Synergistic Hemolysis Associated with Coagulase-Negative Staphylococci Isolated from Bovine Mammary Glands†

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A total of 353 coagulase-negative staphylococcus (CNS) isolates from infected bovine mammary glands were tested for cytolysin production by using the synergistic hemolysin assay (SHA). Overall, 34.6% of CNS isolates were SHA positive. Human-associated, coagulase-negative staphylococcal species contained the greatest number of SHA-positive strains. Milk leukocyte levels expressed as somatic cell counts (SCC) were elevated with SHA-positive Staphylococcus epidermidis, Staphylococcus hominis, and Staphylococcus warneri strains. Elevated SCC levels were associated with strains of Staphylococcus hyicus and Staphylococcus chromogenes. However, no difference in SCC levels was observed between SHA-positive and SHA-negative strains. Results indicated that the SHA was a sensitive test for the detection of cytolysin-producing CNS.

Ecological studies (1, 6, 15, 16, 18) based on current species descriptions have revealed host, body site, and tissue tropism among different coagulase-negative staphylococci (CNS). CNS are involved in disease processes of humans and animals (2, 3, 23, 24), and recent studies (1, 5, 13) have determined that CNS are important mammary gland pathogens.

Polymorphonuclear leukocytes preferentially migrate into the udder in response to bacterial infection (21). Increased polymorphonuclear leukocyte levels expressed as somatic cell counts (SCC) are a highly predictive indicator of infection and are inversely correlated with milk production (11, 22). Past recommendations (22) considered SCC of normal milk to be 5 x 10^3 per ml or less. Since SCC levels associated with CNS have been placed at 462 x 10^3 per ml, these organisms were classified as minor pathogens (4). A recent study (11) indicates that normal SCC may be as low as 5 x 10^4 and that significant milk losses occur at SCC above this level. As a result of these studies, the role of CNS as mastitis pathogens has received increased attention.

Delineation of virulent CNS from nonvirulent resident flora has been difficult because virulence factors have not been well-defined (7, 8). Gemmel and Schumacher-Perdue (8) determined that CNS produce as many as eight toxins and enzymes that could contribute to virulence. Of these toxins and enzymes, a hemolysin resembling Staphylococcus aureus delta-toxin demonstrated the greatest biological activity. Cytotoxicity studies (7) with human embryonic lung fibroblasts and the colony overlay test demonstrated that this CNS cytolysin caused preferential release of cell constituents and cellular swelling. However, these tests are not well adapted for routine use in diagnostic laboratories.

Hébert and Hancock (12) examined the ability of 250 strains representing 17 staphylococcal species to produce a hemolysin capable of potentiating the hemolytic activity of S. aureus beta-toxin. Strains representing nine species produced synergistic hemolysis of human, sheep, and ox erythrocytes. This method, termed the synergistic hemolysin assay (SHA), permits rapid screening of large numbers of CNS strains for cytolysin production. Because of the potential role of CNS in udder pathology and reduction in milk yield, this study was undertaken to determine the prevalence of SHA-positive CNS isolated from bovine mammary glands and to evaluate the utility of the SHA in the detection of pathogenic strains of CNS.

**MATERIALS AND METHODS**

**Cultures.** A total of 353 CNS samples isolated from bovine intramammary glands was used in the study. Of these, 329 were obtained from individual quarter milk samples collected from cows in four dairy herds. Eleven isolates of Staphylococcus hominis and 13 isolates of Staphylococcus warneri were obtained from R. J. Harmon, University of Kentucky, Lexington. Cultures were plated on Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.) supplemented with 5% bovine blood and 0.1% esculin (Sigma Chemical Co., St. Louis, Mo.) and incubated at 35°C for 48 h. The Staph-Trac system (Analytab Products, Plainview, N.Y.) was used to identify all isolates. Accuracy of this system was established previously (27). Isolates demonstrating atypical test results were identified by a previously described conventional methodology (27).

**SHA.** Strains were tested for synergistic hemolysis by the technique of Hébert and Hancock (12), except that a known beta-lysin-producing S. aureus (strain ATCC 29740) was used. The beta-lysin-producing S. aureus strain was streaked down the center of a blood agar plate. Strains of CNS were streaked perpendicular to, but not touching, the central S. aureus streak. From 8 to 10 CNS isolates were tested on each plate. Plates were incubated aerobically at 35°C for 24 h. Isolates exhibiting complete hemolysis within the S. aureus beta-toxin zone were considered SHA positive. A known SHA-positive Staphylococcus epidermidis strain was used to check each batch of media.

**SCC.** 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% (25). Samples were refrigerated at 2 to 8°C for at 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.

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TABLE 1. Synergistic hemolysis exhibited by CNS isolated from bovine mammary glands

<table>
<thead>
<tr>
<th>Organism (no. of strains tested)</th>
<th>No. (%) of strains exhibiting synergistic hemolysis</th>
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<tbody>
<tr>
<td>S. epidermidis (71)</td>
<td>41 (57.7)</td>
</tr>
<tr>
<td>S. hominis (14)</td>
<td>9 (64.3)</td>
</tr>
<tr>
<td>S. warneri (18)</td>
<td>15 (83.3)</td>
</tr>
<tr>
<td>S. haemolyticus (3)</td>
<td>1 (33.3)</td>
</tr>
<tr>
<td>S. capitis (1)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>S. simulans (11)</td>
<td>5 (45.5)</td>
</tr>
<tr>
<td>S. hyicus (156)</td>
<td>37 (23.7)</td>
</tr>
<tr>
<td>S. chromogenes (63)</td>
<td>8 (12.7)</td>
</tr>
<tr>
<td>S. saprophyticus (3)</td>
<td>1 (33.3)</td>
</tr>
<tr>
<td>S. xylosus (9)</td>
<td>3 (33.3)</td>
</tr>
<tr>
<td>S. sciuri (4)</td>
<td>2 (50.0)</td>
</tr>
</tbody>
</table>

Foss, Hilterød, Denmark) and were reported as SCC × 10³ per ml (25).

RESULTS

Distribution of CNS isolates exhibiting synergistic hemolysis is summarized in Table 1. Overall, 122 (34.6%) of 353 CNS isolates were SHA positive. The most frequently encountered organisms were S. epidermidis, Staphylococcus hyicus, and Staphylococcus chromogenes, which constituted 82.1% of total isolates. Of these three species, 52.7% of S. epidermidis strains were SHA positive, compared with 23.7% of S. hyicus strains and 12.7% of S. chromogenes strains.

Synergistic hemolysis was demonstrated with 9 S. hominis strains, 15 S. warneri strains, 3 Staphylococcus haemolyticus strains, and 5 Staphylococcus simulans strains. One Staphylococcus capitis strain was SHA negative. Of the novobiocin-resistant CNS, one Staphylococcus saprophyticus strain, three Staphylococcus xylosus strains, and two Staphylococcus sciuri strains exhibited synergistic hemolysis.

SCC associated with SHA-positive and SHA-negative CNS are summarized in Table 2. Mean SCC associated with SHA-positive S. epidermidis strains was 214.6 compared with 78.6 for SHA-negative strains. SCC for SHA-positive S. hominis and S. warneri strains were 314.4 and 205.5, respectively, compared with 245.2 and 87.3, respectively, for SHA-negative strains. Small differences were observed in SCC values associated with SHA-positive and SHA-negative strains of S. hyicus and S. chromogenes (Table 2). The low frequency of isolation for the remaining CNS prevented comparison of SCC obtained with SHA-positive and SHA-negative strains.

DISCUSSION

The ability of CNS to produce cytolytic toxins, which act synergistically with S. aureus beta-toxin, has long been recognized (14, 19). The CNS cytolsins have been designated previously as delta-toxin because of activity similar to that of classic S. aureus hemolysin (7, 20). However, the nature of cytolytic toxins produced by different CNS species has not been fully elucidated. Gemmell and Thelestam (9) reported that a heat-stable, lecithin-neutralizable, cytolytic toxin was produced by strains of S. epidermidis, S. saprophyticus, and S. haemolyticus. This toxin was considered synonymous with S. aureus delta-toxin (9). Wadstrom (26) determined that a cytolsin elaborated by S. haemolyticus had no relation to S. aureus alpha-, beta-, or delta-toxin. Thus, the nature of CNS cytolsins appears to be strain or species dependent. Since the SHA detects hemolytic effects and does not define the nature of the cytolsins, a phrase such as "SHA positive" may be preferable to a specific toxin designation.

Previous investigators (1, 6) determined that S. epidermidis, a human-associated CNS, and the animal-associated species S. hyicus and S. chromogenes (previously classified as S. hyicus subsp. chromogenes (10)) were the most frequently isolated CNS from milk with abnormal or high SCC. The distribution of isolates obtained from four commercial dairies in this study concurs with findings in these previous reports, since the predominant species isolated were S. epidermidis, S. hyicus, and S. chromogenes. Incidence of SHA-positive CNS in the present study was lower than that reported by Hébert and Hancock (12); human isolates predominated in their study (12). Results of the present study indicate that the human-associated CNS, S. epidermidis, S. hominis, and S. warneri, contain a higher frequency of SHA-positive strains than do the animal-associated species S. hyicus and S. chromogenes.

The species description of S. hyicus (17) indicates that members of this species do not produce alpha-, beta-, or delta-toxin. Hébert and Hancock (12) observed synergistic hemolysis with three S. hyicus reference strains, including the type strain. In the present study, no S. hyicus strains exhibited hemolysis on bovine blood agar. However, 23.7% of 156 strains were SHA positive. Hébert and Hancock (12) reported similar findings and suggested that nonhemolytic strains produced small amounts of cytolsin. Thus, cytolsin production by CNS should be examined by the SHA; otherwise, strains producing small amounts of toxin will go undetected.

Availability of a rapid, convenient procedure for detection of pathogenic CNS strains would permit development of control methods for elimination of these organisms from dairy herds, thereby decreasing SCC and increasing milk production. Higher SCC levels were observed with SHA-positive S. epidermidis and S. warneri strains than with SHA-negative strains. Elevated SCC were associated with all S. hominis strains; SHA-positive strains were associated with higher SCC than were SHA-negative strains. SCC associated with S. hyicus and S. chromogenes were similar, regardless of synergistic hemolysin production. The SHA may be useful for delineation of pathogenic strains of human-associated CNS, but not for animal-associated CNS.

Hébert and Hancock (12) speculated that SHA-positive CNS may act as synergists with more pathogenic microorganisms such as S. aureus. Interactions between toxins of resident CNS and transient S. aureus populations may permit colonization of teat skin and apex by the latter population. This area, once colonized, would serve as a staging area for invasion of the mammary gland.

TABLE 2. SCC associated with CNS isolated from bovine mammary glands

<table>
<thead>
<tr>
<th>Organism</th>
<th>No. of isolates</th>
<th>SHA-positive SCC/ml (mean ± SE) (10³)</th>
<th>No. of isolates</th>
<th>SHA-negative SCC/ml (mean ± SE) (10³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. epidermidis</td>
<td>40</td>
<td>214.6 ± 109</td>
<td>29</td>
<td>78.6 ± 19</td>
</tr>
<tr>
<td>S. hominis</td>
<td>8</td>
<td>314.4 ± 92</td>
<td>5</td>
<td>245.2 ± 112</td>
</tr>
<tr>
<td>S. warneri</td>
<td>12</td>
<td>205.5 ± 32</td>
<td>3</td>
<td>87.3 ± 35</td>
</tr>
<tr>
<td>S. hyicus</td>
<td>37</td>
<td>220.9 ± 47</td>
<td>119</td>
<td>205.5 ± 30</td>
</tr>
<tr>
<td>S. chromogenes</td>
<td>7</td>
<td>237.7 ± 65</td>
<td>47</td>
<td>208.8 ± 55</td>
</tr>
</tbody>
</table>
LITERATURE CITED


19. Marks, J., and A. C. T. Vaughan. 1950. Staphylococcal delta-


