme resembling *Staphylococcus aureus* delta-toxin demonstrated the greatest biological activity (9). Hébert and Hancock (11) determined that this toxin could be readily detected by using a synergistic hemolysin assay. Watts and Owens (23) determined that elevated SCC levels were asso-

ciated with synergistic hemolysin assay-positive strains of *S. epidermidis* but not with strains of animal-associated, coagulase-negative staphylococci such as *S. hyicus* or *S. chromogenes*. Other workers (4, 13, 16, 20) have determined that coagulase-negative staphylococci produce a variety of substances capable of degrading or adhering to normal mammalian tissue components. However, the role of these substances in bovine mastitis has not been elucidated.

The purpose of this study was to determine the ability of coagulase-negative staphylococci isolated from bovine IMI to bind collagen, degrade elastin, and produce slime. Furthermore, SCC associated with collagen binding, elastase production, and slime production were determined.

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MATERIALS AND METHODS

Bacteria. A total of 160 coagulase-negative staphylococci, including 28 *S. epidermidis*, 38 *S. chromogenes*, and 94 *S. hyicus* strains, were used. All isolates were obtained from bovine IMI. The Staph-Trac system (Analytab Products, Plainview, N.Y.) was used to identify all isolates. Accuracy of the system was established previously (22). Isolates demonstrating atypical test results were identified by a previously described conventional method (22).

All isolates were stored in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) supplemented with 20% glycerin at −70°C. Prior to testing, all isolates were subcultured twice on Trypticase soy agar (BBL) supplemented with 5% bovine blood and 0.1% esculin (Sigma Chemical Co., St. Louis, Mo.) for 24 h at 35°C.

Collagen binding. Determination of type I collagen (CN-I) and type II collagen (CN-II) (Collagen Corp., Palo Alto, Calif.) binding was performed as described previously (18). Briefly, CN-I and CN-II were labeled with ¹²⁵I by the
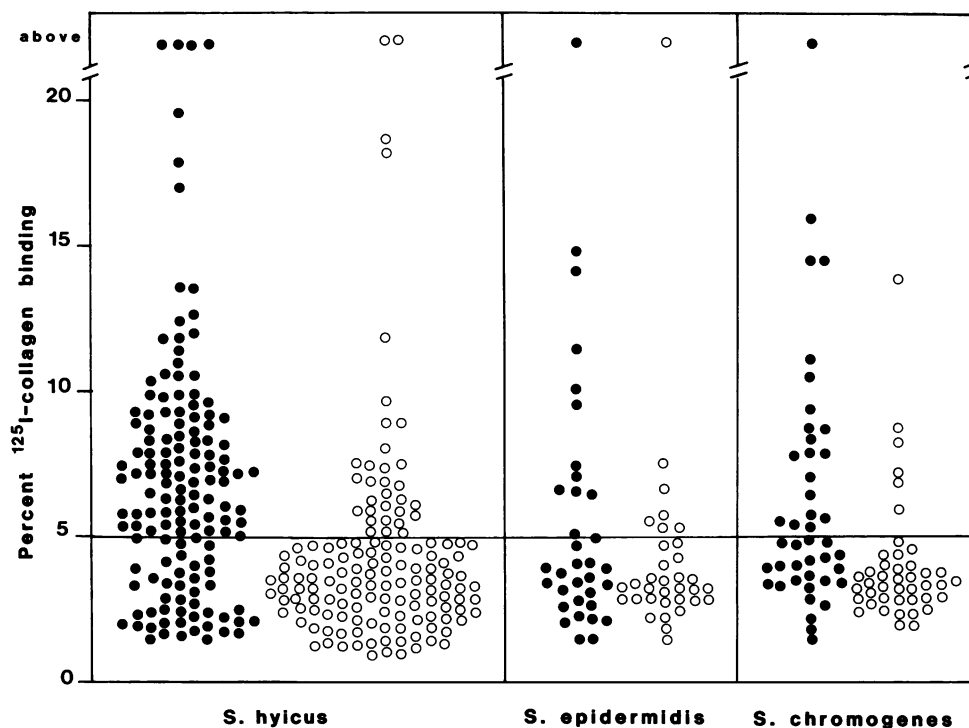

FIG. 1. Binding of ^{125}I-labeled type I (●) and II (○) collagens to three species of coagulase-negative staphylococci isolated from bovine mastitis. Radioisotope protein binding assay was performed with bacterial cells grown in Trypticase soy broth, as described in Materials and Methods. A binding value of <5% of the total added radioactivity was considered negative.

chloramine-T method (10). Binding to staphylococcal cells was quantified by the method of Fröman et al. (7). Bacteria were grown in Trypticase soy broth at 37°C for 18 h with constant shaking. Cells were washed once in 0.15 M phosphate-buffered saline, pH 7.2, and the density was adjusted photometrically to 10^{10} cells per ml. ^{125}I-labeled CN-I and CN-II were diluted to a radioactivity measurement between 15,000 and 25,000 cpm in phosphate-buffered saline, pH 7.2, containing 1% bovine serum albumin. Labeled CN-I or CN-II (50 μl) was mixed with 100 μl of bacterial suspension in a polystyrene centrifuge tube and kept at room temperature for 1 h. After addition of 2 ml of ice-cold phosphate-buffered saline containing 0.05% sodium azide and 0.1% Tween 20, tubes were centrifuged at 4,500 × g for 10 min and supernatants were aspirated. Pellet radioactivity was measured in a 1260-Multigamma counter (LKB-Wallac, Turku, Finland). S. aureus strains Cowan 1, Newman, and Wood 46 and two Micrococcus luteus strains were used as positive and negative controls, respectively, during the binding assay. Binding assays were performed in triplicate and repeated four times.

Elastase production. Elastase production was determined by the method of Janda (13). Briefly, each isolate was streaked radially onto a brain heart infusion agar (BBL) plate supplemented with 0.3% elastin purified from bovine neck ligament (Sigma). A total of eight isolates were streaked on each plate, and plates were incubated for 48 h at 35°C and then transferred to room temperature for an additional 8 days. Plates were observed for elastolytic activity after 24, 48, 72, 120, 168, 216, and 240 h. Clearing of elastin particles adjacent to bacterial colonies was considered positive.

Slime production. Slime production was determined by the method of Christensen et al. (4). Briefly, a single well-isolated colony from a 24-h bovine blood agar culture was inoculated into 10 ml of Trypticase soy broth and incubated overnight at 37°C. The test tubes were emptied and stained with safranin. An isolate was considered positive for slime production when a visible film lined the tube walls. Ring formation at the liquid-air interface was considered negative.

SCC. SCC were performed as described previously (23). After microbiological analysis, milk samples containing sufficient volume were prepared for SCC determination by adding potassium dichromate (Nasco, Fort Atkinson, Wis.) to a final concentration of 0.2%. Samples were refrigerated at 2 to 8°C for a least 24 h to permit proper fixation of the somatic cells. All SCC were performed within 96 h of collection with a Fossomatic milk analyzer (A. S. Foss, Hillerod, Denmark) and reported as SCC × 10^3 per ml.

Statistical analysis. Least-squares analysis of variance was utilized, using the General Linear Models procedure of SAS (SAS Institute, Cary, N.C.). SCC were analyzed for effects of collagen binding, elastase production, and slime production.

RESULTS

Mean CN-I binding values for S. epidermidis, S. chromogenes, and S. hyicus were 5.8, 6.6, and 7.4%, respectively. CN-II binding was lower, with values of 4.3, 4.2, and 4.9% for S. epidermidis, S. chromogenes, and S. hyicus, respectively (Fig. 1). Differences in associated SCC and collagen were observed with S. hyicus strains, which yielded CN-I binding of >5 and CN-II binding of >3, but not with S. epidermidis or S. chromogenes strains (Table 1). Only 7 of 28 (25.0%) S. epidermidis strains yielded CN-I binding values of >5 and CN-II binding values of >3. Mean SCC associated with high collagen binding strains was 59.7 compared with 84.0 for low collagen binding strains. Of 38 S.

TABLE 1. SCC associated with collagen-binding, coagulase-negative staphylococci isolated from bovine IMI

Organism	CN-I > 5, CN-II > 3		CN-I < 5, CN-II < 3	
	No. of isolates	SCC/ml (mean ± SE) (10³)	No. of isolates	SCC/ml (mean ± SE) (10³)
S. epidermidis	7	59.7 ± 32.6	21	84.0 ± 18.25
S. chromogenes	20	182.4 ± 71.5	18	152.5 ± 57.49
S. hyicus	43	320.7 ± 57.77	51	163.9 ± 40.45

TABLE 3. SCC associated with slime-positive and -negative, coagulase-negative staphylococci isolated from bovine IMI

Organism	Slime positive		Slime negative	
	No. of isolates	SCC/ml (mean ± SE) (10³)	No. of isolates	SCC/ml (mean ± SE) (10³)
S. epidermidis	12	85.1 ± 25.51	16	72.6 ± 20.48
S. chromogenes	1	89.0	37	170.4 ± 47.11
S. hyicus	11	257.9 ± 96.06	83	232.7 ± 37.82

chromogenes strains tested, 20 (52.8%) with high collagen binding values had SCC values of 182.4 compared with 152.5 for strains with lower binding affinities. SCC values for 43 (45.7%) S. hyicus strains with high collagen binding values were 320.7 compared with a mean SCC of 163.9 for strains with low collagen binding values. Differences between high and low collagen binding strains for each species were not statistically significant.

Elastase production and associated SCC of coagulase-negative staphylococci isolated from bovine IMI are presented in Table 2. Eleven of 28 (39.3%) S. epidermidis strains were elastase positive compared with only 1 of 38 (2.6%) S. chromogenes strains and 1 of 94 (1.1%) S. hyicus strains. SCC associated with elastase-positive S. epidermidis strains were higher (93.7) than SCC for elastase-negative strains (67.7). A single elastase-positive S. chromogenes strain was associated with an SCC of 89.0 compared with 170.4 for the elastase-negative strains. The elastase-positive S. hyicus strain was associated with an SCC of 656.0 compared with 231.1 for elastase-negative strains. Differences in SCC between elastase-positive and elastase-negative strains were not significant.

Slime production and associated SCC for the coagulase-negative staphlococcal species tested are presented in Table 3. Of the three species tested, 12 (42.9%) S. epidermidis, 1 (2.6%) S. chromogenes, and 11 (11.7%) S. hyicus strains were positive for slime production. However, SCC for the slime production-positive S. epidermidis, S. chromogenes, and S. hyicus strains were 85.1, 89.0, and 257.9 compared with 72.6, 170.4, and 232.7, respectively, for slime-negative strains. These differences were not statistically significant.

DISCUSSION

S. epidermidis, S. chromogenes, and S. hyicus are the most frequently isolated coagulase-negative staphylococcal species from bovine IMI (1, 5, 6; reviewed in reference 21). S. epidermidis is considered a human-associated staphylococcal species and is the coagulase-negative staphylococcal species most often associated with abnormal milk or elevated SCC (1). S. chromogenes and S. hyicus are considered normal flora for the skin of dairy cattle and are frequently

TABLE 2. SCC associated with elastase-positive and -negative, coagulase-negative staphylococci isolated from bovine IMI

Organism	Elastase positive		Elastase negative	
	No. of isolates	SCC/ml (mean ± SE) (10³)	No. of isolates	SCC/ml (mean ± SE) (10³)
S. epidermidis	11	93.7 ± 30.56	17	67.7 ± 17.15
S. chromogenes	1	89.0	37	170.4 ± 47.11
S. hyicus	1	656.0	93	231.1 ± 35.18

isolated from bovine teat skin and teat canals as well as bovine IMI (2, 5, 6).

Results of the present study revealed differences among the three species in ability to bind CN-I and CN-II. Strains of S. hyicus and S. chromogenes demonstrated higher CN-I binding values than S. epidermidis strains. Miedzobrodzki et al. (16) reported that more S. epidermidis strains bound CN-I (76.7%) than did either S. hyicus (74.3%) or S. chromogenes (68.1%) strains. These results may be due to assay differences, as the particle agglutination assay used in the previous study (16) detected CN-I binding whereas the collagen binding assay used in the present study determined the degree of binding.

Mamö et al. (15) determined that the mean binding values for CN-II with three S. hyicus and three S. chromogenes strains were 5.3 and 13.0%, respectively. These findings are similar to results for the 94 S. hyicus strains (4.96%) in the present study. However, these findings are much higher than the 4.19% binding obtained with 38 S. chromogenes strains in the present study. Mamö et al. (15) did not include S. epidermidis strains: our study revealed a 4.29% mean binding value for this species.

No relationship between CN-I and CN-II binding and SCC for S. epidermidis and S. chromogenes was observed. However, SCC for S. hyicus strains with CN-I binding values of >5 and CN-II binding values of >3 were twice as high as for strains with CN-I and CN-II binding values below these thresholds. It appears that collagen binding may play a role in the virulence of S. hyicus but not S. chromogenes or S. epidermidis.

The ability of coagulase-negative staphylococci to degrade elastin may play an important role in tissue invasion. Janda (13) examined 161 staphylococci of human origin for elastolytic activity and determined that, of 12 coagulase-negative staphylococcal species tested, only strains of S. epidermidis were capable of producing elastase. Results of the present study revealed that 39.3% of S. epidermidis strains of bovine origin were elastase positive compared with 100% of strains in the previous study (13). Also, one strain each of S. chromogenes and S. hyicus was elastase positive. Comparison of associated SCC between elastase-positive and -negative S. epidermidis strains revealed only minor differences. These findings indicate that, while elastase may play a role in tissue invasion, this enzyme probably has a minor role in the pathogenicity of S. epidermidis in bovine IMI.

Slime production has been determined to be associated with S. epidermidis in disease and is used for recognition of pathogenic coagulase-negative staphylococci isolated from human sources (4, 11, 12). Christensen et al. (4) determined that slime production was associated with gentamicin resistance and symptomatic infections. Ishak et al. (12) determined that 13 of 14 clinically significant coagulase-negative staphylococci isolates produced slime, whereas only 3 of 13 contaminants and 4 of 27 skin isolates were slime producers.

Furthermore, these workers (12) found that *S. epidermidis* produced slime more frequently than other species. Hébert and Hancock (11) determined that 83% of 672 *S. epidermidis* strains of human origin were slime producers and that the slime production test may be useful in defining clinical significance. Results of the present study showed that a larger percentage of *S. epidermidis* strains (42.9%) than *S. chromogenes* (2.6%) or *S. hyicus* (11.7%) produced slime. Comparison of SCC associated with slime-positive and slime-negative strains revealed only slight differences. Thus, it appeared that slime production was not a reliable indicator of pathogenicity for coagulase-negative staphylococci isolated from bovine IMI.

Virulence factors associated with coagulase-negative staphylococci isolated from bovine IMI remain undefined. Results of the present study indicate that elastase, slime production, and collagen binding were not associated with increased SCC. A previous study (23) determined that production of a delta-like toxin by *S. epidermidis* strains was associated with a threefold increase in SCC. Thus, it appears that toxin production by *S. epidermidis* is the most reliable indicator of pathogenicity. Elastase and slime production occurred with only one *S. chromogenes* strain, and SCC with all strains were low. Furthermore, a previous study (23) indicated that relatively few *S. chromogenes* strains produced a delta-like toxin. Thus, *S. chromogenes* may be relatively nonpathogenic and, since this organism readily colonizes teat canals (2), IMI produced by this organism may be a consequence of opportunity rather than virulence. Increased SCC were observed with *S. hyicus* strains with high collagen binding affinities but not with elastase or slime production in the present study or toxin production in a previous study (23). Whether the increased collagen binding or another substance common to these strains was responsible for virulence has not been elucidated.

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