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Identification of Enterotoxigenic Staphylococci from Sheep and Sheep Cheese

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The total of 127 *Staphylococcus aureus* strains obtained from sheep and sheep cheese were examined for their biochemical activities, biotypes, phage patterns, and ability to produce enterotoxins. Of the 83 staphylococcal strains isolated from animals, 77 (93%) were classified as the C biotype. Of this group of sheep-adapted strains, 61 (79%) were sensitive to phage 78, and 46 (60%) produced enterotoxin C exclusively. The three isolates belonging to the A biotype produced enterotoxin D, and two of the three unclassifiable strains produced enterotoxin A. Of the 44 staphylococcal strains isolated from sheep cheese, there were 37 (84%) identified as the C biotype. From this series, 31 (84%) strains were lysed with phage 78, 6 (16%) strains produced enterotoxin C, and 1 strain produced enterotoxin A. One of the six strains determined as the A biotype produced enterotoxin D. C biotype strains, especially of ovine origin, are an exception among animal staphylococci, because a large number of them are enterotoxigenic. The C antigenic type is the most usual of the known enterotoxins in staphylococci of animal provenance.

One of our previous studies (8) dealt with the frequency of enterotoxigenic strains in staphylococci of different origins. Enterotoxins were shown to be produced by 61% of strains obtained from clinical material and by 38% of strains from healthy persons. All these staphylococcal strains of human origin belonged to the A biotype. Among animal staphylococci of other biotypes, only 1% of enterotoxin-positive strains occurred. There was, however, an exceptional group of 22 C biotype strains isolated from sheep, 18% of which produced enterotoxin C. This solitary case among animal staphylococci stimulated our detailed study of a larger collection of isolates of sheep provenance.

The present paper thus deals with the determination of the biochemical properties, biotypes, and sensitivity to phages of strains isolated from sheep and sheep cheese and their ability to produce enterotoxins.

MATERIALS AND METHODS

Staphylococci. The total of 15 farms from North Moravia and Central and East Slovakia supplied 83 *Staphylococcus aureus* strains isolated from the anterior nares of healthy sheep and from the udders of ewes suffering from purulent mastitis. In addition, 44 *S. aureus* strains were obtained from different specimens of sheep cheese coming from Central Slovakia.

Character determinations. All of the 127 strains were tested for the following characteristics: coagulase activity in rabbit, human, and bovine plasma, fibrinolysin, pigmentation, hemolysins, heat-stable and heat-labile nucleases, clumping factor, lysozyme, egg yolk factor, phosphatase, urease, gelatinase and protease, growth type on crystal violet agar, aerobic and anaerobic utilization of mannitol, tellurite reduction, and sensitivity to penicillin, oxacillin, cephaloridine, tetracycline, chloramphenicol, kanamycin, novobiocin, vancomycin, erythromycin, spiramycin, lincomycin, and rifampicin, as well as to mercuric chloride. The test methods used were described in detail elsewhere (5). Subdivision of the strains into biotypes was performed according to Hájek and Maršálek (7).

Phages. Phage typing was carried out with the standard method of Blair and Williams (2). All the strains were tested with the basic sets of phages for typing both bovine (20) and human (21) staphylococci. The additional phages AC_1, 42F, 108, 111, and 187 were included. Strains nontypable with the routine test dilution (RTD) were retested with 100 × RTD.

Enterotoxin assay. The cellophane-over-agar technique was used for enterotoxin production in the examined strains. Enterotoxins were determined by the slide-gel double-diffusion test as stated before (8). Reference enterotoxins A, B, C, D, and E and their corresponding specific antisera were kindly supplied by M. S. Bergdoll, Food Research Institute, University of Wisconsin, Madison. The concentration of the control toxins for optimum reactions was 2.0 μg/ml. Final serum dilutions were as follows: anti-A, 1:20; anti-B, 1:50; anti-C, 1:30; and both anti-D and anti-E, 1:20.

RESULTS

Biotyping. All of the 127 *S. aureus* strains producing heat-stable nuclease and coagulating rabbit plasma were characterized by high numbers of positive biochemical reactions.

Biotype could be defined in 80 (96.4%) strains

of the 83 isolates obtained from sheep. On the whole, 77 (92.8%) strains belonged to the C biotype. They coagulated human and bovine plasma, the latter mostly with a delay of 24 to 72 h. They produced pigment and beta hemolysin but lacked fibrinolysin and alpha hemolysin. On crystal violet agar, all but two of them grew in negative-type (violet) colonies. Two strains were identified as pigment-deficient variants of the C biotype.

Three more isolates were classified as the A biotype. They coagulated human though not bovine plasma, produced fibrinolysin, pigment, and alpha hemolysin, grew in positive-type (orange) colonies on crystal violet agar, and lacked beta hemolysin. The remaining three strains could not be biotyped (Table 1).

Of the group of 44 strains harvested from sheep cheese, 43 (97.7%) could be classified into biotypes. Thirty-seven (84.1%) of them had properties corresponding to the C biotype, which was mentioned above. Only three strains grew on crystal violet agar in positive-type (orange) colonies.

Six more strains were determined as the A biotype, and a single strain was unclassifiable (Table 1).

Phage typing. A total of 75 (90.4%) strains of the 83 isolates from animals were typable with phages of both sets. Sixty-one (79.2%) strains of the C biotype were sensitive to phage 78 of the bovine set, 19 strains at RTD and 23 strains at 100 × RTD; another 19 strains gave only weak reactions at 100 × RTD. Forty-three (55.8%)

TABLE 1. *Biochemical properties of S. aureus strains from sheep and sheep cheese*

Property			No. of strains					
			Sheep			Cheese		
			C[a] [77 strains (%)]	A[a] (3 strains)	—[b] (3 strains)	C[a] [37 strains (%)]	A[a] (6 strains)	—[b] (1 strain)
Coagulation of plasma	rabbit	1 h	65 (84.4)	3	0	30 (81.1)	6	1
		3 h	12 (15.6)		3	7 (19.9)		
	human	1 h	77	3	3	37	6	1
	bovine	1 h	0	0	0	0	0	0
		3 h	27 (35.1)	0	1	27 (73.0)	0	1
		24 h	18 (23.4)	0	2	4 (10.8)	0	
		72 h	32 (41.6)	0		6 (16.2)	0	
Fibrinolysin			0	3	3	0	6	1
Pigment			75 (97.4)	3	3	37	6	1
Hemolysin	alpha		0	3	0	0	6	0
	beta		77	0	3	37	1	1
	delta		77	3	3	19 (51.3)	6	0
Crystal Violet type	orange	(positive)	2 (2.6)	3	0	3 (8.1)	2	0
	violet	(negative)	75 (97.4)	0	3	34 (91.9)	4	1
Heat-stable nuclease			77	3	3	37	6	1
Heat-labile nuclease			77	3	3	37	6	1
Mannitol aerobically			77	3	3	37	6	1
Mannitol anaerobically		5 days	33 (42.9)	0	0	9 (24.3)	4	0
		7 days	18 (23.4)	3	1	23 (62.2)	2	1
		10 days	23 (29.9)		2	5 (13.5)		
Clumping factor			77	3	3	34 (91.9)	6	1
Lysozyme			77	3	3	37	6	1
Reduction of tellurite			72 (93.5)	3	3	34 (91.9)	6	0
Egg yolk factor	weak reaction		58 (75.3)	3	3	20 (54.1)	0	1
	strong reaction		5 (6.5)			4 (10.8)	6	
Tween-80			6 (7.8)	0	1	4 (10.8)	6	1
Phosphatase			77	3	3	37	6	1
Urease			77	3	0	36 (97.3)	6	1
Gelatinase			77	3	3	37	6	1
Protease			77	3	3	37	3	1
Resistance	to penicillin		0	3	0	5 (13.5)	6	0
	to chloramphenicol		0	3	0	0	0	0
	to other antibiotics		0	0	0	0	0	0
	to mercuric chloride		0	0	0	0	0	0

[a] Biotype.
[b] —, Unclassifiable strains.

strains were lysed by the phage 78 by itself. With phages of the human set, only 28 (36.4%) strains were typable at 100 × RTD, many of them giving only weak reactions. The majority of these strains were sensitive to phages 3A, 3C, and 55 of group II.

The three isolates identified as the A biotype were lysed at RTD by group III phages from the human set, being thus quite different from the others. The three unclassifiable strains were registered as nontypable (Table 2).

Forty-two (95.5%) of the 44 strains obtained from cheese were typable. Thirty-one (83.8%) of the 37 C biotype strains were sensitive to Davidson's phage 78, 6 at RTD and 15 at 100 × RTD, with the remaining 10 strains giving weak reactions at 100 × RTD only. A total of 13 (35.1%) strains were typable with human phages at 100 × RTD, many of them giving weak reactions. Three strains characterized by positive-type (orange) growth on crystal violet agar having the phage pattern 42D,102,107,117,-42F,108,111 were utterly different from the others.

The six A biotype strains were lysed by phages of the human set, five of them by phage 3C at 100 × RTD. The single unclassifiable strain was resistant to the used phages (Table 3).

Frequency of enterotoxin producers. Surprisingly high was the number of enterotoxigenic strains isolated from the nares or udders of ewes. Out of the 77 C biotype strains, 46 (59.7%) produced enterotoxin C exclusively. All three of the A biotype isolates formed enterotoxin D, and two unclassifiable strains formed enterotoxin A.

The demonstration of enterotoxin C producers was also relatively frequent, with 44 staphylococcal strains from sheep cheese. Among the 37 C biotype strains, the ability to produce this antigenic type of toxin was observed in 6 (16.2%) cases, and furthermore, 1 strain (2.7%) produced enterotoxin A. Out of the six A biotype strains, one was D enterotoxin positive (Table 4).

TABLE 2. *Phage patterns of 83 S. aureus strains from sheep*

Phage pattern[a]	No. of C biotype strains
78	14
78,(54)w	1
78,(3A,3C,55)w	1
78,(3A,3C)	1
78,(3A,55),(3C)w	1
78,55,(3A,3C,42E)	1
(78)	15
(78),(55)w	2
(78),(81)w	1
(78),(3A,55)w	1
(78),(3A,3C,71)w	1
(78,55),(119)w	1
(78,55),(3A,3C,54)w	1
55,(78),(3A,6)w	1
(78)w	14
(78,3A)w	1
(78,55)w	1
(78,119,55)w	1
(78,3A,55)w	1
(55),(78,3A)w	1
(116,3A,55)	1
(3A,55),(116,119)w	1
(55)	8
(AC_1)w	1
NT	5

[a] Total number of C biotype strains was 77. The three A biotype strains had the following patterns: 47,53,54,75,(42E),(117)w; 47,53,54,75,(117)w; and 47,-53,54,75. The three unclassifiable strains were nontypable. In parentheses: lysis at 100 × RTD. w, Weak reaction (<50 plaques); NT, nontypable.

DISCUSSION

Biochemical examination proved that a vast majority of the *S. aureus* strains from sheep cheese and from ewes' nares and udders had similar features corresponding to the C biotype (7, 9). They coagulated human plasma and bovine plasma (the latter mostly with a delay of 24 to 72 h) produced pigment and beta hemolysin, lacked fibrinolysin and alpha hemolysin, and grew in violet (negative-growth-type) colonies on crystal violet agar. Sensitivity to phage 78, characteristic of ovine staphylococci (1, 14), was

TABLE 3. *Phage patterns of 44 S. aureus strains from sheep cheese*

Phage pattern[a]	No. of C biotype strains
78	4
78,(81)w	1
78,(3C),(117,52,80,3A,55,54,96)w	1
(78)	10
(78),(29)w	1
(78),(3C)w	1
(78),(116,3C)w	1
(78),(3A,3C,6,42E,54)w	1
(78,3C),(81)w	1
(78)w	8
(78,116,79,85)w	1
119,94,(116,54,96),(78)w	1
(119)w	1
(3C)w	1
42D,102,107,117,42F,108,111,(42E,47, 77),(AC_1,6,96,81)w	3
NT	1

[a] Total number of C biotype strains was 37. The six A biotype strains had the following patterns: two strains, (3C); two strains, (3C), (116,55,71)w; one strain, (3C)w; one strain, 6,47,53,83A,(42E,96),(111,95,81)w. The one unclassifiable strain was nontypable. In parentheses: lysis at 100 × RTD. w, Weak reaction (<50 plaques); NT, nontypable.

TABLE 4. *Incidence of enterotoxigenic S. aureus strains from sheep and sheep cheese*

Origin of strains	Biotype	No. of strains	No. of producers elaborating enterotoxin				
			A	B	C (%)	D	E
Sheep	C	77	0	0	46 (59.7)	0	0
	A	3	0	0	0	3	0
	—[a]	3	2	0	0	0	0
Sheep cheese	C	37	1	0	6 (16.2)	0	0
	A	6	0	0	0	1	0
	—[a]	1	0	0	0	0	0

[a] —, Unclassifiable strains.

proved in the tested C biotype strains, also. On the grounds of the presented data, a large majority of staphylococcal strains isolated from cheese (84%) can be expected to be of ovine origin.

There was an interesting finding of three strains from cheese differing from the others by growing in orange (positive-growth-type) colonies on crystal violet agar and by sensitivity to bovine phages 42D, 102, 107, and 117 of group IV. These features make them belong to the bovine forma specialis within the scope of the C biotype (6, 7, 9, 15). Their presence in the cheese means that some of the examined products were most probably adulterated by addition of cow's milk to sheep milk on some of the farms. Accidental contamination of the cheese by bovine staphylococci is much less likely because sheep cheese is produced on specialized sheep farms where cows are not kept, and the technology of sheep cheese production is rather different from the manufacture of cow's cheese.

Characteristic of the isolated strains of the A biotype was their ability to coagulate human but not bovine plasma and to produce fibrinolysin, pigment, and alpha hemolysin. They were resistant to bovine phages, including phage 78. In their properties, they corresponded to staphylococci commonly parasitic in man (9). The presence of individual A biotype strains among staphylococci from the nares of sheep indicates their transfer from man during manipulation with animals. Identification of A biotype strains in the other group of isolates suggests contamination of the cheese with staphylococci of human origin during manufacture or distribution.

The examination of enterotoxin production showed the staphylococci obtained from sheep to be a special group among animal isolates. One of our previous papers (8) demonstrated sporadic (1%) presence of enterotoxigenic populations among staphylococci of animal origin. Only a small group of 22 sheep isolates was found to have 18% of C enterotoxin-positive strains. The surprisingly large proportion (60%) of 77 sheep-adapted strains that produced enterotoxin supports our hypothesis that enterotoxin type C is the form most frequently found in animal staphylococci, especially in the ovine forma specialis characterized within the scope of the C biotype by violet (negative-growth-type) colonies on crystal violet agar and by sensitivity to phage 78. Nevertheless, further investigations of ovine C biotype strains from different geographic areas are desirable.

No work has so far been published dealing with enterotoxin production and biotyping in sheep staphylococci in detail. More attention has been concentrated on staphylococcal strains of bovine origin as potential sources of human enterotoxicosis. A number of authors have, however, shown a limited occurrence of enterotoxin-positive strains from bovine mastitis and milk specimens without any remarkable predominance of some of the antigenic types of toxins: Casman et al. (3), 2 or 9%, respectively; Sinell (19) and Untermann and Sinell (27), 4%; Untermann (25), 5%; Wieneke, (28) 6%; Terplan and Zaadhof (23), 8%; Olson et al. (16), 14%; and Mayer (11), 15%. Only Untermann et al. (26) could observe among their 120 strains of bovine provenance 5 (4%) producing solely C enterotoxin. These studies have not, however, relied on subdivision of enterotoxin-positive strains into biotypes and, in this way, reliable determination of their primary host as the source of infection. Staphylococci adapted to cattle belong, like ovine staphylococci, to the C biotype (7, 9). The above discussed studies on enterotoxigenic bovine staphylococci can, however, be expected to include also strains of other than the C biotype, particularly those of the human A biotype. Thus Hájek and Maršálek (8) have found only 1% of C enterotoxin-positive strains in the group of staphylococci isolated from cows, but it belonged to the human A biotype. Mochmann et al. (13) have identified five (8%) A enterotoxin-positive strains in their bovine material, but four of them were determined as the A biotype or human variety according to Meyer (12), only one of them being the C biotype or bovine variety.

Many investigators dealing with the distribution of enterotoxin producers among staphylo-

cocci isolated from food showed that in most cases it was enterotoxin A or A and D that prevailed (3, 4, 10, 13, 17, 18, 22, 24, 25, 28). The results reported by Mochmann et al. (13) indicate that a large majority of these enterotoxin-positive strains most probably belong to human staphylococci.

The present study shows that in staphylococcal isolates from sheep cheese, C enterotoxin producers predominated in accordance with the highest frequency of sheep-adapted staphylococci.

From the above discussed findings it is obvious that C biotype staphylococci contained in sheep milk resist, during the period of cheese-making, unfavorable environmental effects (pH value, NaCl concentration, water activity, temperature, and competitive growth of other microorganisms). They keep their ability to multiply and to produce C enterotoxin even in the final products, which can, when improperly stored, represent the public health hazard of intoxication.

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