Production of Enterotoxins and Toxic Shock Syndrome Toxin by Bovine Mammary Isolates of *Staphylococcus aureus*

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The production of staphylococcal enterotoxin A (SEA), SEB, SEC, SED, and SEE and toxic shock syndrome toxin 1 by bovine mammary isolates of *Staphylococcus aureus* was evaluated. Enterotoxin secretion was detected by immunodiffusion using specific polyclonal antisera. Of 262 isolates examined, 75 (28.6%) produced one or more toxins. The most common pattern was secretion of both SEC and SED and toxic shock syndrome toxin 1. No isolates secreted SEE, one produced SEA, and seven secreted SEB.

The staphylococcal enterotoxins are a group of low-molecular-weight proteins which have been classified on the basis of serological differences (1). They are designated staphylococcal enterotoxin A (SEA), SEB, SEC, SED, and SEE. On the basis of minor serological differences, SEC is divided into three subtypes designated SEC-1, SEC-2, and SEC-3. These molecules were initially discovered because they caused food poisoning (1). Toxic shock syndrome toxin 1 (TSST-1) is the causative agent in toxic shock syndrome in humans (4). These proteins have been found to have a wide spectrum of effects on cells of the immune system (20). When human isolates of *Staphylococcus aureus* from cases of septicemia were compared with those isolated from the nares of healthy carriers, it was found that the secretion of enterotoxins A, B, and C was a feature of bacteria cultured from patients with septicemia (10). Enterotoxins may thus play a role in staphylococcal disease states, in addition to their ability to cause food poisoning.

*S. aureus* is a frequent cause of bovine mastitis, a major disease of dairy cattle. There are differences between human and bovine strains. Human strains of this bacterium belong to biotype A and secrete staphylokinase, while bovine strains belong to biotype C, typically produce β-hemolysin, and are capable of coagulating bovine plasma (15). Phage typing can be used to help differentiate the origins of strains. Four isolates from a herd of cows experiencing severe mastitis which was unresponsive to therapy were found to produce enterotoxin C and TSST-1 (12). Examination of 57 isolates of *S. aureus* from cases of bovine mastitis in Spain found that 4 isolates (7%) were enterotoxigenic (7). Three isolates secreted SEC, and one isolate produced SED. No strains produced SEA, SEB, or SEE. In a study of 127 isolates from cases of subclinical mastitis in Brazil, 6 (4.7%) were found to secrete enterotoxin, and only SEA and SEC were produced (13). These latter studies did not screen for TSST-1. It was decided to conduct a study to evaluate production of enterotoxins and TSST-1 by a large panel of bovine mammary isolates from New York State.

**Bacterial isolates and culture methods.** A total of 262 isolates of *S. aureus* cultured from mammary secretions of cows located throughout New York State were used. Bacteria were maintained on tryptic soy agar (Difco Laboratories, Detroit, Mich.) plates containing 5% bovine blood. A single colony was transferred to 10 ml of casein hydrolysate-yeast extract medium and incubated at 37°C for 18 h (2). Enterotoxins were produced by the membrane-over-agar technique of Hallander (8). A dialysis membrane with a molecular weight cutoff of 6,000 to 8,000 (Spectra Por 1; VWR Scientific, Rochester, N.Y.) was laid on the surface of 10-cm-diameter tryptic soy agar plates. Broth culture (100 μl) was added to the plate and spread with a glass spreader. The plate was incubated at 37°C for 20 to 24 h, and the surface, containing toxins and bacteria, was harvested in 2 ml of 10 mM phosphate buffer, pH 7.2. The plate was washed with an additional 2 ml of buffer. Bacteria were removed by centrifugation, and the resulting supernatant was transferred to a glass vial, frozen at −70°C, and then lyophilized.

**Detection of enterotoxin.** Enterotoxins and TSST-1 were detected by immunodiffusion (18). Sheep antisera specific for enterotoxins A, B, C, D, and E and TSST-1 were obtained from Toxin Technology Inc. (Sarasota, Fla.). Standard preparations of purified toxins were obtained from the same source and adjusted to a concentration of 5 μg/ml in phosphate-buffered saline (PBS) (0.145 M NaCl, 0.01 M NaH2PO4; pH 7.4) containing 0.01% (vol/vol) thimerosal. Preliminary immunodiffusion analysis revealed that antisera could be used at a dilution of 1:32, except for the sera specific for enterotoxins B and E, which were used at a dilution of 1:16.

Immunodiffusion plates measuring 10 by 5 cm (ICN, Costa Mesa, Calif.) were filled with 7.5 ml of a 1.2% solution of noble agar (Difco Laboratories) in PBS. Wells 4.5 mm in diameter were punched with a template and aspirated. The lyophilized material was resuspended in 0.3 ml of PBS. For each toxin being assayed, specific antisera were placed in the center well, while test samples and standards were placed in the surrounding wells. A volume of 25 μl was used throughout. After being incubated for 24 h at 37°C in a humid chamber, the plates were examined for precipitin lines which gave lines of identity with the standards.

The testing technique employed was quite rapid and sensitive to 2.5 to 5.0 μg/ml on the basis of the distinct precipitin lines obtained with standard enterotoxin preparations. Antisera did not display cross-reactions to other enterotoxins. Of the 262 isolates examined, 75 (28.6%) secreted one or more enterotoxins. One isolate secreted SEA. SED was the sole enterotoxin produced by seven isolates. No isolates secreted SEE. SEC, SED, and TSST-1 were secreted either solely or with other enterotoxins by 44, 58, and 50 isolates, respectively. The actual pattern of

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enterotoxin production is outlined in Table 1. The most common pattern is the secretion of both SEC and SED and TSST-1. In this study, only SEC-3 reagents which cross-react with SEC-1 and SEC-2 were used.

Using a 3% N-Z amine, a 3% protein hydrolysate medium, or brain heart infusion medium, Robbins et al. found that enterotoxin was secreted at concentrations ranging from 1 to 185 μg/ml when the cellophane-over-agar method was used (18). SEC-1 was secreted at levels of 86 to 185 μg/ml, while SEA and SEB were found at concentrations of 19 to 32 and 1 to 35 μg/ml, respectively. The value for SED was 4 to 5 μg/ml, while SED was found at 16 to 32 μg/ml. These values were obtained by washing the plate with 2.5 ml of 0.01 M Na₂HPO₄ and using this supernatant directly in immunodiffusion. In the current study, the plate was washed with 4 ml of PBS and after bacteria were removed, the supernatant was lyophilized and resuspended in a volume of 0.3 ml, resulting in a protein concentration about eight times that used by Robbins et al. (18).

The value of 28.6% of bovine isolates secreting one or more enterotoxins is much greater than those of previous studies and may be a consequence of the concentration of proteins prior to immunodiffusion or may reflect differences between North American isolates and those of Europe and South America. An early study of 157 strains isolated from cows with mastitis found that 25 isolates (14.6%) were positive for enterotoxins, of which 11 secreted SEC, 11 elaborated SED, and 1 produced both (16). No strains produced SEA or SEB, and neither SED nor TSST-1 was assayed for.

It is evident that mastitis can be caused by strains which lack the capacity to secrete either enterotoxins or TSST-1. These proteins exert mitogenic effects on T cells, can induce populations of suppressor cells, and act to suppress humoral immunity (3, 6, 17). It is probable that these effects are mediated in part through cytokines released by T lymphocytes or monocytes (19, 20). TSST-1 induces the release of tumor necrosis factor from human monocytes and stimulates human mononuclear cells to release gamma interferon and interleukin-1α (5, 11, 14). Unlike those of ovine and caprine strains, the TSST-1 secreted by bovine strains is similar to that of human strains (9). Bovine mammary strains which secrete these toxins may induce the release of such cytokines and promote inflammation in mammary tissue or enhance the chronicity of this disease. The secretion of enterotoxins is also important in the context of public health and food safety.

It is possible to classify cases of bovine S. aureus mastitis into subclinical cases, severe clinical cases, and clinical cases which are unresponsive to antibiotics. Concurrent screening of isolates from these cows for enterotoxins may establish whether strains which produce enterotoxins are associated with severe clinical cases or cases which respond poorly to therapy. In the current study, there were insufficient farm records to permit such classification.

REFERENCES