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Slime Production by Bovine Milk *Staphylococcus aureus* and Identification of Coagulase-Negative Staphylococcal Isolates

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Staphylococcus aureus **isolates from bovine milk were assessed for capsule or slime production. When pure *S. aureus* cultures in milk were inoculated directly into serum-soft agar constituted with a modified staphylococcus 110 medium, 100% of the isolates grew with diffuse colony morphology. Diffuse colony morphology was rapidly lost on subculture and was more rapidly lost in brain heart infusion-serum-soft agar. No evidence was seen for encapsulation in India ink preparations or by the clumping factor test. It was concluded that freshly isolated *S. aureus* strains produce slime, not true capsules. During examination of the 84 milk samples that grew staphylococci in addition to *S. aureus* (27.4%), a significant number of coagulase-negative staphylococcal species were encountered and identified by conventional tests as *S. simulans* (41.7%), *S. xylosus* (11.9%), *S. epidermidis* (3.6%), *S. saprophyticus* (3.6%), *S. hyicus* (2.9%), *S. cohnii* (1.2%), *S. haemolyticus* (1.2%), and *S. warneri* (1.2%). Five isolates (6.0%) were not identified. Attempts were also made to identify the isolates by the API Staph-Ident system, which gave an overall accuracy of 45.2%. The susceptibilities of the isolates to a variety of antibiotics were determined, and they appeared to be less resistant than human clinical isolates.**

Norcross and Opdebeeck (22, 23) reported that 93% of *Staphylococcus aureus* isolates from lactating cows were encapsulated when cultured directly from milk, as determined by diffuse growth in serum-soft agar (10) constituted with a modified staphylococcus 110 medium (33). Limited subculture of isolates resulted in decreased encapsulation. Modified 110 medium is made by dialyzing yeast extract and peptone against 3.0% (wt/vol) NaCl followed by the addition of mannitol, lactose, and K_2HPO_4 (33). Growth in this medium encourages the production of mucoid or viscid material that is readily removed from cells by washing with saline (33). This material is known as slime and is chemically and morphologically distinct from staphylococcal capsules (28). Thus, it appears possible that Norcross and Opdebeeck (22, 23) actually observed slime production rather than encapsulation. To demonstrate true capsule formation, a variety of other criteria can be used, including observation in India ink preparations and diffuse growth in media more normal than 110 medium, such as brain heart infusion (BHI) or tryptic soy broth media (28). Accordingly, we set out to examine fresh *S. aureus* isolates from bovine milk for encapsulation or slime production. Capsules and slimes are significant staphylococcal virulence factors (28), and antibodies against them could have protective activity.

On culturing milk samples, we encountered a significant number of coagulase-negative staphylococci. Because few studies have identified such strains at the species level, we proceeded to do so by using the simplified scheme of Kloos and Schleifer (15). We compared the identification given by use of the API Staph-Ident system to that by the Kloos and Schleifer scheme (15).

After completion of our experimental work, Anderson (2) concluded that mastitis strains that had been maintained on artificial medium for 1.5 to 3 years were not encapsulated, although 28.8% of them grew as diffuse colonies in serum-soft agar. Langlois et al. (19) reported that the API Staph-Ident system identified correctly only 41.8% of non-*S. aureus* bovine staphylococci from quarter milk isolates.

MATERIALS AND METHODS

Strains. In a 6-month period, 142 composite, individual milk samples were aseptically collected in the morning before milking and refrigerated in 5-ml plastic tubes (Fischer Scientific Co., Springfield, N.J.), as recommended by the National Mastitis Council (21). The samples were collected from two herds in the Flanagan, Ill., area. On the same day, the samples were mixed, and one loopful from each tube was streaked on quadrants of MacConkey (Difco Laboratories, Detroit, Mich.), Columbia CNA (Difco), mannitol-salt (8), and blood agar plates. Blood agar plates were made by adding 5% (vol/vol) sheep blood to tryptic soy agar (Difco). The plates were incubated for 48 h at 37°C. The milk samples were frozen at −20°C after the initial culturing. Suspected staphylococcal isolates were purified by restreaking on mannitol-salt plates. Gram stains were performed, and the identification of the isolates as staphylococci was verified by aerobic acid production from glycerol in the presence of erythromycin (25) and their lysostaphin susceptibility and lysozyme resistance (17). The organisms were grown overnight in BHI medium (Difco), sterile glycerol was added to a final concentration of 15% (vol/vol), and the strains were stored at −70°C.

Coagulase production. Isolates were tested for coagulase production by the tube test with citrated rabbit plasma according to the instructions of the manufacturer (Difco).

Identification of staphylococcal species by conventional tests. Strains were identified by the simplified scheme of Kloos and Schleifer (15, 29).

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TABLE 1. Colony morphology in serum-soft agar of *S. aureus* cultivated directly from milk and after subculture

Subculture no.	% of Isolates with the indicated colony morphology in the following medium:			
	BHI-serum-soft agar		110-serum-soft agar	
	Diffuse	Compact	Diffuse	Compact
0[a]	85.7	14.3	100	0
1	0	100	78.6	21.4
2	0	100	50	50
3	0	100	7.1	92.0

[a] Taken directly from milk; 14 strains were examined.

Identification of staphylococcal isolates by the API Staph-Ident system. API Staph-Ident strips (Analytab Products, Plainview, N.Y.) were inoculated according to the manufacturer's instructions using an overnight culture on a blood agar plate. Tubes containing 2 ml of 0.85% (vol/vol) NaCl were inoculated to a density equivalent to a McFarland turbidity standard of 3. A cotton swab was used to remove organisms from the plate.

Antibiotic susceptibility. Susceptibility to antibiotics was determined by the disk diffusion method on Mueller-Hinton plates (BBL Microbiology Systems, Cockeysville, Md.) (3).

Colony morphology in serum-soft agar. Milk samples containing pure *S. aureus* cultures were used to directly inoculate (one loopful) serum-soft agar tubes (10) constituted with 1% (vol/vol) normal rabbit serum and 0.15% (wt/vol) agar with either BHI broth or modified staphylococcus 110 medium (22, 23, 33). Further subcultures were performed overnight at 37°C on P agar (17). Serum-soft agar was inoculated with a wire dipped into a suspension of one loopful of subcultured organisms in 5 ml of 0.9% (wt/vol) NaCl. This was found to give a convenient number of colonies in the tubes for determination of morphology.

India ink preparations and clumping factor. To further evaluate *S. aureus* strains for evidence of encapsulation the following tests were performed (28). India ink preparations were done as described by Cruickshank et al. (6) using Pelikan no. 17 black India ink (Pelikan AG, Hannover, Federal Republic of Germany). Clumping factor was determined by using citrated rabbit plasma as described by the manufacturer (Difco).

RESULTS

Colony morphology of *S. aureus* in serum-soft agar. When milk samples were streaked, 84 (59.2%) grew staphylococci in various amounts from a few colonies per plate to heavy pure cultures. On the basis of coagulase production and other characteristics (see below), 23 strains (27.3%) of *S. aureus* were obtained. Fourteen of these strains, which were present in relatively pure culture, were chosen for examination of colony morphology in serum-soft agar when cultivated directly from milk. The results are shown in Table 1. On initial culture from milk, 100% of the isolates had a diffuse colony morphology in 110-serum-soft agar, confirming the result of Norcross and Opdebeeck (22, 23), and 85.7% had diffuse morphology in BHI-serum-soft agar. However, after one subculture, 100% of the isolates gave a compact colony morphology in BHI-serum-soft agar, which was maintained through two more subcultures. The percentage of strains giving diffuse colonies in 110-serum-soft agar declined to 78.6% after one subculture, and only one strain still gave diffuse colonies after three subcultures.

Diffuse colonies in serum-soft agar indicate either capsule or slime production (28). The fact that the isolates lost their diffuse colony morphology in BHI-serum-soft agar after one subculture argues that the morphology was not due to capsule production but rather to slime production. Some of the isolates were further examined for evidence of encapsulation in India ink preparations and by clumping factor tests, with appropriate positive controls (28), and the results were negative.

Identification of staphylococcal species. Conventional tests. On initial examination of the strains, it was noted, perhaps somewhat surprisingly, that the majority (61 of 84; 72.6%) were coagulase negative, i.e., not *S. aureus*. It was of interest to identify these strains at the species level because relatively few studies have so classified bovine milk isolates. The simplified scheme of Kloos and Schleifer (15) was used; the results are shown in Table 2. The most abundant species was *Staphylococcus simulans* (41.7%), followed by *S. aureus* (27.4%), and *Staphylococcus xylosus* (11.9%).

Thirty-five of the isolates were identified as *S. simulans*. For the most part, the strains correlated well with the characteristics described by Kloos and Schleifer, although 31 strains produced acid from galactose. Three of the strains were novobiocin resistant, but they classified best as *S. simulans* rather than as any of the recognized novobiocin-resistant species (15). Novobiocin resistance in staphylococcal species other than *S. cohnii*, *S. saprophyticus*, and *S. xylosus* was noted previously (29). One of the *S. aureus* isolates was coagulase negative (29). Forty percent of the *S. xylosus* isolates did not produce acid from xylose, and one strain was novobiocin susceptible. Not all *S. xylosus* strains produce acid from xylose (16). Two of the *S. saprophyticus* strains produced acid from both galactose and ribose. Five of the strains could not be classified as any of the recognized species. However, it must be remembered that the scheme of Kloos and Schleifer was designed for human, not animal, isolates.

API Staph-Ident system. The API Staph-Ident system is designed for the rapid identification of staphylococcal species. Several studies have documented its use in identifying human staphylococcal species (1, 5, 18), but only one study has investigated its applicability to bovine isolates (19). Assuming the identifications given by the Kloos and Schleifer scheme are correct, the species most often correctly identified by the API system was *S. aureus* (82.3%). Overall, the accuracy was 45.2%, but it was only 31.1% when coagulase-negative strains alone were considered.

TABLE 2. Identification of staphylococci by the simplified scheme of Kloos and Schleifer[a] and by the API Staph-Ident system

Staphylococcal species	No. (%) identified by simplified scheme	% Correctly identified by API system
S. simulans	35 (41.7)	25.7
S. aureus	23 (27.4)	82.3
S. xylosus	10 (11.9)	50
S. epidermidis	3 (3.6)	66.7
S. saprophyticus	3 (3.6)	0
S. hyicus	2 (2.9)	100
S. cohnii	1 (1.2)	0
S. haemolyticus	1 (1.2)	0
S. warneri	1 (1.2)	100
Unidentified	5 (6.0)	NA[b]

[a] Reference 15.
[b] NA, Not applicable.

TABLE 3. Susceptibility of bovine staphylococcal isolates to various antibiotics

Staphylococcal species and no. of isolates	% Resistant[a]				
	Penicillin	Methicillin	Tetracycline	Erythromycin	Novobiocin
S. simulans, 35	14.3	5.7	2.9	5.7	8.6
S. aureus, 23	21.7	8.7	0	4.3	0
S. xylosus, 10	10	30	20	0	90
S. epidermidis, 3	0	0	0	0	0
S. saprophyticus, 3	100	66.7	66.7	66.7	100
S. hyicus, 2	0	0	0	0	0
S. cohnii, 1	0	100	0	0	100
S. haemolyticus, 1	0	0	0	0	0
S. warneri, 1	0	0	0	0	0

[a] No isolates were resistant to chloramphenicol, amikacin, and gentamicin.

Antibiotic susceptibility. The results of susceptibility testing are shown in Table 3. All the isolates were susceptible to chloramphenicol, amikacin, and gentamicin. In general, these bovine isolates showed less antibiotic resistance than do human clinical isolates (12, 29).

DISCUSSION

Slime production. All *S. aureus* isolates tested grew diffusely in 110-serum-soft agar when cultivated directly from milk. Subcultivation on artificial medium quickly led to loss of diffuse colony morphology. These results confirm those of Opdebeeck and Norcross (23), who found that 93% of *S. aureus* isolates grew diffusely upon initial cultivation from milk and that diffuse colony morphology was quickly lost. These workers assigned diffuse colony morphology to being due to encapsulation. However, we believe that the diffuse colony morphology is due to slime rather than capsule production. Yoshida and Ekstedt (33) found that strains showed mucoid or viscid growth on 110-agar but not on BHI-agar and that strains showing diffuse colony morphology in 110-serum-soft agar had a compact morphology in BHI-serum-soft agar. An exception was the classically encapsulated Smith diffuse strain (13, 28), which grew diffusely in both media (33). Characterized by high levels of NaCl and carbohydrate, 110 medium has been used to encourage slime production (4, 9, 28). Upon initial cultivation in BHI-serum-soft agar, 85.7% of the strains grew diffusely, but the strains quickly reverted to compact colony morphology.

To confirm encapsulation, microscopic visualization should be used, and strains typically are examined in India ink preparations (28). We could find no evidence for capsules when the strains were examined directly in this manner. Also, the strains were positive in the clumping factor test; a negative clumping factor test is an indicator of encapsulation (28). Thus, we conclude that freshly isolated milk strains produce slime but not capsules and that this ability is readily lost upon subculture. Anderson (2) also concluded that mastitis *S. aureus* strains are not encapsulated because unstained halos could not be shown in India ink preparations, and viable numbers were readily reduced in the peritoneal cavity of mice, in contrast to the case with the truly encapsulated M strain. Although the strains had been maintained on artificial medium in the study of Anderson, they all grew as diffuse colonies in 110-serum-soft agar, in contrast to our results and those of Norcross and Opdebeeck (22, 23). Yokomizo et al. (32) found that 1.2% of 1,598 *S. aureus* strains isolated from bovine mastitis milk exhibited diffuse growth in serum-soft agar.

Recently, Sompolinsky et al. (26) studied the encapsulation of a large number of *S. aureus* isolates from various sources. Nonagglutinability with antiteichoic acid antiserum was used as the main criterion for encapsulation, and over 90% of all isolates were reported to be encapsulated. Seventeen bovine mastitis milk isolates were examined, and most were encapsulated but nontypeable with the 11 existing antisera. Clearly, there is a difference between *S. aureus* strains that have a classical capsule defined as a covering layer outside the cell wall, demonstrable by the light microscope, thus having a thickness greater than 200 nm and a definite external surface (28, 31), and most of the strains described by Sompolinsky et al. (26). Heavily encapsulated strains, exemplified by the M and Smith diffuse strains, are also mouse lethal, non-phage typeable, and clumping factor negative and give diffuse colonies in serum-soft agar, owing to the capsule (28). Most of the strains described by Sompolinsky et al. (26) would probably be more accurately described as having microcapsules, defined as sub-light-microscopic layers outside the cell wall that are chemically and immunologically distinct from the cell wall (28, 31).

The mechanism of diffuse colony formation by encapsulated and slime-producing *S. aureus* strains is not fully understood. It has been suggested that compact colony formation by unencapsulated strains is due to a reaction between protein A and the Fc of immunoglobulin G molecules, which results in the adherence of daughter cells during colony development (10, 11). The staphylococcal capsule is readily penetrated by high-molecular-weight proteins (14, 30), and one of us has proposed (28) that immunoglobulin G reacts with cell wall protein A and becomes buried in the mass of the capsule. This probably applies equally well to slime. Thus, immunoglobulin G molecules are not exposed at the cell surface and are unable to cause adherence of daughter cells.

An alternative view of the staphylococcal substance responsible for compact colony formation is that of Yoshida et al. (34) and Usui et al. (27). These workers isolated a polysaccharide similar to but distinct from teichoic acid that absorbs the serum reacting-factor, causing compact colonies from protein A- and non-protein A-producing strains. This is not incompatible with the general mechanism proposed for diffuse colony formation, i.e., lack of surface exposure of the serum protein, causing adherence of daughter cells and, hence, compact colonies.

It seems likely that repeated subcultivation on artificial medium results in the repression of slime synthesis, the degree of expression also being influenced by the medium in which slime production is tested.

Identification of coagulase-negative species. We encountered a significant number of coagulase-negative staphylococcal strains in our examination of the milk samples. The

most frequently encountered species were *S. simulans* and *S. xylosus*, with six other species being found. In the study of DeVries (7), the most commonly encountered species were *S. xylosus*, *S. epidermidis*, *S. sciuri*, and *S. haemolyticus*. Thus, we found a significantly different distribution. *S. epidermidis* made up the highest proportion of the isolates in the study by Langlois et al. (19), with a variety of other species being found. In addition to *S. aureus*, *S. simulans* was often present in pure or heavy culture in our milk samples, which may indicate that this species has the ability to cause mastitis.

The API Staph-Ident system, which was developed for the identification of human staphylococcal strains, correctly identified only 45.2% of these bovine isolates. This may have been due to biochemical differences between bovine and human staphylococci (19). Also, the results were difficult to interpret at times, with borderline reactions, especially in the carbohydrate tests, that were difficult to score as positive or negative. These results confirm the findings of Langlois et al. (19), who found that the API Staph-Ident system correctly identified only 54% of bovine staphylococci from quarter milk samples. However, the same workers (20) found that the DMS Staph-Trac system, which uses more biochemical tests than the API Staph-Ident system, correctly identified 91.2% of the bovine isolates studied. In our study, most of the *S. aureus* isolates had the same profile number, which might indicate that one strain of this species was passed through the herd.

It appears that the significance of slime production deserves to be studied further, perhaps with particular reference to the development of immunization schemes for protection against infection (2, 24). Opdebeeck and Norcross (24) used the encapsulated *S. aureus* Smith diffuse strain grown in 110 medium as the basis of their vaccine preparations for the immunization of pregnant cows. It is likely that antibodies were produced against both capsular and slime polymers. The coagulase-negative strains could not be examined for slime production by using the serum-soft agar test because these species give diffuse colonies in this test (10). This and their significance as pathogens deserve further examination.

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