

HEMOLYSIN AND LYSOZYME PRODUCTION BY STAPHYLOCOCCI ISOLATED FROM BOVINE UDDERS

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SUMMARY

A study was made on the distribution of hemolytic patterns and frequency of lysozyme production among 912 coagulase positive staphylococci strains of bovine udder origin. The alpha-beta-delta pattern was seen by more than 50 p. 100 of the strains and all other patterns were noted. Lysozyme was produced by 98 p. 100 of the strains.

There was no difference in the occurrence of the lysins in strains from clinical mastitis, subclinical mastitis and normal quarters.

Nine out of 18 lysozyme-negative strains produced only beta lysin.

Of the many extracellular substances produced by the Micrococcaceae, coagulase is universally accepted as being the most important in identifying potentially pathogenic strains. Closely associated with coagulase production as *in vitro* test of pathogenicity is alpha hemolysin production by strains of human origin (ELEK, 1959), while in strains of animal origin, especially in bovine mastitis, presence of beta hemolysin is considered to be very strong evidence for the presence of a pathogenic strain (JOSHI and DALE, 1963; LOKEN and HOYT, 1962; MINETT, 1937; SCHALM and WOODS, 1953; SLANETZ and BARTLEY, 1962). The delta hemolysin is now receiving increased attention in connection with its possible relationship to pathogenicity (EDWARDS and RIPPON, 1957; ELEK and LEVY, 1950; JACKSON and LITTLE, 1957; McLEOD, 1963; REID and WILSON, 1959), but its manifestations on blood agar plates have not been established with certainty (EDWARDS and RIPPON, 1957; JACKSON and LITTLE, 1958; MARKS and VAUGHAN, 1950). The presence of epsilon hemolysin and its distinction from the delta hemolysin is still uncertain (MARKS, 1952).

One of the most recent addition to the long list of micrococcal extracellular substances having association with coagulase production and perhaps with patho-

genicity, is lysozyme. A rather vast literature exists on the effects of lysozyme on bacteria, dating back to 1922 when FLEMING first reported the discovery of this substance (FLEMING, 1922; SALTON, 1957). Far fewer reports, however, exist on the production of this enzyme by bacteria and only lately was staphylococcal lysozyme production associated with pathogenicity (KASHIBA, NUZU, TAMAKA, NOZU and AMANO, 1959; OMORI, KATO and LIDA, 1960). It was suggested that lysozyme production is either a good biochemical index or determinant of pathogenicity in the Micrococcaceæ (GOLDBACH and HAEMEL, 1964; GROSSGEBAUER, SCHMIDT and LANGMAACK, 1968; JAY, 1966).

It was reported that about 97-100 p. 100 of coagulase positive staphylococci of human origin were able to produce lysozyme (HAWIGER, 1968). All 85 isolates of animal origin, from cases of diarrhea or dermatitis produced lysozyme (GROSSEGEBAUER *et al.*, 1968) while the incidence of lysozyme production by isolates recovered from outbreaks of staphylococcal food poisoning was 90-95 p. 100 (JAY, 1966). No information is available on the frequency of lysozyme production in staphylococci isolated from the bovine udder.

This work describes the frequencies of hemolysin and lysozyme production by staphylococci isolated from normal and clinically infected bovine udders.

MATERIALS AND METHODS

Nine hundred and twelve strains of *Staph. aureus* were isolated from milk samples collected from 42 dairy herds. The methods of sampling and culture have been described elsewhere (SCHALM and ZIV-SILBERMAN, 1968). All strains were coagulase positive when tested by the tube method with rabbit plasma diluted 1 in 5. Three hundred and seventeen strains were from quarter samples on which the somatic cell content was estimated by the California Mastitis Test (CMT) (SCHALM and NOORLANDER, 1957). This somatic cell content reflects the pathological damage to the udder, and on this basis quarters were divided into normal (CMT scores of N and T which estimate cell count of less than 1 000 000 per ml) or affected with subclinical Mastitis (CMT scores of 1 and higher which estimate cell count greater than 1 000 000 per ml). Mastitis was defined as clinical if the milk was grossly abnormal at the time of sampling. Thus the udder irritation was considered as an index of pathogenicity for the infecting strain.

Hemolytic activity was examined by a modification (JASPER and JAIN, 1966) of the plate method of ELEK and LEVY (ELEK and LEVY, 1950) using nutrient agar with 4 p. 100 washed sheep, rabbit and horse erythrocytes. Cultures were incubated at 37°C for 48 hours, one set of plates incubated in air and a duplicate set in 10 p. 100 (v/v) CO₂. Alpha lysin was identified by specific neutralization with anti-alpha serum (1) which was incorporated onto the sheep blood agar plates by streaks made with cotton wool swabs saturated with the undiluted serum. Alpha lysin was further characterized by « angle-lines » inhibition of the alpha lysin by the beta lysin, on sheep blood agar (JASPER and JAIN, 1966). Beta lysin was identified by its typical appearance on sheep blood agar and by the CAMP reaction. Delta lysin was recognized by potentiation of its hemolytic activity in conjunction with the beta lysin, on sheep blood agar and by hemolysis of horse erythrocytes. Strains showing hemolysis which was not inhibited by anti-alpha serum, not potentiated by beta lysin and negative to the CAMP test were considered as producers of other types of hemolysins.

Lysozyme production was determined on plates prepared by adding lysozyme substrate (Difco), which contained ultraviolet killed cells of *M. lysodeikticus*, to heart infusion (HI) Agar (Difco) at the rate of 1 mg/ml, sterilizing at 121°C for 15 mn and pouring about 12 ml into sterile plates. Broth cultures, grown overnight at 37°C, were spot inoculated onto the hardened plates and incubated at 37°C for 48 hours in 10 p. 100 (v/v) CO₂. Lysozyme production was indicated by a zone of definite clearing surrounding the area of growth. A lysozyme producing *Staph. aureus* strain and a coagulase negative micrococcus strain served as positive and negative controls.

(1) Burroughs Wellcome and Co. Ltd., London, containing 150 IU alpha antitoxin per ml.

RESULTS AND DISCUSSION

The production of hemolysins and lysozyme by 912 strains was examined. The distribution of the possible hemolytic patterns is shown in table 1. The distribution of the lytic patterns among selected strains from clinical mastitis, subclinical mastitis and normal quarters is given in table 2 and the types of lysins produced by lysozyme-negative strains is presented in table 3.

TABLE I

Distribution of lytic patterns among 912 Staphylococcus aureus strains isolated from bovine udders
Distribution des types hémolytiques parmi 912 souches de Staphylococcus aureus isolées de la mamelle de vaches laitières

Lytic pattern Type hémolytique	Alpha Bêta Delta	Alpha Bêta	Alpha Delta	Bêta Delta	Alpha	Bêta	Delta	No lysis pas de lyse	Total
No. of strains	459 (a)	121 (b)	42 (a)	132 (c)	2	126 (d)	28	2	912
Nombre de souches									
(%)	50,5	13,3	4,5	14,5	0,2	13,8	3,0	0,2	100,0

(a) 2 strains produced other hemolysins.
2 souches produisent d'autres hémolysines.

(b) 1 strain produced other hemolysins.
1 souche produit d'autres hémolysines.

(c) 54 strains produced other hemolysins.
54 souches produisent d'autres hémolysines.

(d) 12 strains produced other hemolysins.
12 souches produisent d'autres hémolysines.

Data from table 1 indicate that 68.5 p. 100 of the strains examined produced alpha hemolysin, 92.1 p. 100 produced beta hemolysin and 72.5 p. 100 produced delta hemolysin, either alone or in combination with other lysins. All combinations of the three lysins were seen, the most common combinations were alpha-beta-delta, alpha-beta and beta-delta. Sixty nine strains (7.5 p. 100) produced additional hemolysin (s), which were mostly produced by strains producing the beta-delta combinations.

It has been suggested (ELEK and LEVY, 1954) that the possession of the full complement of alpha, beta and delta hemolysins is characteristic of coagulase positive staphylococci and any other pattern represents a loss of one or more hemolytic characters due to mutation. The relative frequency with which bovine staphylococci produce beta lysin has been regarded as a basic difference between human and bovine strains. Only 11 p. 100 of ELEK and LEVY'S human strains (ELEK, 1959) produced

this lysin while 96 p. 100 produced alpha and delta, respectively. FLEK (FLEK, 1959) suggested that selection may explain the low frequency of beta lysin in human strains. With regard to the bovine strains of udder origin it has been postulated (LOKEN and HOYT, 1962) that although alpha hemolysin producing strains may exist in a dairy herd for several years, the prolonged maintenance of the carrier state may result in the loss of ability to produce alpha hemolysin by many strains.

TABLE 2

Distribution of lytic patterns among selected Staphylococcus aureus strains from clinical mastitis, subclinical mastitis and from normal quarters
Distribution des types hémolytiques parmi des souches de Staphylococcus aureus obtenues à partir de cas de mammite clinique ou subclinique et de glande mammaire normale

Lytic pattern Type hémolytique	Alpha Bêta Delta	Alpha Bêta	Alpha Delta	Bêta Delta	Alpha	Bêta	Delta	Total
Clinical mastitis Mammite clinique	17	4		6		4	1	32
% of strains % des souches	53,1	12,5		18,7		12,5	3,2	100,0
Subclinical mastitis Mammite subclinique	120	12	13	37	2	35	4	223
% of strains % des souches	53,8	5,4	5,8	16,6	0,9	15,7	1,8	100,0
Normal quarters Quartiers normaux	36	8		6		6		62
% of strains % des souches	58,0	12,9	4,85	9,7		9,7	4,85	100,0

There were essentially very little differences in the frequency of lytic patterns produced by strains isolated from clinical mastitis, subclinical mastitis and from normal quarters (table 2) and the most common lytic pattern was the production of the 3 lysins together, regardless of the origin of the culture. The data, therefore, does not seem to agree with an earlier suggestion (LOKEN and HOYT, 1962) that strains producing both delta and beta lysins produce more udder irritation than those producing beta hemolysin alone. It rather agrees with recent observations (FROST, 1967) that the hemolytic patterns of the strains and the degree of udder irritation, which may reflect the virulence of the strains toward the tissues of the hosts, are independent characteristics.

Lysozyme was produced by 98 p. 100 of the strains. The 18 lysozyme-negative strains exhibited slow and irregular coagulase production, some strains coagulating rabbit plasma only after 48-73 hours of incubation. Nine of the 18 lysozyme-negative strains produced only beta lysin (table 3) while only 2 strains produced all the 3 lysins. Differences were observed in the rate of lysozyme production. Certain strains which

produce less than the full complement of lysins showed rather small diameters of zones of clearing. Smallest zones were observed by strains lacking alpha or delta lysins, or both. It should be recalled, however, that the plate method for testing lysozyme production is a qualitative and not a quantitative one. No conclusions could yet be drawn by relating the rate of lysozyme production to the hemolytic patterns but these findings merit further studies.

TABLE 3

Distribution of lytic patterns among 18 lysozyme-negative Staphylococcus aureus strains isolated from bovine udders

Distribution des types hémolytiques parmi 18 souches de Staphylococcus aureus « Lysozymes négatives », isolées de mamelle de vache laitière

Lytic pattern Type hémolytique	Alpha Bêta Delta	Alpha Bêta	Alpha Delta	Bêta Delta	Alpha	Bêta	Delta	No lysis pas de lyse	Total
No. of isolates Nombre d'isolements	2	5				9	1	1	18

Information on the state of udder irritation was available on only 11 of the 18 quarters from which lysozyme-negative strains were isolated. Ten of these quarters were with subclinical mastitis and one quarter was classified as normal. No definite conclusions could be drawn on the relationship between ability or inability to produce lysozyme and the degree of udder irritation but judging from our data it appears that the 2 parameters are probably independent.

The value of lysozyme production over alpha hemolysin production as an ancilliary test of staphylococcal pathogenicity, in strains of human origin, has been pointed out recently (GROSSGEBAUER *et al.*, 1968 ; JAY, 1966). In this context our findings could only indicate that lysozyme production and coagulase production are indicators of equal importance in testing potentially pathogenic strains of bovine udder origin. It should, however, be very desirable to obtain data on the frequency of lysozyme production among coagulase negative staphylococci of known or suspected virulence for the bovine udder.

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RÉSUMÉ

PRODUCTION D'HÉMOLYSINE ET DE LYSOZYME PAR DES STAPHYLOCOQUES ISOLÉS DE MAMELLE DE VACHES LAITIÈRES

La distribution des types hémolytiques et la fréquence de la production du lysozyme ont été étudiées parmi 912 souches de staphylocoques « coagulase positifs », isolés de la mamelle de vaches laitières. Le type alpha-bêta-delta a été rencontré dans plus de 50 p. 100 des souches ; tous les autres types ont été notés (tabl. 1). Le lysozyme est produit par 98 p. 100 des souches.

La distribution des hémolysines dans les souches provenant de mammites cliniques, subcliniques ou de glandes mammaires normales n'est pas différente (tabl. 2).

Parmi les souches « lysozyme négatives », 9 seulement sur 18 sont « hémolysine bêta positives », contre 92 parmi les « lysozyme positives ».

La production de lysozyme et l'irritation de la glande mammaire apparaissent comme des paramètres indépendants.

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