A Quantitative Study of Enterotoxin Production by Sheep Milk Staphylococci

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Of 124 staphylococcal strains isolated from sheep milk, 78 produced enterotoxin A, B, C, or D when evaluated by an enzyme-linked immunosorbent assay. Enterotoxins A and D, elaborated by 44 and 43 strains, respectively, showed the highest incidence. Enterotoxin production by coagulase-negative strains (one Staphylococcus cohnii, three S. epidermidis, five S. haemolyticus, and four S. xylosus) was detected. Linear and logarithmic-logarithmic regressions of optical density on enterotoxin concentration yielded the best-fitting equations for enterotoxin quantitation. A significantly higher incidence of enterotoxin producers and significantly higher levels of enterotoxins produced were recorded for coagulase-positive, thermostable nuclease-positive, hemolysis-positive, or mannitol-positive strains. Mannitol utilization was the best test for discriminating between enterotoxigenic and nonenterotoxigenic staphylococci.

Staphylococcal intoxication worldwide stands out as one of the main food-borne diseases, with a frequency in incidents of microbiological origin second only to salmonellosis (35, 36). Milk and milk products are common vehicles for staphylococcal food poisoning (7).

A low incidence (2.6 to 9.4%) of enterotoxin producers in staphylococci isolated from raw bovine milk has generally been recorded (9, 10, 20). Staphylococcal enterotoxin A (SEA) and SEB (9, SEC (20), and SED (10) are the most common serotypes. Mastitic udders of cows constitute a major reservoir for staphylococci, with a frequency of enterotoxigenic strains ranging from 0 to 14.6% (1, 9, 10, 17, 25) and a distinct preponderance of strains producing SEC, SED, or both.

Staphylococcal enterotoxins have been detected in cow’s milk cheeses (33, 39), with a relatively high frequency (2.8 to 5.4%) of contaminated samples. Enterotoxin production in different cheese varieties manufactured from cow’s milk inoculated with Staphylococcus aureus has been demonstrated (31, 32, 37). Levels of S. aureus over 10^5 CFU/g were in most cases necessary for the production of detectable amounts of enterotoxin.

Sheep milk is generally used for cheese manufacture, in many cases without a previous heat treatment. Staphylococci from sheep milk have been characterized, and frequencies of 61.4 to 81.8% enterotoxigenic strains have been recorded (18, 19, 26), most of them SEC producers. Staphylococcus counts from single herd samples investigated at our laboratory (2) frequently exceeded 10^6 CFU/ml, with coagulase-positive strains accounting for 62.3% of the isolates obtained in spring months, and populations of S. aureus over 10^6 CFU/g were found in sheep milk Manchego cheese (P. Gaya, M. Medina, L. Bautista, and M. Nuñez, J. Appl. Bacteriol. 57; xvii, 1984). Staphylococcal intoxications after consumption of sheep milk cheese have recently occurred in France (8) and the United Kingdom (38). SEA and/or SED was found in the cheeses responsible for these outbreaks.

This considerable risk of staphylococcal intoxication due to sheep milk cheese consumption has renewed interest in enterotoxin production by staphylococci present in milk from this species. Previous studies (18, 19, 26) dealt only with strains isolated from sheep or mastitic ovine milk and did not investigate the amount of enterotoxin produced. Both the reliability and the sensitivity of staphylococcal enterotoxin detection have increased since enzyme-linked immunosorbent assay (ELISA) techniques have been available (15, 16, 28, 34). The objectives of the present work were the quantification of the enterotoxins produced by sheep milk staphylococci with ELISA techniques and the search for correlations of the enterotoxigenicity with the physiological characteristics of our strains.

MATERIALS AND METHODS

Bacterial cultures. Staphylococci isolated from sheep milk and characterized in a previous work (2) as S. aureus (87 strains), S. capitis (6 strains), S. cohnii (4 strains), S. epidermidis (6 strains), S. haemolyticus (6 strains), S. hominis (3 strains), S. simulans (1 strain), S. warneri (2 strains), S. xylosus (5 strains), and Staphylococcus spp. (4 coagulase-positive, maltose-negative strains) were studied.

Enterotoxin production. The sac culture dialysis technique (11) was used as recommended by the International Commission on Microbiological Specifications for Foods (22).

Enterotoxin assay. SEA, SEB, SEC, and SED were determined by using an ELISA diagnostic kit obtained from W. Bommel (Bern, Switzerland), with polystyrene spheres as the sorbent (14). ELISAs were performed in polystyrene tubes, and the reactions were quenched by adding 100 μl of 2 N sodium hydroxide to each tube. An Dynatech microplate reader (model MR 600) was used to read optical density at 410 nm after 200 μl was pipetted into the wells of a microdilution plate. From the optical densities of the four control spheres, the value y + 3s was obtained and taken as the threshold for positive-negative reaction (99.7% confidence limit). Standard enterotoxin solutions of SEA, SEB, SEC, and SED (1.25, 2.5, 5, 10, 15, 20, and 25 ng/ml) were prepared from culture supernatants containing 1 μg of enterotoxin per ml obtained from W. Bommel, and their optical densities (ELISAs in triplicate) were used to calculate standard curves. Optical densities from standard solutions containing 0.625 ng/ml did not differ significantly from controls and were not included in standard curves.

Statistical analysis. Determination coefficients and regression equations for enterotoxins were calculated by means of
programs Curve and Regress (Sigstat, Provo, Utah). The chi-square test (30) was used for the comparison of frequencies. Comparison of means by the Student-Newman-Keuls test was performed with program ANOVA 12 (Sigstat, Provo, Utah).

RESULTS AND DISCUSSION

Enterotoxigenicity of sheep milk staphylococci. Of 124 staphylococcal strains isolated from sheep milk, 78 (62.9%) produced enterotoxin (Table 1). This incidence of enterotoxigenic strains is similar to the 61.4% (19) and 71.8% (18) levels previously reported for sheep and milk staphylococci and considerably higher than levels obtained for cow's milk staphylococci (9, 10, 20). In the present work the most frequently detected enterotoxins were SEA and SED, produced by 44 and 43 strains, respectively, whereas SEB and SEC were elaborated only by 8 and 27 strains, respectively. Our results are in disagreement with data published by Hájek (19), who found 2 SEA producers, 46 SEC producers, and 3 SED producers out of 83 staphylococci from sheep nares or udders, and by Gutierrez et al. (18), who detected 4 SEA producers, 48 SEC producers, and 1 SED producer out of 71 staphylococci from mastitic ovine milk.

The origin of our strains, isolates from single herd samples of sheep milk instead of nares or udder isolates (19) or mastitic ovine milk isolates (18), may account for differences in predominant enterotoxin serotypes. The use of the ELISA, which is more sensitive than the slide-gel double-diffusion test (19) or the optimal sensitivity plate method (18), might also explain the higher incidence of SEA and SED producers, in agreement with the results obtained by Lenz et al. (24) for staphylococci of food origin.

Our enterotoxigenic strains had been characterized (2) as S. aureus (65 coagulase-positive strains) or S. cohnii, S. epidermidis, S. haemolyticus, and S. xylosus (1, 3, 5, and 4 coagulase-negative strains, respectively). Enterotoxin production by coagulase-negative staphylococci such as S. capitis (27), S. epidermidis (5, 6, 21, 27), S. haemolyticus (12, 27), and S. haemolyticus subsp. hyicus (21) has been detected. In the present study, the occurrence of enterotoxigenic strains from two coagulase-negative species which had not been previously described as enterotoxin producers, S. cohnii and S. xylosus, is reported.

Quantification of enterotoxin production. Different variable transformations have been used to calculate staphylococcal enterotoxin concentration from optical density values obtained by means of ELISAs (4, 13, 16, 29, 34).

Determination coefficients for our data obtained with different variable transformations are shown in Table 2. The linear-linear equation was superior to the rest of equations for SEA and SED, performing better than linear-logarithmic (4, 13), logit-logarithmic (16), and logarithmic-logarithmic (29). This linear-linear equation was proposed by Thompson et al. (34) for monoclonal and polyclonal antibody ELISAs. Since most of our enterotoxigenic staphylococci were SEA and SED producers, the linear-linear regression equation was also preferred for SED and SEC quantification, in spite of the higher determination coefficients obtained for these enterotoxins with the logarithmic-logarithmic transformation. The equations employed in this work for the quantification of enterotoxins are presented in Table 3. The highest amounts of the various enterotoxins produced by our strains were 288.7 ng of SEA per ml by an S. haemolyticus strain, 10.4 ng of SED per ml by an S. aureus strain, 212.7 ng of SEC per ml by an S. aureus strain, and 779.0 ng of SED per ml by an S. aureus strain.

Physiological characteristics and enterotoxin production. The enterotoxigenicity of staphylococci positive or negative for various physiological characteristics is shown in Table 4. When coagulase activity was considered, chi-square tests detected significantly higher \((P < 0.001)\) frequencies of coagulase-positive strains than of coagulase-negative strains for SEA production and enterotoxigenicity. Thermostable nuclease-positive staphylococci yielded a significantly higher \((P < 0.001)\) rate of SEA producers than did nuclease-negative staphylococci.

### Table 1. Enterotoxigenic Staphylococcus isolates from sheep milk

<table>
<thead>
<tr>
<th>Species</th>
<th>No. of strains</th>
<th>SEA</th>
<th>SEB</th>
<th>SEC</th>
<th>SED</th>
<th>SEAB</th>
<th>SEAD</th>
<th>SEBD</th>
<th>SECD</th>
<th>SEACD</th>
<th>SEBCD</th>
<th>SEABCD</th>
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<tr>
<td>S. aureus</td>
<td>87</td>
<td>26</td>
<td>5</td>
<td>13</td>
<td>1</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>11</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. cohnii</td>
<td>4</td>
<td>1</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. epidermidis</td>
<td>6</td>
<td>1</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>S. haemolyticus</td>
<td>6</td>
<td></td>
<td>1</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. xylosus</td>
<td>5</td>
<td></td>
<td></td>
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<td></td>
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</tr>
</tbody>
</table>

*a S. capitis, S. hominis, S. simulans, S. warneri, and Staphylococcus spp. (6, 3, 1, 2, and 4 strains tested, respectively) did not produce detectable amounts of any enterotoxin.

### Table 2. Comparison of determination coefficients \((r^2)\) obtained after different variable transformations for the regression of optical density on staphylococcal enterotoxin concentration

<table>
<thead>
<tr>
<th>x</th>
<th>y</th>
<th>SEA</th>
<th>SEB</th>
<th>SEC</th>
<th>SED</th>
<th>SEAB</th>
<th>SEAD</th>
<th>SEBD</th>
<th>SECD</th>
<th>SEACD</th>
<th>SEBCD</th>
<th>SEABCD</th>
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<tbody>
<tr>
<td>T</td>
<td>OD</td>
<td>0.968</td>
<td>0.929</td>
<td>0.955</td>
<td>0.906</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>1/T</td>
<td>1/OD</td>
<td>0.607</td>
<td>0.962</td>
<td>0.922</td>
<td>0.856</td>
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<tr>
<td>T</td>
<td>Log OD</td>
<td>0.790</td>
<td>0.837</td>
<td>0.845</td>
<td>0.806</td>
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<tr>
<td>T</td>
<td>Logit OD</td>
<td>0.823</td>
<td>0.874</td>
<td>0.880</td>
<td>0.830</td>
<td></td>
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<td></td>
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<tr>
<td>Log T</td>
<td>OD</td>
<td>0.897</td>
<td>0.867</td>
<td>0.846</td>
<td>0.824</td>
<td></td>
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</tr>
<tr>
<td>Log T</td>
<td>Log OD</td>
<td>0.933</td>
<td>0.962</td>
<td>0.971</td>
<td>0.904</td>
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</tr>
<tr>
<td>Log T</td>
<td>Logit OD</td>
<td>0.943</td>
<td>0.955</td>
<td>0.968</td>
<td>0.904</td>
<td></td>
<td></td>
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</tbody>
</table>

*a Enterotoxin concentration (nanograms per milliliter).

### Table 3. Linear regression equations of optical density on enterotoxin concentration

<table>
<thead>
<tr>
<th>Enterotoxin</th>
<th>Equation</th>
<th>(OD = )</th>
</tr>
</thead>
<tbody>
<tr>
<td>SEA</td>
<td>(0.01315 + 0.01142T)</td>
<td></td>
</tr>
<tr>
<td>SEB</td>
<td>(0.04142 + 0.01762T)</td>
<td></td>
</tr>
<tr>
<td>SEC</td>
<td>(0.00982 + 0.01576T)</td>
<td></td>
</tr>
<tr>
<td>SED</td>
<td>(0.03452 + 0.00956T)</td>
<td></td>
</tr>
</tbody>
</table>

*a Optical density measured at 410 nm.

*b Enterotoxin concentration (nanograms per milliliter).
frequency of SEA producers (P < 0.05) and of enterotoxigenic strains (P < 0.001) than nonhemolytic staphylococci. Acetoin production was not a reliable test to differentiate enterotoxigenic from nonenterotoxigenic staphylococci. Significantly higher (P < 0.001) rates of SEA producers and of enterotoxigenic strains were detected in manniitol-positive staphylococci when compared with manniitol-negative staphylococci.

Indirect tests such as the tube coagulase test and the thermostable nuclease test are useful to detect potentially enterotoxigenic staphylococci (21), although some authors (3) concluded that physiological characteristics do not differentiate enterotoxigenic from nonenterotoxigenic S. aureus. The data in Table 4 suggest that hemolysis production and manniitol utilization are at least as reliable as the thermostable nuclease test when used as indicators of enterotoxigenicity by sheep milk staphylococci.

Sixty-five coagulase-positive strains were enterotoxigenic. When the 33 coagulase-negative strains were examined, no additional enterotoxigenic staphylococci were found among thermostable nuclease-positive strains, but 10 additional enterotoxigenic staphylococci were detected among hemolytic strains, 10 were detected among acetoin-positive strains, and 3 were detected among manniitol-positive strains. Seventy-one hemolytic strains were enterotoxigenic. In the 24 nonhemolytic strains, 4 additional enterotoxigenic staphylococci were found among coagulase-positive strains, 4 were detected among nuclease-positive strains, 7 were detected among acetoin-positive strains, and 4 were detected among manniitol-positive strains. Sixty-six manniitol-negative strains were enterotoxigenic. When the 34 manniitol-negative strains were taken into account, 2 additional enterotoxigenic staphylococci were detected among coagulase-positive strains, 1 was detected among nuclease-positive strains, 9 were detected among hemolytic strains, and 10 were detected among acetoin-positive strains.

From 78 enterotoxigenic staphylococci studied in the present work, the highest recoveries (number recovered/total) were obtained with the following pairs of tests: hemolysis-acetoin production (78/111), manniitol utilization-acetoin production (76/112), and hemolysis-manniitol utilization (75/112), whereas the widely accepted tandem coagulase production-thermostable nuclease production only recovered 65 enterotoxigenic strains from a total of 98 strains. The potential enterotoxigenicity of non-S. aureus staphylococci or of coagulase-negative, thermostable nuclease-negative staphylococci has commonly been underestimated, even though a high incidence (8 of 15) of enterotoxigenic staphylococci among strains with these physiological characteristics was reported in an early work by Lachica et al. (23).

Mean levels of SEA (Table 5) were significantly higher for coagulase-positive, nuclease-positive, hemolysis-positive, or manniitol-positive strains than for the strains with a negative reaction in these tests. Mannitol utilization exhibited the highest degree of significance (P < 0.001) in the differentiation of enterotoxigenic and nonenterotoxigenic sheep milk staphylococci. Production of SEB and SEC was generally low and independent of staphylococci physiological characteristics. Mean levels of SED tended to be higher in strains with a positive reaction in at least one of the physiological characteristics considered, but differences between means were not statistically significant. The total amount of enterotoxins was significantly higher for coagulase-positive (P < 0.05), nuclease-positive (P < 0.05), and manniitol-positive (P < 0.05) strains.

These results confirm the conclusion drawn from the data in Table 4 that not only the tube coagulase test and the thermostable nuclease test but also hemolysis production and manniitol utilization are useful to differentiate enterotoxigenic from nonenterotoxigenic sheep milk staphylococci.

ACKNOWLEDGMENT
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LITERATURE CITED
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