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NUCLEASE OF STAPHYLOCOCCUS EPIDERMIDIS ISOLATED FROM MASTITIC MILK

PRODUCTION AND SOME PROPERTIES

By

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GUDDING, R.: *Nuclease of Staphylococcus epidermidis isolated from mastitic milk. Production and some properties.* Acta vet. scand. 1980, *21*, 267—277. — Nuclease was produced by 47 % of the Staphylococcus epidermidis strains isolated from bovine quarter milk samples. The quantity of enzyme produced by different strains varied considerably. The nuclease of bovine S. epidermidis strains was heat-stable, the average D-value at 120°C being estimated to be 19 min. The nucleases of S. epidermidis and S. aureus could be identified, and consequently differentiated by serological methods.

S. epidermidis was a less severe udder pathogen than S. aureus. However, no difference was observed in udder pathogenicity between S. epidermidis strains with low or high in vitro nuclease production.

Staphylococcus epidermidis; nuclease.

Staphylococcus epidermidis seems to have a dual effect in relation to bovine mastitis. On one hand, the organism is considered to be an udder pathogen, producing mastitis both naturally and experimentally (*Holmberg* 1973). On the other hand, however, a protective effect has been attributed to S. epidermidis, the presence of this organism either preventing or modifying the course of S. aureus mastitis (*Linde et al.* 1975, *Anderson* 1978).

The production of coagulase has been considered the key criterion when differentiating S. aureus from S. epidermidis. The ability to produce heat-stable nuclease has been found to correlate well with coagulase production, and this test is included in the scheme for identification of staphylococci in Bergey's Manual of Determinative Bacteriology (*Baird-Parker* 1974).

Staphylococci may also be classified by serological differentiation of proteinases (*Sandvik & Fossum* 1965). Antibodies

neutralizing the S. epidermidis nuclease can be demonstrated in bovine serum (*Gudding* 1980b).

In the present study, the production of nuclease by bovine strains of S. epidermidis was examined, and related to systems for biochemical differentiation of the organism as well as to its udder pathogenicity. The heat stability of the nuclease of bovine strains of S. epidermidis was also investigated as was the differentiation of bovine staphylococci by serological examination of the nuclease.

MATERIALS AND METHODS

Samples

The material was based on quarter milk samples examined at the National Veterinary Institute over a period of about 2 years. Most of the strains originated from herds participating in a mastitis research programme in which quarter samples were collected from the herds twice a year. The results of these examinations were used to assess the udder pathogenicity of S. epidermidis and S. aureus.

Bacteriological and enzymological examinations

Routine bacteriological examinations and tests for nuclease activity in the quarter samples were performed as described by *Gudding* (1980a).

Non-β-toxic staphylococci were selected and examined for the production of coagulase and nuclease. The coagulase test was performed according to recommendations of the *Subcommittee on taxonomy of staphylococci and micrococci* (1965). The production of nuclease by the strains was tested on DNase Test Agar (Difco)* and on a TDA (*Lachica et al.* 1971a) with added peptone 1.0 %, meat extract 0.5 % and yeast extract 0.1 %. A large colony mass was used when inoculating this "enriched" TDA. The nuclease activity in the DNase Test Agar and the "enriched" TDA was expressed by values from 0 to 3, the highest figure applying to at least 25 mm wide pink zones along the streak of organisms.

The further differentiation of coagulase negative staphylococci was based on the production of phosphatase and acetoin, aerobic and anaerobic acid production from glucose and man-

* Difco Laboratories Inc., Detroit, Michigan, USA.

nitol, aerobic acid production from lactose and maltose, and sensitivity to novobiocin. The oxidation fermentation test with glucose and mannitol was performed according to the *Subcommittee* (1965). The same basis substrate was also used when testing acid production from lactose and maltose. Acetoin production was tested as described by *Baird-Parker* (1966), and phosphatase production according to *Dornbusch et al.* (1976). Novobiocin sensitivity was tested and evaluated as described by *Digranes & Oeding* (1975).

Enzymo-serological examinations

Antibodies against the nuclease of S. aureus and S. epidermidis were produced by immunizing rabbits according to the procedure described by *Gudding* (1979). Antinuclease activity was demonstrated using the cross-wise inhibition test (*Sandvik* 1974).

Thermostability and activity at different pH levels

Nuclease activity before and after heating broth cultures at 100°C for 10 min was tested for 661 strains of S. epidermidis. In addition, the resistance to heat of the nuclease of 5 strains of S. epidermidis and 3 strains of S. aureus was tested as described by *Gudding* (1979) by submerging ampoules with enzyme solution in water (45—95°C) and glycerol (100—120°C) for 2 min, and by heating ampoules at temperatures of 100°C, 110°C and 120°C for periods ranging from 1 to 60 min.

The effect of pH on enzyme activity was tested by the agar diffusion test, and by the turbidimetric method of *Erickson & Deibel* (1973b).

Data processing

A computer was used for assessing the data concerning the udder pathogenic effect of S. epidermidis.

RESULTS

Nuclease production

Nuclease activity was demonstrated in 10 % of non-heated and 21 % of heated quarter samples from cows with a clinical S. epidermidis mastitis (*Gudding* 1980c).

When examined on the DNase Test Agar, 47 % of the S. epi-

dermidis strains exhibited nuclease activity, the corresponding figure on "enriched" TDA being 46 % (Table 1). The values indicating the quantity of nuclease production in the 2 plate methods showed high correlation ($r = 0.90$). Concentrations of nuclease determined in broth cultures were also correlated to those obtained when growing the organisms on the DNase Test Agar ($r = 0.39$) and "enriched" TDA ($r = 0.43$) by statistically significant coefficients ($P < 0.001$).

Table 1. Percentage distribution of 1871 Staphylococcus epidermidis strains according to the amount of in vitro nuclease produced.

Test medium	No production	Amount of nuclease produced		
		small	medium	high
DNase Test Agar	53	5	5	37
"Enriched" TDA*	54	8	8	30

* Toluidine Blue DNA Agar enriched with 1.0 % peptone, 0.5 % meat extract and 0.1 % yeast extract.

Heat stability and pH optimum

Concentrations of nuclease demonstrated in broth cultures which had been heated at 100°C for 10 min were generally the same as or slightly less than those recorded before heat treatment. Post-heating enzyme activity was recorded in samples from 647 of the 661 strains which produced detectable amounts of the enzyme when cultivated in a nutrient broth. The enzyme production of all the 14 strains producing an apparently heat-sensitive nuclease was very low, the highest concentration recorded being 20 diffusion units per 0.1 ml. There was good correlation between concentrations of nuclease in broth before and after heating, the correlation coefficient being $r = 0.94$.

In Fig. 1 the logarithms of the nuclease activity of 2 S. epidermidis strains are shown plotted against the duration of heat treatment at 110°C and 120°C, respectively. The calculated average D-values (time at a given temperature to effect 1 log decrease in enzyme activity) at 120°C for the nuclease of 5 S. epidermidis and of 3 S. aureus strains were 19 and 26 min, respectively. One of the S. epidermidis strains examined (NVH 3360) produced less nuclease in vitro than the remainder (250 versus approx. 1500 diffusion units per 0.1 ml). The nuclease of this

particular strain was less heat-stable than that of the others (Fig. 1). The nuclease activity of the 5 S. epidermidis and 3 S. aureus strains was influenced only slightly, if at all by heat treatment for 2 min in the temperature range 45—120°C.

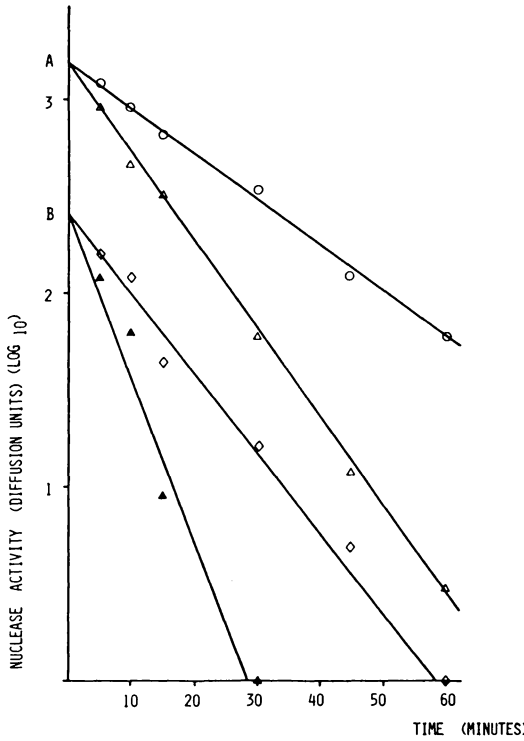

Figure 1. Thermal-destruction-rate curve for the nuclease of 2 strains of Staphylococcus epidermidis (A: NVH 3359, B: NVH 3360) at 110°C (strain A ○———○, strain B ◇———◇) and 120°C (A △———△, B ▲———▲).

The activity of S. epidermidis nuclease was highest in the pH range 8.5 to 9.0, both when tested by the agar diffusion method and by the turbidimetric method.

Biochemical reactions

As seen from Table 2, only 16 of the in all 82 S. epidermidis strains which were examined could be classified according to Baird-Parker's system, the remainder being classified into subgroups according to *Holmberg* (1973). The in vitro nuclease

production of the strains varied considerably, both between and within the subgroups. Nuclease production was most common and abundant in phosphatase positive and acetoin negative strains (subgroups III, c and e), 93 % of these strains producing the nuclease, most of them in fairly high concentrations. Initially, 11 strains, all showing abundant in vitro nuclease production (zone diameter $\geq$ 16 mm), were classified as subgroup a. However, in the enzymo-serological examinations, all these strains turned out to be S. aureus. In a repeated coagulase test they were assessed to be coagulase positive, clots scoring 2 + and 3 + on the scale described by *Turner & Schwartz* (1958) being observed.

Table 2. Distribution of 82 strains of S. epidermidis according to biochemical properties and amount of nuclease produced.

Subgroup*	Total number	Concentration of nuclease in broth culture**				
		number of strains				
		0	1—5	6—10	11—15	$\geq$16
II	5	2			1	2
III	3				2	1
IV	6	2		1	3	
VI	2	2				
c	8	1		2	1	4
e	35	2	1	12	12	8
g	10	2	5	3		
h	2	1	1			
i	11	3	5	1	2	

* Subgroups II—VI according to the system of Baird-Parker, subgroups c—i according to the system of Holmberg.
** Expressed as mm zone diameter in TDA (minus well diameter).

Enzymo-serological examination

The nuclease of 25 S. epidermidis strains showing the most abundant in vitro nuclease production as presented in Table 2, was inhibited by rabbit antibodies against the nuclease of 3 different S. epidermidis strains, but not by S. aureus antinucleases. As inferred above, the nuclease of 11 S. aureus strains, initially classified as S. epidermidis subgroup a, was not inhibited by antibodies against the nuclease of S. epidermidis. The S. epidermidis strains could not be differentiated on the basis of the results of the enzymo-serological examinations.

Pathogenicity

S. epidermidis strains isolated from cases of clinical mastitis produced nuclease more frequently and in higher concentrations than those isolated from subclinical cases, when cultivated in a nutrient broth. However, the difference was moderate.

With 1 exception, no statistically significant correlation was established between the amount of nuclease produced in vitro on one hand and the clinical status (1 to 4) or temperature of the cow recorded by the practising veterinarians on the other. However, the temperature of the cow at the onset of the mastitis was correlated to the nuclease production as determined on DNase Test Agar by a coefficient of $r = 0.28$ ($P < 0.05$).

At the laboratory examinations carried out 1 to 9 months after a subclinical S. aureus or S. epidermidis mastitis, the same species of organism was isolated from the previously affected quarters in 52 % and 14 % of the cows, respectively. Cows with S. epidermidis mastitis were also classified according to the amount of in vitro nuclease produced by the strains. The frequency with which S. epidermidis was re-isolated from identical quarters in the 3 groups is shown in Table 3. Approx. 3 out of

Table 3. Percentage distribution of cows according to udder health status after subclinical mastitis caused by Staphylococcus aureus or Staphylococcus epidermidis.

Diagnosis at the subsequent examination	Diagnosis at the first examination: mastitis caused by Staphylococcus aureus n = 1461	Staphylococcus epidermidis: no nuclease production n = 150	Staphylococcus epidermidis: low or medium production n = 52	Staphylococcus epidermidis: high nuclease production n = 34
Non-specific mastitis (microorganisms not isolated)	7	7	6	3
S. aureus mastitis and latent infection	52	4	8	12
S. epidermidis mastitis and latent infection	3	16	10	9
Normal milk	32	71	75	74

* Examined 1 to 9 months after the first examination. Diagnoses other than those listed were excluded.

every 4 cows with S. epidermidis mastitis had no infection or cytological reaction of the same quarter when examined 1 to 9 months after the subclinical mastitis.

DISCUSSION

In contrast to S. aureus in which nuclease production is a general characteristic, less than half of the examined bovine S. epidermidis strains excreted a nuclease when grown on a solid or in a liquid medium. (This compares with the 34 % of nuclease positive bovine S. epidermidis strains found by *Holmberg* 1973). Furthermore, great variation in the quantity of nuclease produced by different S. epidermidis strains seemed to be characteristic.

Previous reports on the heat stability of S. epidermidis nuclease are conflicting. Whereas *Lachica et al.* (1971b) found the nuclease of S. epidermidis to be heat-sensitive, the existence of a heat-stable nuclease of S. epidermidis strains isolated from man as well as animals was subsequently demonstrated by *Devriese & Oeding* (1975) and *Dornbusch et al.* (1976). In the present study the nuclease of S. epidermidis strains isolated from bovine milk was found to be heat-stable. The average D-value of S. epidermidis nuclease at 120°C of 19 min was slightly lower than the corresponding value found for S. aureus nuclease which was 26 min in this study and 34 min in the study presented by *Erickson & Deibel* (1973a).

The lack of nuclease activity after heating the broth cultures of 14 strains does not provide a basis for asserting that the nuclease of these strains was heat-sensitive. It is more likely to be due to weak production of nuclease by these strains, the heat treatment thus reducing the activity to below the limit of detection.

There may be several reasons to explain the contradictory results obtained regarding the heat resistance of S. epidermidis nuclease. In addition to strains isolated from different animal species having deviating properties, the use, described in most papers, of different methods for the demonstration of nuclease production and for testing of nuclease activity after heat treatment may constitute a source of error. Furthermore, previous investigations failed to include an examination of the concentration of the nuclease or a quantitative estimate of heat stability such as the D-values.

The finding that nuclease production was most abundant in strains belonging to subgroup III and other phosphatase positive and acetoin negative strains (subgroups c and e) is in agreement with the results of *Holmberg* that nuclease positive strains occur more frequently in these subgroups. Production of a heat stable nuclease was also a key characteristic for 1 of the subspecies of Staphylococcus hyicus (*Devriese et al.* 1978) which seems to correspond biochemically with subgroup III (biotype 2) of S. epidermidis (*Baird-Parker* 1974).

The nuclease of bovine strains of S. aureus and S. epidermidis was found to be serologically different. This observation is in accordance with the results of *Gudding* (1980b) who found no correlation between the titres of antinucleases against S. epidermidis and S. aureus.

As the coagulase test alone may sometimes be inadequate to distinguish S. aureus from other staphylococci, there is a need for supplementary tests. In addition to the nuclease test, serological identification of biocatalysts such as nuclease or proteinase (*Sandvik & Fossum* 1965) provides a simple system for the reliable differentiation of S. aureus and S. epidermidis.

In contrast to the results of *Sandvik & Fossum, Brown et al.* (1967) and *Brown & Scherer* (1978), it was not possible to further classify S. epidermidis on an enzymo-serological basis as the nucleases investigated in the present study proved to be serologically identical or closely related. However, the selection of strains for immunization was based on the quantity of nuclease excreted and not on biochemical characteristics, and all sera were produced against phosphatase positive and acetoin negative strains. According to *Brown & Scherer*, most phosphatase positive and acetoin negative strains belong to group B in the serological system, a factor which indicates that bovine strains of subgroup III, c, d and e are similar.

The data concerning recovery rate after a previous subclinical S. aureus or S. epidermidis mastitis provide numerical expression for the difference in the virulence of these 2 staphylococcal species. However, there is no indication from the results shown in Table 3 that the nuclease contributes to the udder pathogenicity of S. epidermidis. Though this observation is not in contradiction with the conclusion of *Gudding* (1980a) on the significance of the S. aureus nuclease for udder pathogenicity, it does provide another indication that the S. aureus nuclease exerts its effect only after prior action of other extracellular substances.

In conclusion, it may be stated that the value of S. epidermidis nuclease determinations in mastitis bacteriology is primarily related to its significance for classifying strains of staphylococci with different degrees of udder pathogenicity. In routine investigational work, the coagulase test should be supplemented by tests for nuclease production. Dubious strains may be successfully identified by enzymo-serological methods.

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SAMMENDRAG

Nuklease hos Staphylococcus epidermidis isolert fra mastittsekret. Produksjon og noen egenskaper.

Nuklease ble produsert av 47 % av stammene av Staphylococcus epidermidis isolert fra speneprøver hos ku. Det var en betydelig variasjon i mengden av enzym produsert hos forskjellige stammer. S. epidermidis-nukleasen viste stor varmestabilitet, idet D-verdien ved 120°C i gjennomsnitt ble beregnet til 19 minutter. Nukleasene produsert av S. epidermidis og S. aureus kunne identifiseres og adskilles ved hjelp av serologiske metoder.

S. epidermidis var mindre jurpatogen enn S. aureus. Det var imidlertid ingen forskjell mellom stammer av S. epidermidis med høy og lav in vitro nukleaseproduksjon.

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