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Characterization of Staphylococci Isolated from Mastitic Cows in Spain

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A total of 57 gram-positive, catalase-positive cocci, considered etiological agents of clinical and subclinical bovine mastitis, were tested for glucose and mannitol fermentation, coagulase and thermonuclease production, sensitivity to lysostaphin, gelatin hydrolysis, lysozyme, phosphatase and egg yolk factor production, hemolytic properties, antibiotic sensitivity, susceptibility to human and bovine phages, and enterotoxin production. All 57 strains were identified as staphylococci. A good correlation was found between 3+ and 4+ coagulase reactions, thermonuclease production, and high sensitivity to lysostaphin. Neither mannitol fermentation nor production of other enzymes appeared to be a specific property of bovine *Staphylococcus aureus* strains. β- and δ-hemolysins were more frequently found than α-hemolysin. Nearly 40% of the strains were penicillin resistant. Strains were lysed by phage 42E from the human phage set more frequently than by phage 42D, whereas with the bovine set, strains were more sensitive to specific bovine phages. Three strains produced enterotoxin C, and one strain produced enterotoxin D.

Staphylococcal strains from animals have not been studied as extensively as strains associated with human infections. Staphylococci of bovine origin have been investigated more frequently than those from other animals because staphylococci are some of the most important etiological agents of mastitis in cattle (24). There are remarkable differences in human and animal staphylococci, but both release a large number of enzymes. Some of these, such as coagulase, thermonuclease, phosphatase, proteinases, or lysozyme, are considered indices of pathogenicity. Other characteristics commonly associated with staphylococci are mannitol fermentation, high sensitivity to lysostaphin, and egg yolk factor and hemolysin production.

Although coagulase production is commonly used for predicting virulence or toxicity, there is no single standard criterion for determining which clotting intensity can be considered positive. Some workers consider staphylococcal strains to be coagulase positive only when the plasma is completely coagulated (26), whereas others consider any degree of clotting as a positive reaction. The production of thermonuclease and mannitol fermentation are accepted as reliable properties of *Staphylococcus aureus* (5, 12); however, a considerable number of bovine strains are mannitol negative (8, 18, 34). Sensitivity to lysostaphin is considered a characteristic of staphylococci but not of micrococci (10; R. V. F. Lachicha and C. Genigeorgis, Bacteriol. Proc., p. 17, 1970). The production of phosphatase, lysozyme, and egg yolk factor, gelatin hydrolysis, and hemolysin production are often used to estimate the virulence and toxicity of *S. aureus* strains.

The public health significance of staphylococci isolated from milk and dairy products is important. It has been suggested that cattle can be a source of antibiotic-resistant strains for humans (30), with a possible interchange of staphylococci strains between humans and animals (33). Phage typing and antibiotic sensitivity have been used in attempts to determine the origin of strains and to investigate the importance of antibiotic-resistant strains. Dairy foods are frequently contaminated with staphylococci, and mastitic milk may be an important source of these strains. Data reported by several authors show that some of these strains are enterotoxigenic (17, 18, 32).

One purpose of this work was to investigate the importance of staphylococci isolated from mastitic milk as a possible source of food poisoning in Spain. We attempted to characterize these strains with special attention to some conflicting points and to establish the relationship between enterotoxigenicity and some properties considered to be indices of toxicity.

MATERIALS AND METHODS

Cultures. Milk samples (168) from 143 cows with clinical or subclinical mastitis were tested for the

presence of staphylococci. The animals were distributed among 85 herds in different areas of Leon, Spain. For the isolation of strains, 0.1 ml of milk was streaked on a tryptose blood agar base (Difco Laboratories, Detroit, Mich.) with 5% calf blood and on Baird-Parker medium (Oxoid Ltd., London, England). After incubation for 24 to 48 h, any colonies formed on the plates were examined by Gram staining.

Catalase. Catalase activity was tested for by growing the staphylococci on tryptone-yeast extract agar with 1% glucose for 24 h at 37°C, followed by flooding the plates with 3% H_2O_2.

Anaerobic fermentation of glucose and mannitol. Aerobic and anaerobic fermentation of glucose and mannitol were carried out according to the recommendations of the Subcommittee on Taxonomy of Staphylococci and Micrococci (27). For determination of the pH, the cultures were incubated in the same medium for 5 days in a GasPak anaerobic jar (BBL Microbiology Systems, Cockeysville, Md.).

Coagulase. The production of coagulase was studied by the tube method. An overnight culture (0.1 ml) on brain heart infusion broth (Difco) was added to 0.3 ml of rabbit plasma (coagulase plasma ethylenediaminetetraacetic acid [EDTA]; Difco). The tubes were incubated at 37°C. Readings were made after 30 min and 2, 4, and 24 h. The results were tabulated by the scheme of Turner and Schwartz (31) as follows: 1+, small unorganized clots; 2+, small organized clot; 3+, large organized clot; 4+, complete clot. The authors considered the absence of fibrin formation to be negative.

Thermonuclease. The production of thermonuclease was detected by a plate technique with toluidine blue-deoxyribonucleic acid-agar, as described by Lachica et al. (11).

Lysostaphin susceptibility. The sensitivity of strains to lysostaphin was determined by a method similar to that of Zygmut (personal communication). Lysostaphin (1 U/ml) (Schwarz/Mann, Orangeburg, N.Y.) was added to standardized suspensions of strains and to a control strain (FDA 209P). The reactions were monitored at 620 nm (1-cm light path) with a Perkin-Elmer 200 spectrophotometer at 37°C (thermostatically controlled). Optical density readings were taken at 0, 5, 10, and 20 min. Lysostaphin susceptibility was measured as the percent decrease in optical density after 20 min of incubation. Strains were considered highly sensitive when the percent reduction in turbidity was 85% or more and slightly sensitive when the percent reduction in turbidity was less than 20%.

Phosphatase production. Phosphatase production was determined by the procedure of Barber and Kuper (2).

Gelatinase production. The liquefaction of gelatin was tested for by streaking overnight cultures in brain heart infusion broth on Chapman Stone medium (Difco). After incubation for 48 h at 30°C, the clear zones surrounding the growth were measured. A lytic zone larger than 2 mm from the edge of the growth area was recorded as positive.

Lysozyme production. Lysozyme production was determined by the procedure of Roskey and Hamdy (22). The formation of a clear zone larger than 2 mm from the edge of the growth area was considered positive.

Egg yolk reaction. The egg yolk reaction was studied on Baird-Parker medium containing 5% egg yolk-tellurite emulsion (Oxoid).

Nitrate reduction. The reduction of nitrate was tested with tryptic nitrate medium (Difco).

Hemolysins. The production of α-, β-, and δ-hemolysins was studied by the method of Nakagawa (15). Washed rabbit, sheep, horse, and human erythrocytes were employed, and filter paper strips soaked in anti-α-hemolysin (Burroughs Wellcome Co., London, England) were used (6).

Antibiotic susceptibility testing. The agar dilution method of Barry (3) was used to determine the antibiotic susceptibility of the staphylococcal strains. All strains were tested against penicillin G, cephalothin (sulfate), kanamycin (sulfate), tetracycline (chlorhydrate), streptomycin (sulfate) (all from Antiobioticos S.A., Leon, Spain), methicillin (sodium) (Beecham Research Laboratories, Madrid, Spain), chloramphenicol (Laboratories Park Davis S.A., Madrid, Spain), novobiocin (The Upjohn Co., Kalamazoo, Mich.), and erythromycin (Lilly Indiana de Espana, Alcobendas, Spain). Cultures were considered sensitive or resistant according to the standards of Barry (3), except that the method of Baird-Parker (5) was used for novobiocin. Antibiograms were also performed by the disk diffusion method recommended by the National Committee for Clinical Laboratory Standards Subcommittee on Antimicrobial Susceptibility Testing (16). Disks (Difco) containing the same antibiotics listed above and sulfadiazine, sulfathiazole, and sulfamerazine were used. *S. aureus* strain ATCC 25923 and *Escherichia coli* strain ATCC 25922 were included in each series of tests as controls.

Phage typing. Bacteriophage typing was carried out by the method of Blair and Williams (4) as modified by Parker (19) with the phages of the international basic set for typing *S. aureus* strains (28) and the 16 phages accepted as the international basic set for typing *S. aureus* strains from bovine sources (35). When a culture was negative at the routine test dilution, it was retested at 100 times the routine test dilution.

Enterotoxin detection. All strains were examined for enterotoxins A, B, C, D, and E. The cellophane-over-agar method for enterotoxin production and the optimal sensitivity plate method for enterotoxin detection were used (21).

RESULTS

Staphylococci. A total of 57 gram-positive, catalase-positive strains of cocci, either in pure culture or in numbers sufficient to be considered the cause of mastitis, were isolated.

Anaerobic fermentation of glucose. A total of 56 strains fermented glucose anaerobically, but 6 strains were weak producers of acid (pH 5.8 to 6.0) (Table 1). One strain (classified as *Staphylococcus saprophyticus*) failed to produce detectable acid (pH 6.6), but it was able to

TABLE 1. *Properties of staphylococci isolated from mastitic milk*

Property	*S. aureus* (*n* = 46)	*S. intermedius* (*n* = 1)	*S. hyicus* (*n* = 1)	*S. epidermidis* (*n* = 6)	*S. saprophyticus* (*n* = 1)	Unclassified (*n* = 2)	
						No. 39	No. 138
Coagulase 4+	40						
Coagulase 3+	5	+					+
Coagulase 2+	1		+	4		+	
Coagulase 1+				2	+		
Thermonuclease	46+	+	+	6−	−	−	−
Mannitol (anaerobic)[a]	40+, 3±, 3−	−	−	6−	−	−	−
Glucose (anaerobic)	46+	+	+	6+	−	+	+
Lysostaphin sensitivity[b]	46HS	HS	HS	1MS, 5SS	SS	MS	HS
Hemolysis	46+	−	−	5+, 1−	+	+	−
Phage typable[c]	45+, 1−	−	−	−	−	+	−
Novobiocin resistance	46−	−	−	−	+	−	−
Phosphatase	46+	+	+	1+, 5−	+	+	+
Gelatinase	42+, 4−	+	+	1+, 5−	±	+	+
Lysozyme	46+	+	+	3±, 3−	+	±	+
Egg yolk	21+, 25−	−	−	4+, 2−	−	+	−
Nitrate reduction	46+	+	+	6+	−	+	+

[a] +, pH below 6.2; ±, pH 6.2 to 6.4; −, pH 6.5 or above.

[b] HS, High sensitivity (85.3 to 97.6% lysis); MS, moderate sensitivity (27.5 to 40.5% lysis); SS, slight sensitivity (10.0 to 17.5 % lysis).

[c] Typable by human or bovine set of phages.

grow under anaerobic conditions. From these results and from those from the lysostaphin sensitivity study (Table 1), all strains were classified as staphylococci.

Other characteristics. The results of the coagulase and thermonuclease tests, mannitol fermentation, phosphatase, gelatinase, and lysozyme production, egg yolk factor production, and nitrate reduction are given in Table 1.

Hemolysins. Only 4 of the 57 cultures failed to produce any of the hemolysins (see Table 1 for classification). δ-Hemolysin was produced by 47 strains, β-hemolysin was produced by 45 strains, and α-hemolysin was produced by 30 strains. The hemolysin patterns found most frequently were as follows: α-β-δ (25 strains), β-δ (14 strains), and δ (6 strains).

Antibiotic sensitivity. The results of the antibiotic sensitivity studies are given in Table 2.

Phage typing. All but one of the *S. aureus* strains were phage typable (Table 3). Only one other strain (unclassified strain 39) was typable. A total of 44 strains (29 at the routine test dilution and 15 at 100 times the routine test dilution) were lysed by phages in the human basic set (Table 3). Of the 18 different phage patterns, the ones most frequently found were 42E/42D (13 strains) and 42E (9 strains). No strains were lysed by phages 71, 47, 83A, 84, 85, or 187. A total of 46 strains (44 at the routine test dilution and 2 at 100 times the routine test dilution) were lysed by phages in the bovine set. Of the 24 different phage patterns, the ones most frequently found were 102 (8 strains) and 42D/102 (6 strains). A total of 37 strains were lysed by phage 102, 22 strains were lysed by phage 42E, 19 strains were lysed by phage 107, and 17 strains were lysed by phage 117. No strains were lysed by phage 84 or 116. A total of 45 strains were lysed by the seven specific bovine phages, and 40 strains were lysed by phage 102, 107, or 117, all in group IV.

Enterotoxin production. Three strains produced enterotoxin C, and one strain produced enterotoxin D. All four strains gave 4+ coagulase reactions, produced thermonuclease, fermented mannitol, and were highly sensitive to lysostaphin. Three strains produced β- and δ-hemolysins, and the other strain produced α-β-δ-hemolysins. Only one strain was resistant to penicillin. One of the enterotoxin C producers was not lysed by any of the phages tested, whereas the other enterotoxin C producers were lysed by phages from more than one group. The enterotoxin D producer was lysed by phages in group I.

DISCUSSION

The high frequency of staphylococcal mastitis found in this study (39.9%) confirms the general agreement that staphylococci are very important factors in this disease.

On the basis of glucose fermentation and lysostaphin sensitivity, all strains could be placed in the genus *Staphylococcus*. Several authors have reported that the criteria proposed by the Subcommittee on Taxonomy of Staphylococci and Micrococci (27) do not permit the identification of certain species of staphylococci (14, 25); however, the results with our strains show that strains isolated from mastitic milk are able to produce acid from glucose anaerobically.

Various criteria have been used to classify a

TABLE 2. *Antibiotic sensitivity of staphylococci from mastitic milk*

Antibiotic	S (MIC) or R (MIC)[a]						
	S. aureus (*n* = 46)	*S. intermedius* (*n* = 1)	*S. hyicus* (*n* = 1)	*S. epidermidis* (*n* = 6)	*S. saprophyticus* (*n* = 1)	Unclassified (*n* = 2)	
						No. 39	No. 138
Penicillin	31S (0.015–0.1) 16R (0.25–8)	S (0.03)	S (0.03)	3S (0.03) 3R (0.5–1.0)	R (0.125)	R (1.0)	S (0.03)
Methicillin	46S (0.5–4)	S (1.0)	S (0.5)	6S (1.0–2.0)	S (2.0)	S (2.0)	S (0.5)
Chloramphenicol	44S (2–4) 2R (64)	S (4.0)	S (2.0)	6S (2–4)	S (4.0)	S (4.0)	S (4.0)
Novobiocin	46S (0.03–0.5)	S (0.06)	S (0.03)	6S (0.062–0.25)	R (64)	S (0.125)	S (0.125)
Cephalothin	46S (0.06–0.25)	S (0.12)	S (0.12)	6S (0.12–0.25)	S (0.25)	S (0.125)	S (0.06)
Kanamycin	46S (0.5–4)	S (1.0)	S (1.0)	6S (0.5–2.0)	S (1.0)	S (2.0)	S (1.0)
Tetracycline	46S (0.06–0.12)	S (0.125)	S (0.12)	6S (0.06–0.125)	S (0.125)	S (0.125)	S (0.12)
Erythromycin	46S (0.06–0.25)	S (0.125)	S (0.06)	3S (0.125) 3R (128)	S (0.125)	S (0.125)	S (0.125)
Streptomycin	42S (2–4) 4R (64–128)	R (8.0)	S (4.0)	4S (2–4) 2R (64–128)	S (4.0)	S (2.0)	S (4.0)

[a] S (MIC), Sensitive (minimal inhibitory concentration in micrograms per milliliter or international units per milliliter); R (MIC), resistant (minimal inhibitory concentration in micrograms per milliliter or international units per milliliter).

TABLE 3. *Phage typing of S. aureus strains from mastitic milk*

Phage group	No. of strains		
	Human set	Bovine set	Specific bovine phages
I	2	1	
III	10		
IV	3	18	39
M		1	3
Mixed			
I-M	1	2	
I-II-III	1		
I-III-IV	1		
I-III-M	1		
I-II-III-IV	1		
II-III	4		
III-IV	17	18	
III-IV-M	2	1	
II-III-IV		2	
II-IV		1	
I-II-III-IV		1	
IV-M			2
Not typable		1	2

strain as coagulase positive, from small unorganized clots (1+) to complete clotting (4+). The data presented in Table 1 show that of the strains studied in this investigation, all that gave 3+ and 4+ coagulase reactions were highly sensitive to lysostaphin and, with one exception (strain 138), produced thermonuclease. All but strain 138 and the strain classed as *Staphylococcus intermedius* were classified as *S. aureus*. Only one strain classified as *S. aureus* gave a 2+ coagulase reaction, but its other properties (thermonuclease positivity, phage typability, hemolysin positivity, sensitivity to lysostaphin, and lysozyme positivity) were those of *S. aureus*. The one *S. aureus* strain that was untypable was mannitol positive anaerobically, and its other characteristics were those of *S. aureus*. All of the strains classed as *Staphylococcus epidermidis* gave 1+ or 2+ coagulase reactions, but their properties (Table 1) clearly mark them as *S. epidermidis* (1). These results indicate to us that a coagulase reaction of less than 3+ should be considered negative or borderline, depending on the other properties of the strain. The classification of staphylococci on the basis of one characteristic such as coagulase production, which is often done, is not sufficient.

Two strains had identical properties, except for the coagulase reaction; one strain gave a 2+ reaction, and the other strain gave a 3+ reaction. Both strains might be classed as *Staphylococcus hyicus*, but because of the differences in the coagulase reaction one strain was classed as *S. hyicus*, and the other strain was classed as *S. intermedius* (Table 1). One strain was classed as *S. saprophyticus* because it was resistant to novobiocin and did not ferment glucose anaerobically. Two strains (39 and 138) were unclassified because of their borderline properties. Strain 39 gave a 2+ coagulase reaction, was negative for thermonuclease, had medium sensitivity to lysostaphin, was hemolytic, and was phage typable. Strain 138 gave a 3+ coagulase reaction, was thermonuclease negative, had high sensitivity to lysostaphyin, was nonhemolytic, and was untypable.

The fact that three *S. aureus* strains did not ferment mannitol and three gave a ± reaction is in agreement with the findings of Hajek and Marsalek (8) that this is not uncommon among animal strains. Whereas there is a good correlation between 3+ and 4+ coagulase reactions and thermonuclease production, only two of seven strains that gave 2+ coagulase reactions produced this enzyme. Similar observations have been reported by Rayman (20). It is widely accepted that lysostaphin sensitivity is useful in

the separation of staphylococci from micrococci (5). Also, it has been reported that only a small number of coagulase-negative staphylococcal strains are as sensitive to this enzyme as coagulase-positive cultures are. In our study all strains highly sensitive to lysostaphin possessed one or more specific characteristics of *S. aureus* (coagulase production, thermonuclease production), whereas strains with low or only moderate sensitivity did not possess these characteristics. Our findings that phosphatase, gelatinase, and lysozyme production are not specific characteristics of *S. aureus* strains are in agreement with others (17, 23). The egg yolk reaction is often considered characteristic of *S. aureus* strains; however, this property was demonstrated by only 21 of the 46 strains classified as *S. aureus*. This low number of positive strains is similar to that reported by Marandon and Oeding (13) but differs from those found by Hajek and Marsalek (7) and Olson et al. (18).

A large percentage (90%) of our *S. aureus* strains produced β-hemolysin, whereas a smaller percentage (61%) produced α-hemolysin. This is in accordance with the observations of other investigators who have worked with *S. aureus* strains from animals (6, 7, 32). Five of the six strains classed as *S. epidermidis* produced δ-hemolysin (only), whereas one unclassified strain (39) produced both β- and δ-hemolysin.

Penicillin resistance was noted in nearly 40% of our strains, including three of the *S. epidermidis* strains, with a smaller percentage showing resistance to streptomycin (12.3%), erythromycin (5.3%), and chloramphenicol (3.5%). The variety of tests used by other investigators makes it difficult to compare our results with what they found with staphylococci isolated from bovine milk. The presence of penicillin-resistant staphylococci in milk from cows with mastitis is not uncommon, but the incidence varies from location to location (9). Thatcher and Simon (30) suggested that milk can serve as a vehicle for the dissemination of antibiotic-resistant staphylococci; however, our results show that with the exception of penicillin resistance, milk is not an important vehicle for the dissemination of antibiotic-resistant staphylococci in Spain.

Bovine strains isolated by other investigators were found to be susceptible to phage 42D (group IV), whereas our strains were more susceptible to phage 42E (group III). Even so, our strains were more susceptible to specific phages than to those from the human set. On the basis of the phage typing, the majority of the strains isolated could be classed as bovine strains.

Only 7% of the strains produced enterotoxin, a finding similar to those of others (18, 29, 32). The enterotoxins produced by our strains (enterotoxin C and enterotoxin D) are the ones reported by other investigators to be produced by bovine strains. The low percentage of enterotoxigenic strains makes it impossible to relate this property to any of the other characteristics of the staphylococci. The results reported here indicate that milk from cows with mastitis could be a source of staphylococcal food poisoning, but the risk is relatively low.

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