Characterization and Enterotoxigenicity of Staphylococci Isolated from Mastitic Ovine Milk in Spain

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(Received for publication June 16, 1982)

ABSTRACT

On the basis of glucose fermentation and lysostaphin sensitivity, 71 gram-positive, catalase-positive cocci, isolated from mastitic ovine milk in Spain, were classified as members of the genus Staphylococcus. Identification at the species level was accomplished by complete characterization of the staphylococcal strains. Fifty-nine of the isolates were classified as S. aureus, 5 as S. simulans, 5 as S. epidermidis, a as S. haemolyticus and 5 could not be classified as any accepted or newly proposed species. The number of strains lysed by phages of S. aureus of human and bovine origin was 8 and 40, respectively. The phage pattern most frequently found was 78 (34 strains). Fifty of the S. aureus strains belonged to biotype C. Forty-nine of the S. aureus strains and 2 of the unclassified ones produced enterotoxin. 46 produced enterotoxin C, 2 produced enterotoxin A, 1 produced enterotoxin D and 2 produced both enterotoxins A and C. Forty-one of the 46 enterotoxin C producers belonged to biotype C, and 31 of these were lysed by phage 78.

The staphylococci are among the most important etiological agents of ovine mastitis, a disease of great sanitary and economic significance in Spain and other Mediterranean and Balkan countries where some breeds of sheep are kept mainly for milk production. (10,31,33,38,41). The number of investigations dealing with characterization of staphylococci isolated from mastitic ovine milk is limited (14,31,41). Information available indicates that ovine and bovine staphylococci may form a specific group of organisms, which sets them apart from those associated with other animal species (17). Additional information indicates ovine and bovine strains may be identifiable one from the other by phage typing and determination of their enterotoxigenicity (14).

Staphylococcal food poisoning is probably the main cause of foodborne disease in Spain and other countries. Dairy foods, especially cheese, are responsible for some of the outbreaks. Although the frequency of the involvement of sheep cheese in Spain is not known, it must be pointed out that in this country most of the ewes' milk is converted into cheese and that, in spite of legal requirements, milk sometimes does not receive an appropriate heat treatment.

The purpose of this study was to identify and characterize 71 strains of staphylococci from mastitic ovine milk and to determine their ability to produce enterotoxins. This paper also deals with the usefulness of biotyping, phage typing and enterotoxin production in identifying the ovine Staphylococcus aureus strains as species specific.

MATERIALS AND METHODS

Taxonomic schemes

Staphylococci were identified using the criterion of the Subcommittee on the Taxonomy of Staphylococci and Micrococci (39) and by lysostaphin susceptibility testing (1). The schemes employed for classification of coagulase-positive staphylococci were those of Baird-Parker et al. (1) taking into account key characters of Staphylococcus intermedius (13) and Staphylococcus hyicus (8). The coagulase-negative strains were classified according to the criteria of Kloos and Schleifer (20,21), Schleifer and Kloos (37) and Kloos et al. (22,23). Subdivision of coagulase-positive strains into biotypes was done according to the scheme by Hajek and Marsalek (16,18).

Cultures

The number of strains studied was 71. Fifty-four of them were isolated in the laboratory of the Department of Food Hygiene and Food Microbiology, Veterinary Faculty, Leon (Spain), and the remaining 17 strains were provided by Dr. F. Rejas, Oviedo Laboratories, Leon (Spain). In both instances, the strains were isolated from mastitic ovine milk, and each culture was obtained from one sample of milk taken from an individual ewe suffering from acute, gangrenous or chronic mastitis. Each strain was incubated in brain heart infusion broth (BHI) and streaked on BHI agar. One colony was picked, and the procedure was repeated three times.

Anaerobic fermentation of glucose and mannitol

Aerobic use of glucose and mannitol as well as fermentation of these sugars were tested by the method recommended by the Subcommittee of Taxonomy of Staphylococci and Micrococci (39). Final pH values were determined after incubation of the cultures in the same medium for 5 d in a GasPak anaerobic jar (BBL, Microbiology Systems, Cockeysville, MD).

Coagulase

Coagulase activity was studied by the tube method using desiccated rabbit plasma [coagulase plasma ethylendiaminetetraacetic acid (EDTA), Difco] and fresh pig, human and bovine plasmas containing 0.1% EDTA. The test was performed by adding 0.1 ml of an overnight culture grown in BHI to 0.3 ml of plasma. The tubes were incubated in a waterbath at 37°C and examined after 30 min, 2, 4 and 24 h. Reactions were tabulated according to the scheme of Turner and Schwartz (42). Only +, 3+ and 2+ reactions were considered as positive evidence of coagulase production.

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JOURNAL OF FOOD PROTECTION, VOL. 45, DECEMBER 1982
Thermonuclease  
Production of heat-stable and heat-labile nucleases was investigated by the plate method (5) using toluidine blue-deoxyribonuclease acid agar, as described by Lachica et al. (25).

Acetoin  
Acetoin production from carbohydrates was considered as sensitive or resistant according to the criterion of Baird-Parker (9).

Phosphatase production  
Phosphatase production was detected by the method of Barber and Kuper (3).

Hemolysins  
Production of α-, β- and δ-hemolysins was investigated by the plate method of Nakagawa (30) using tryptose blood agar base (Difco) supplemented with 1% (final concentration) filter-sterilized carbohydrate solution, as described by Kloos et al. (24).

Novobiocin susceptibility  
The minimal inhibitory concentrations of novobiocin (The Upjohn Co., Kalamazoo, MI) were determined by the agar dilution method of Barry (4). Cultures were considered as sensitive or resistant according to the criterion of Baird-Parker (1). Medium without novobiocin and activity with S. aureus strain ATCC 25923 and Escherichia coli strain ATCC 25922 were used as controls.

Lysostaphin susceptibility  
Sensitivity of the strains to the lytic action of 1 U (final concentration) of lysostaphin/ml (Schwarz/Mann, Orangeburg, NY) was determined by a turbidimetric method described previously (12). Optical density readings were made at 0.5, 20, 30 and 60 min. Susceptibility to lysostaphin was measured on the basis of the percent decrease in optical density at 20 min. Strains were considered as highly sensitive (HS) when the reduction in turbidity was 80% or higher and slightly sensitive (SS) when this was 20% or lower. Cultures showingpercentages between 80 and 20% were considered as moderately sensitive (MS). For control purposes, the autolytic activity of each strain was determined, and cell suspensions of S. aureus strain FDA 209P, Micrococcus luteus strain NCTC 2665 and Sarcina lutea strain 9341 were tested also.

Lysosome production  
Lysosome production was detected by the plate method of Roskey and Handly (36) using lysozyme substrate (Difco). After incubation for 48 h at 37°C, formation of a lytic zone larger than 2 mm from the edge of the growth area was interpreted as a positive test.

Gelatinase production  
Gelatinase activity was determined by streaking overnight cultures in BHI broth on Chapman Stone medium (Difco) and examining for clear zones after 48 h of incubation at 37°C. A clear zone larger than 2 mm from the edge of the growth area was considered positive for gelatinase activity.

Caseinase activity  
This test was performed on buffered caseinate agar medium (Standard Methods caseinate agar, SMCA), as recommended by Martley et al. (27). After incubation at 30 and 37°C for 48 h, the strains were classified into one of the five groups (A-E) described by Martley et al. (28) on the basis of the types of precipitation zones.

Egg yolk reaction  
Egg yolk factor production was investigated on Baird-Parker medium (Oxoid, Ltd., London, England) containing 5% egg yolk tellurite emulsion (Oxoid).

Lipase activity  
Lipase activity was examined on blood agar base (Oxoid) containing 0.01% CaCl₂·2H₂O and 1% Tween 20, 40, 60 and 80. Opaque zones surrounding the growth after 18 h of incubation at 37°C and after 24 h at room temperature were recorded as positive evidence of Tween splitting.

Fibrinolysin production  
The agar plate method of Christie and Wilson (7) was used.

Pigment production  
The color of colonies was investigated on BHI agar (Difco) incubated for 5 d at 35°C.

Crystal violet type  
This test was done as described by Meyer (29).

Phage typing  
Phage typing was carried out by the method of Blair and Williams (6) as modified by Parker (34) with the base set of phages for typing human (40) and bovine (44). S. aureus strains. When a culture was negative at the routine test dilution (RTD), it was retested at 100 × RTD.

Enterotoxin assay  
All strains were assayed for enterotoxins A (SEA), B (SEB), C (SEC), D (SED) and E (SEE). The cellophage-over-agar method was used for enterotoxin production (35), the enterotoxins were detected and identified by the optimal sensitivity plate method (35).

RESULTS  
Classification as staphylococci  
The 71 strains were classified in the genus Staphylococcus. They used glucose anaerobically and were sensitive to lysostaphin. The pH values after 5 d in the glucose medium were between 4.9 and 5.0. Only two strains gave a higher pH value (5.8 and 6.4). Results of the lysostaphin sensitivity test are given in Table 1. The average percent reductions in turbidity in the coagulase-positive and coagulase-negative groups were 88.3 and 64.6%, respectively.

Coagulase production  
In rabbit plasma, 56 strains gave a 4+ coagulase reaction, 3 gave a 3+ reaction, 1 gave a 2+ reaction and 11 gave a 1+ reaction. Human, pig and bovine plasma were coagulated by 60, 61 and 63 strains, respectively.

Identification of species  
The biochemical patterns obtained with the different strains and the species into which they were classified are given in Table 1. Five strains could not be classified.

Other characteristics  
The hemolysin, lysozyme, gelatinase production, egg yolk and lipolytic activities and the types of precipitation zones on SMCA are given in Table 1.

Biotyping and enterotoxin production  
Fifty-one of the 59 S. aureus strains belonged to biotype C. They coagulated both human and bovine plasmas, produced pigment, produced alpha- and beta-hemolysins; all, except 4, gave biotype C growth (negative) on crystal violet agar. Forty-one of these strains produced SEC, the only enterotoxin produced by any of the biotype C strains. Of the
<table>
<thead>
<tr>
<th>Property</th>
<th>S. aureus (n=59)</th>
<th>S. simulans (n=1)</th>
<th>S. haemolyticus (n=1)</th>
<th>S. epidermidis (n=5)</th>
<th>No. 29</th>
<th>No. 53</th>
<th>No. 70</th>
<th>No. 11</th>
<th>No. 6</th>
</tr>
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<tbody>
<tr>
<td>Coagulase</td>
<td>59+</td>
<td>-</td>
<td>-</td>
<td>5-</td>
<td>+</td>
<td>+a</td>
<td>-</td>
<td>+b</td>
<td>+b</td>
</tr>
<tr>
<td>Thermonuclease</td>
<td>58+, 1w&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-</td>
<td>-</td>
<td>5-</td>
<td>+</td>
<td>w</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Mannitol (anaerobic)</td>
<td>42+, 13w, 4-</td>
<td>w</td>
<td>-</td>
<td>5-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>Lysostaphin sensitivity&lt;sup&gt;d&lt;/sup&gt;</td>
<td>56HS, 3MS</td>
<td>HS</td>
<td>MS</td>
<td>1HS, 4MS</td>
<td>HS</td>
<td>HS</td>
<td>MS</td>
<td>MS</td>
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<td>Hemolysin</td>
<td>10αβ, 48αβδ, 1βδ</td>
<td>αβ</td>
<td>δ</td>
<td>38, 2</td>
<td>αβ</td>
<td>α</td>
<td>αδ</td>
<td>δ</td>
<td>δ</td>
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<td>Phosphatase</td>
<td>59+</td>
<td>-</td>
<td>-</td>
<td>5+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Novobiocin sensitivity</td>
<td>59+</td>
<td>+</td>
<td>-</td>
<td>5+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>Lysozyme</td>
<td>35+, 24w</td>
<td>+</td>
<td>-</td>
<td>2w, 3</td>
<td>w</td>
<td>w</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Gelatinase</td>
<td>46+, 13w</td>
<td>w</td>
<td>-</td>
<td>5+</td>
<td>-</td>
<td>+</td>
<td>-</td>
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<tr>
<td>Egg yolk</td>
<td>31+, 28-</td>
<td>-</td>
<td>3+, 2</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
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<tr>
<td>Acetoin</td>
<td>59+</td>
<td>-</td>
<td>-</td>
<td>5+</td>
<td>+</td>
<td>-</td>
<td>+</td>
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<td>+</td>
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<td>Trehalose (acrobic)</td>
<td>56+, 3-</td>
<td>+</td>
<td>-</td>
<td>5-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Sucrose (acrobic)</td>
<td>58+, 1-</td>
<td>+</td>
<td>-</td>
<td>5+</td>
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<td>+</td>
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<td>Mannitol (acrobic)</td>
<td>58+, 1-</td>
<td>+</td>
<td>-</td>
<td>5-</td>
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<td>-</td>
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<td>5-</td>
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<td>Caseinase activity (30°C)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>49C, 8D, 2E</td>
<td>D</td>
<td>E</td>
<td>5A</td>
<td>E</td>
<td>A</td>
<td>E</td>
<td>A</td>
<td>C</td>
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<tr>
<td>Caseinase activity (37°C)</td>
<td>49A, 9C, 1E</td>
<td>C</td>
<td>C</td>
<td>5A</td>
<td>D</td>
<td>A</td>
<td>E</td>
<td>A</td>
<td>C</td>
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<td>Lipase activity-Tween 20</td>
<td>57+, 2-</td>
<td>+</td>
<td>-</td>
<td>5+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Tween 40</td>
<td>51+, 8-</td>
<td>+</td>
<td>-</td>
<td>4+, 1</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Tween 60</td>
<td>40+, 19-</td>
<td>+</td>
<td>1+, 4</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Tween 80</td>
<td>6+, 53-</td>
<td>+</td>
<td>3+, 2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>-</td>
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<tr>
<td>Enterotoxin&lt;sup&gt;f&lt;/sup&gt;</td>
<td>2 SEA, 45 SEC, 1 SED, 1 SEA &amp; SEC, 10 neg.</td>
<td>-</td>
<td>-</td>
<td>5-</td>
<td>-</td>
<td>SEC</td>
<td>SEA, SEC</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a</sup>4+, pig plasma.
<sup>b</sup>4+, bovine plasma.
<sup>c</sup>W, weak reaction.
<sup>d</sup>HS, High sensitivity (81.3 to 99.3% lysis); MS, moderate sensitivity (25 to 75% lysis).
<sup>e</sup>A, B, C, D, E represent types of precipitation.
<sup>f</sup>Enterotoxins A (SEA), C (SEC), D (SED).
remaining *S. aureus* strains, 5 had properties of both biotypes A and C (2 produced SEA, 2 produced SEC, 1 produced both SEA and SEC), 1 was similar to biotype C (produced SED) and 2 were similar to biotype F (lacked one property; both produced SEC). One of the unclassified strains (strain 29) belonged to biotype C (nonenterotoxigenic).

**Phage typing**

Only 8 strains were lysed by phages in the basic set of phages from *S. aureus* of human origin (at 100 × RTD) while 40 were lysed by phages (phage 102, 1 strain; phage 107, 1 strain; phages 102 and 107, 4 strains; and phage 78, 34 strains) of the bovine set (25 at 100 × RTD).

**DISCUSSION**

Only 5 of the 71 staphylococcal strains isolated from mastitic ovine milk were unclassifiable according to species. Strain 29 ordinarily would be classified as *S. aureus*, but was not because of its failure to oxidize trehalose, sucrose and mannitol (Table 1). Strain 6 was not classified as *S. intermedius* because it produced acetoin and did not coagulate rabbit plasma. Strain 70 correlated best with *S. intermedius* but it did not coagulate rabbit plasma (Table 1). Strain 11 was enterotoxigenic and coagulated bovine plasma but it did not produce alpha- or beta-hemolysin or acid from trehalose aerobically. Also, there were significant differences between this strain and those classified as *S. intermedius* and *S. hyicus*. Strain 53 had characteristics related to *S. hyicus* subsp. *chromogens*, *Staphylococcus epidermidis* and *Staphylococcus capitis*, but because it coagulated pig plasma it was not classified.

The high percentage of the strains that were α-hemolytic is similar to the results obtained with staphylococci isolated from ovine mastitic milk by Lernau et al. (26) and Tamarin (41), but differs from the results reported by Oeding at al. (31) and Hajek (14). None of Hajek's strains produced α-hemolysin.

Forty-one (80%) of the 51 *S. aureus* strains that were classified as biotype C produced SEC and 34 (67%) of the 51 strains were sensitive to phage 78. Hajek (14) found that 46 (60%) of the 77 *S. aureus* biotype C strains of ovine origin he studied produced SEC and 61 (79%) of the 77 were sensitive to phage 78. Hajek's and our results indicate that ovine *S. aureus* strains form a special group of staphylococci characterized by their belonging to biotype C, their sensitivity to phage 78 and their ability to produce SEC. Production of only one enterotoxin type by a specific group of staphylococci has not been noted previously.

Results of several investigations (11,15,19,32,43) on *S. aureus* strains isolated from mastitic cows show these strains to differ in some respects from the sheep strains. The difference in enterotoxin production of the bovine and ovine strains is quite marked. Of 439 *S. aureus* strains that were isolated from mastitic cows in five countries (United States, Czechoslovakia, Germany, New Zealand, Spain) only 34 (8%) produced enterotoxin as compared to 100 (70%) of 142 sheep strains. Twenty of the bovine strains produced SEC, 13 produced SED and one produced both enterotoxins. Only the bovine strains from Czechoslovakia were biotyped, with 74 of 79 belonging to biotype C (15). It is expected that most bovine strains isolated in other places would belong to biotype C also. Enterotoxin D has been associated with bovine strains, but it is not known whether the strains that produce it are biotype C strains. Only four of the ovine strains produced SED and none were classified as biotype C. The general properties of the ovine and bovine strains are quite similar with the exception of their sensitivity to phages and their enterotoxin production. The ovine strains are sensitive to phage 78 while the bovine strains are sensitive to phage 102 (11). There apparently is very little crossover of strains between the animals as most of the strains isolated from sheep and cows in the same general area of Spain show these differences (11).

Food poisoning outbreaks in the United States and other countries have been traced to milk and dairy products, and often the enterotoxins involved have been either SEC or SED, the ones that have been associated with milk. Sheep cheese is a common item of food in Europe and much of it is made by individuals on a small scale. Undoubtedly, food poisoning outbreaks do occur from consumption of toxic sheep cheese, but because of the nature of the disease and the lack of surveillance systems the frequency of the disease is not known. The fact that most *S. aureus* strains associated with sheep mastitis are enterotoxigenic should be of concern as the opportunity for contamination with enterotoxigenic staphylococci is enhanced.

**ACKNOWLEDGMENTS**

This research was supported in part by the College of Agricultural and Life Sciences, University of Wisconsin-Madison, Madison, Wisconsin.

**REFERENCES**


