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NUCLEASES OF SOME UDDER PATHOGENIC ORGANISMS

IN VIVO AND IN VITRO PRODUCTION

By

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GUDDING, R.: *Nucleases of some udder pathogenic organisms. In vivo and in vitro production.* Acta vet. scand. 1980, *21*, 256—266. — The nuclease activity in quarter milk samples from cows with mastitis, and in vitro nuclease production by some udder pathogenic microorganisms were tested. Results concerning Staphylococcus aureus are, however, dealt with in a separate paper. Streptococcus dysgalactiae was found to be a vigorous and frequent producer of nuclease both in vivo and in vitro. The production by S. agalactiae was quantitatively inferior to that by S. dysgalactiae. Nearly half of the S. epidermidis strains were found to excrete a nuclease, and this enzyme was also demonstrated in the milk of cows with S. epidermidis mastitis.

Comparatively high nuclease activity was recorded in the udder secretions of some cows with summer mastitis. All the tested strains of Corynebacterium pyogenes were nuclease positive when tested by agar plate methods.

nuclease; udder pathogenic microorganisms; milk.

Enzymes which hydrolyse DNA (nucleases, DNases) are produced by a variety of microorganisms (*Lehman* 1971), some of which are associated with bovine mastitis. In addition, nucleases are biological catalysts which are normally present in tissues and biological fluids (*Bernandi* 1971, *Gudding* 1979 b).

The Staphylococcus aureus nuclease has been demonstrated in quarter milk samples from cows with acute and chronic mastitis (*Gudding* 1976, 1980 a, b). The aim of the present work was to survey the occurrence of other nucleases in samples of normal and mastitic milk, and to study in vitro nuclease production by certain microorganisms associated with bovine mastitis.

MATERIALS AND METHODS

Samples

The milk samples originated from 1533 cows with clinical or subclinical mastitis. Of these, cows with S. aureus mastitis are dealt with separately (*Gudding* 1980 b). The present investigation also includes the testing of the in vitro nuclease production of 1070 strains of streptococci, 1871 strains of Staphylococcus epidermidis and 35 strains of various other microorganisms (Table 3).

Bacteriological investigations

Quarter milk samples were examined according to the methods presented by *Klastrup & Schmidt Madsen* (1974). The preliminary identification procedure for streptococci included the CAMP test and esculin hydrolysis test, the final diagnosis being based on a serological examination of esculin negative strains. The term "Streptococcus uberis" in the present paper comprises exclusively CAMP and esculin positive strains which do not react serologically with antisera against groups E and G. "Esculin positive streptococci" are in this paper a heterogenous group of streptococci generally believed to belong to the faecal, the viridans and the lactic streptococci.

Nuclease examinations

The determination of nuclease and heated nuclease (nuclease activity after heating the milk at 100°C) in the milk samples was performed as described by *Gudding* (1980 a).

The in vitro production of nuclease by the streptococcal strains was tested in a culture of Todd Hewitt broth incubated for 24 h at 37°C. The concentration of nuclease was measured in a Toluidine Blue DNA Agar (TDA) (*Lachica et al.* 1971), modified for streptococcal nucleases (*Gudding* 1979 a) before and after heating the broth culture at 100°C for 10 min. Nuclease production, except that of streptococci, was also examined on an "enriched" TDA as described by *Gudding* (1980 d) and on DNase Test Agar (Difco)*.

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

Data analyses

In the computerized data analyses, the concentrations of nuclease and heated nuclease were converted to square roots before calculation of the average values.

RESULTS

Streptococci

Enzymes which hydrolyzed DNA were sometimes detected in quarter milk samples from which streptococci were isolated. The frequency of demonstration and amounts of enzyme measured in the samples are presented in Table 1. Detectable amounts of the nuclease of Streptococcus agalactiae and S. dysgalactiae were found more frequently in heated than in non-heated milk samples. Nuclease activity was found infrequently in non-heated as well as heated samples of mastitic milk from which S. uberis

Table 1. Occurrence of nuclease and heated nuclease in quarter milk samples from mastitic cows.

Microorganism	Number of samples	Nuclease			Heated nuclease		
		percentage of positive samples	concentration of nuclease* (diffusion units per 0.1 ml)		percentage of positive samples	concentration of nuclease* (diffusion units per 0.1 ml)	
			average of positive samples	average of all samples		average of positive samples	average of all samples
Streptococcus agalactiae	26	15	484	11	35	210	12
Streptococcus dysgalactiae	104	13	571	9	44	249	9
Streptococcus zooepidemicus	2	100	93	93	50	10	5
Streptococcus group G	2	0			50	9	5
Streptococcus group L	5	40	90	40	60	74	42
Streptococcus uberis	29	3	9	1	3	6	1
"Esculin positive streptococci"	136	2	11	1	2	12	1
Staphylococcus epidermidis	193	10	530	6	21	1256	100
Escherichia coli	173	18	20	1	3	23	1
Coliform bacteria	10	10	24	1	10	6	1
Corynebacterium pyogenes	31	13	295	5	26	240	17
Bacillus cereus	4	25	10	1	0		
Other Bacillus sp.	15	20	7	1	7	36	1
Clostridium perfringens	1	0			0		
Pseudomonas aeruginosa	1	0			0		
Yeast and mould	2	0			0		

* The figures were converted to square roots before calculation of averages. This applies also to Table 2.

and "esculin positive streptococci" were isolated. As seen from Table 1 the concentration of nuclease in the few positive samples was low.

S. epidermidis

Nuclease and heated nuclease were demonstrated in respectively 10 % and 21 % of the quarter milk samples from which S. epidermidis were isolated (Table 1). The concentration of the enzyme in positive samples was generally higher than that found for other nucleases, except the S. aureus nuclease (*Gudding* 1980 b).

Escherichia coli and other enterobacteria

Nuclease was detected in 18 % of the quarter milk samples from cows with an E. coli mastitis (Table 1). In contrast to staphylococci and most streptococci, the nuclease activity in these cases was demonstrated more frequently in the milk samples which were not heated than in those which were. The nuclease activity displayed by the positive samples was generally very moderate (Table 1).

Corynebacterium pyogenes

Heated nuclease was present in 26 % of the samples from the affected quarters of cows with C. pyogenes mastitis. Concentrations of enzyme in the samples were comparatively high (Table 1).

Bacilli, clostridia, pseudomonades, yeasts and moulds

Nuclease activity was not detected in udder secretions from 4 cows with mastitis caused by respectively Clostridium perfringens, Pseudomonas aeruginosa, a yeast species and a mould species. A few of the samples from quarters with mastitis caused by bacilli were nuclease positive. However, the nuclease activity recorded was generally moderate.

Age and stage of lactation of affected cows and severity of the mastitis

No characteristic trend was observed concerning the distribution of affected cows, or the occurrence of nuclease or heated

nuclease in the milk, in relation to the age of the cow or the stage of lactation at the onset of the mastitis.

No statistically significant differences were found in the concentrations of nuclease and heated nuclease between cows with chronic, subacute and acute mastitis. However, cows with summer mastitis seemed to represent an exception, as no nuclease activity was demonstrated in the udder secretions of any of the cows with "chronic summer mastitis".

In vitro production of nuclease

As seen in Table 2, most strains of S. dysgalactiae produced a nuclease, the in vitro excretion of which was vigorous. In contrast, S. uberis and the heterogenous group "esculin positive streptococci" generally did not display nuclease activity.

Table 2. In vitro nuclease production by streptococci isolated from milk samples.

Microorganism	Number of strains	Percentage of positive strains	Concentration of nuclease (diffusion units per 0.1 ml) average of positive strains	Concentration of nuclease (diffusion units per 0.1 ml) average of all strains
Streptococcus agalactiae	180	77	171	127
Streptococcus dysgalactiae	266	97	3946	3765
Streptococcus zooepidemicus	7	57	18	6
Streptococcus group G	38	13	34	4
Streptococcus group L	31	90	48	39
Streptococcus uberis	438	3	6	1
"Esculin positive streptococci"	102	2	8	1
"Esculin negative streptococci"*	8	88	188	145

* Not groups B, C, G or L.

After heating on a boiling water-bath for 10 min, the nuclease activity shown by most streptococci decreased by approx. 10 %. The nucleases produced by strains of S. zooepidemicus were generally less thermostable than those produced by other streptococci.

When examined on a solid test medium (DNase Test Agar), 47 % of the 1871 strains of S. epidermidis examined were found to produce a nuclease. Nearly all positive strains also produced the enzyme when cultivated in a meat extract broth. The nu-

clease of S. epidermidis was generally found to be heat-stable (*Gudding* 1980 d).

Nuclease production by a selection of possible udder pathogenic microorganisms is presented in Table 3. There was conformity between the results obtained with the "enriched" TDA with those obtained with the DNase Test Agar. One of the nuclease producing strains of E. coli was haemolytic in bovine blood agar. The enzyme production of all these microorganisms, except Serratia marcescens, was found to be sparse. With the exception of S. marcescens, nuclease activity in broth cultures of the same organisms was either absent or insignificant.

Table 3. Production of nuclease by some udder pathogenic microorganisms on DNase Test Agar (Difco) and "enriched" TDA.

Organism	Number of strains	
	total	producing nuclease
Corynebacterium pyogenes	7	7
Corynebacterium ulcerans	1	1
Escherichia coli	10	2
Klebsiella pneumoniae	1	0
Proteus sp.	2	2
Serratia marcescens	2	2
Pseudomonas aeruginosa	4	4
Bacillus cereus	2	2
Bacillus subtilis	2	2
Clostridium perfringens	1	1
Nocardia asteroides	1	0
Yeast species	2	0

Non-mastitic milk

No nuclease activity was detected in the samples of cytologically normal milk from which no udder pathogenic organisms could be isolated. However, milk samples stored 3 to 5 days at room temperature and with a contaminant microflora, sometimes produced zones in the TDA.

Streptococci of groups B, C, G and L were isolated from 23 quarter samples of normal milk as judged by the CMT-score. Nuclease and heated nuclease could not be demonstrated in any of these samples.

Small amounts of nuclease were demonstrated in approx. 5 % of the quarter samples with a CMT-score of 1 or 2 and with S.

epidermidis as bacterial isolate, though this was only after the samples had been heated.

All 91 samples of normal milk with S. uberis, "esculin positive streptococci", and E. coli were nuclease negative.

Samples from cows in the dry period

Nuclease activity in TDA was demonstrated in non-mastitic quarter samples from 32 % of the cows which were in the dry period at the time of sampling. The average concentration of the positive samples was 18 diffusion units per 0.1 ml. After heating, no nuclease activity was recorded in any of the samples.

If 1 or more of the quarters of cows in the dry period had an udder inflammation, the absence of nuclease activity in the non-heated samples from the mastitic quarters was found to be a characteristic feature. This observation applied to the samples from 21 of 37 dry cows in which nuclease activity had been detected in the non-mastitic quarters, and did not seem to depend on the causative microorganism, 7 of the most common udder pathogenic microorganisms including S. aureus, being isolated from the samples.

DISCUSSION

Among the microorganisms causing mastitis, S. aureus has been found to be a vigorous producer of nuclease both in vitro and in vivo (*Gudding* 1980 a, b). However, as shown in the present study, nucleases can also be demonstrated in milk samples from which mastitis organisms other than S. aureus are isolated, though frequency of occurrence and amount vary.

S. dysgalactiae seems to exhibit similarities with S. aureus regarding the production of the nuclease and the occurrence of the nuclease and heated nuclease in quarter milk samples. Nearly all strains of S. dysgalactiae produce nuclease. The enzyme is found in quarters with mastitis caused by the organism although the demonstration of the S. dysgalactiae nuclease is less frequent than that of S. aureus (*Gudding* 1980 b). In vitro and in vivo production is vigorous compared with that of other streptococci, though inferior to that of S. aureus. As with S. aureus, the enzyme is demonstrated most frequently in heated samples. There are also antibodies against the nuclease of S. dysgalactiae which can neutralize the activity of the enzyme in the milk (*Gudding* 1980 c).

The in vitro production of nucleases by S. agalactiae is generally sparse and infrequent as compared with that of S. dysgalactiae. However, nuclease excretion by these streptococcal species during growth in the inflamed udder seems to be qualitatively and quantitatively similar. The nuclease and heated nuclease examinations were performed with a TDA originally described for staphylococcal nuclease. This agar is less suitable for the demonstration of the nuclease of S. agalactiae than for that of S. dysgalactiae (*Gudding* 1979 a).

The data for S. zooepidemicus and the streptococci of groups G and L are based on only a few observations and should thus be assessed critically. The nuclease of S. zooepidemicus has previously been found to be heat-labile (*Gudding* 1979 a), and this property was confirmed for 1 of the strains. The demonstration of nuclease activity after heating in 1 of the samples from which S. zooepidemicus was isolated may indicate that the property of heat sensitivity does not apply to all strains of this organism.

The scheme for routine examination of streptococci in the author's laboratory includes the CAMP-test, the test for esculin hydrolysis and serological grouping, primarily of esculin negative strains. A general feature of streptococci which hydrolyse esculin seems to be that they do not produce a nuclease neither in vivo nor in vitro (Table 1 and 2). The few strains showing nuclease activity excrete only small quantities.

S. uberis and "esculin positive streptococci" are normal inhabitants of various organs of the cow, such as the alimentary tract and the skin, and are also sometimes isolated from udder inflammations. However, the udder pathogenicity of these organisms is less marked than that of S. agalactiae or S. dysgalactiae, which, together with S. aureus, are considered to be the most important organisms in the aetiology of bovine mastitis. It is noticeable that the demarcation between groups of organisms with different degrees of udder pathogenicity coincides with the character of nuclease production. The results of the present study may indicate that the nuclease may contribute to the udder pathogenic properties of S. dysgalactiae and possibly also of S. agalactiae.

In a comprehensive study of S. epidermidis, *Holmberg* (1973) found that 34 % of the strains produced a nuclease. The corresponding figure in the present study was 47 %. The S. epidermidis nuclease may appear in fairly high concentrations in milk

samples. Though this enzyme may represent a diagnostic problem versus the S. aureus nuclease, the 2 enzymes can be separated enzymo-serologically (*Gudding* 1980 d).

The observation that, when applied in wells in TDA, neither broth cultures of E. coli nor udder secretions from cows with E. coli mastitis produce zones in the TDA or only produce zones of a moderate size, seems to disagree with accepted findings (*Lehman* 1971). The pH and the composition of TDA may partially provide an explanation for this discrepancy. However, it may also be explained by differences in DNA cleavage. According to *Lehman*, E. coli produces several nucleases, primarily exonucleases, though endonucleases are also produced. As visual determination of nuclease activity in the TDA is based on metachromasia, it seems logical that an exonucleolytic attack, producing mononucleotides, does not lead to a change in the wavelength with maximal absorption, such a change being the biophysical basis of metachromasia. According to *Lehman*, E. coli endonuclease I also promotes the liberation of small obligonucleotides, presumably by an exonucleolytic mode of attack.

The small pink zones measured when examining 18 % of the cows with E. coli mastitis were most probably a result of DNA cleavage. A characteristic of the nuclease of E. coli seems to be heat sensitivity, in contrast to most other bacterial nucleases.

Previous papers have reported the excretion of nuclease by P. aeruginosa (*Streitfeld et al.* 1962), Bacillus subtilis (*Birnboim* 1966), S. marcescens (*Schreier* 1969), C. perfringens (*Porschen & Sonntag* 1974) and by certain yeast species (*Cazin jr. et al.* 1969). The production of an extracellular nuclease by C. pyogenes seems, however, not to have been reported previously. Although the origin of the nuclease activity demonstrated in the udder secretions of cows with "summer mastitis" has not been substantiated, it is presumably due to a C. pyogenes nuclease.

In spite of the inhibition of bacterial growth, especially of Gram positive organisms, the "enriched" TDA was found to be suitable for the direct demonstration of nuclease production by most microorganisms, except streptococci. The inhibitory effect of Toluidine Blue, can be minimised by a heavy inoculate.

In the present study nucleases were demonstrated with varying frequency in the udder secretions of cows with mastitis caused by different udder pathogenic microorganisms. However, in normal milk samples, with and without the same micro-

organisms, these enzymes were generally not demonstrated. The quarter samples of cows in the dry period represented an exception, but the normally occurring nucleases in these samples were easily distinguished from most other bacterial nucleases, primarily by their heat sensitivity.

REFERENCES

Bernandi, G.: Spleen acid deoxyribonuclease. *In* Boyer, P. D. (ed.): The Enzymes. Acad. Press, New York and London 1971, *4*, 271—287.

Birnboim, H. C.: Cellular site in Bacillus subtilis of a nuclease which preferentially degrades single-stranded nucleic acids. J. Bact. 1966, *91*, 1004—1011.

Cazin jr., J., T. R. Kozel, D. M. Lupan & W. R. Burt: Extracellular deoxyribonuclease production by yeasts. J. Bact. 1969, *100*, 760—762.

Gudding, R.: Heat stable nuclease in mastitic milk. Acta vet. scand. 1976, *17*, 501—502.

Gudding, R.: The demonstration and characterization of deoxyribonucleases of streptococci group A, B, C, G and L. Acta vet. scand. 1979 a, *20*, 102—121.

Gudding, R.: DNases in milk and blood sera from different species. Acta vet. scand. 1979 b, *20*, 404—416.

Gudding, R.: Staphylococcal nuclease in udder secretions of cows with acute mastitis. Acta vet. scand. 1980 a, *21*, 79—95.

Gudding, R.: Measurement of Staphylococcus aureus nuclease and antinucleases. Applicability for the assessment of mastitic milk. Acta vet. scand. 1980 b, *21*, 229—241.

Gudding, R.: Antibodies against staphylococcal and streptococcal nucleases in bovine blood serum and milk. Acta vet. scand. 1980 c, *21*, 242—255.

Gudding, R.: Nuclease of Staphylococcus epidermidis isolated from mastitic milk. Production and some properties. Acta vet. scand. 1980 d, *21*, 267—277.

Holmberg, O.: Staphylococcus epidermidis isolated from bovine milk. Acta vet. scand. 1973, Suppl. *45*, 1—144.

Klastrup, O. & P. Schmidt Madsen: Nordiske rekommendationer vedrørende mastitisundersøgelser af kirtelprøver. (Nordic recommendations concerning mastitis examinations of quarter samples). Nord. Vet.-Med. 1974, *26*, 197—204.

Lachica, R. V. F., C. Genigeorgis & P. D. Hoeprich: Metachromatic agar-diffusion methods for detecting staphylococcal nuclease activity. Appl. Microbiol. 1971, *21*, 585—587.

Lehman, I. R.: Bacterial deoxyribonucleases. *In* Boyer P. D. (ed.): The Enzymes. Acad. Press, New York and London 1971, *4*, 251—270.

Porschen, R. K. & S. Sonntag: Extracellular deoxyribonuclease production by anaerobic bacteria. Appl. Microbiol. 1974, *27*, 1031—1033.

Schreier, J. B.: Modification of deoxyribonuclease test medium for rapid identification of Serratia marcescens. Amer. J. clin. Path. 1969, *51*, 711—716.

Streitfeld, M. M., E. M. Hoffmann & H. M. Janklow: Evaluation of extracellular deoxyribonuclease activity in Pseudomonas. J. Bact. 1962, *84*, 77—80.

SAMMENDRAG

In vivo og in vitro nukleaseproduksjon hos enkelte jurpatogene mikroorganismer.

Innholdet av nuklease i kjertelprøver fra kyr med mastitt og in vitro produksjon av nuklease hos enkelte mastittbakterier er blitt undersøkt. Resultater vedrørende Staphylococcus aureus er imidlertid omtalt i en egen artikkel. De fleste stammene av Streptococcus dysgalactiae produserte nuklease in vivo og in vitro og produksjonen av enzymet hos denne bakterien var generelt sterkere enn hos de fleste andre av de undersøkte bakteriene. Streptococcus agalactiae produserte mindre nuklease enn S. dysgalactiae. Nesten halvparten av S. epidermidis-stammene var nuklease positive og dette enzymet ble også påvist i melk fra enkelte kyr med mastitt forårsaket av S. epidermidis.

I jursekret fra enkelte kyr med sommermastitt ble det funnet relativt høy nukleaseaktivitet. Alle de undersøkte stammene av Corynebacterium pyogenes produserte nuklease.

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