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MEASUREMENT OF STAPHYLOCOCCUS AUREUS NUCLEASE AND ANTINUCLEASES

APPLICABILITY FOR THE ASSESSMENT OF MASTITIC MILK

By

Roar Gudding

GUDDING, R.: *Measurement of Staphylococcus aureus nuclease and antinucleases. Applicability for the assessment of mastitic milk.* Acta vet. scand. 1980, *21*, 229—241. — The concentration of the nuclease produced by Staphylococcus aureus and titres of antinucleases were determined in the udder secretions of 539 cows, most of them with clinical S. aureus mastitis. On average, the nuclease was detected in 85 % of the samples from mastitic quarters. In milk from most cows, antinucleases neutralize the activity of the nuclease, and a successful demonstration of this thermostable enzyme presupposed the inactivation of antibodies by heating the samples on boiling waterbath. The nuclease was demonstrated most frequently, and at highest concentration, in samples from cows with a severe mastitis and from cows which did not recover completely.

The test for nuclease, performed on both non-heated and heated quarter samples, and the antinuclease test, may supplement conventional laboratory methods. The advantages and limitations of the methods in the diagnosis of mastitis are discussed.

bovine mastitis; nuclease; heated nuclease; antinucleases.

Various products are excreted during the growth of Staphylococcus aureus in vitro and in vivo, among these the staphylococcal nuclease (DNase). The interest for this enzyme is related both to its diagnostic value and its role in microbial pathogenicity. Using the Toluidine Blue DNA Agar (TDA) described by *Lachica et al.* (1971) the nuclease has been demonstrated in quarter milk samples from cows with acute and chronic mastitis caused by S. aureus (*Gudding* 1976, 1980 a).

The nuclease acts as an antigen, stimulating the production of neutralizing antibodies (antinucleases) which have even been demonstrated in bovine milk (*Sandvik* 1975).

The aim of the present work was to study the diagnostic value of the determination of S. aureus nuclease and antinucleases in bovine milk. This was done by quantitative analyses of the nuclease and its neutralizing antibodies in quarter milk samples of cows with S. aureus udder infection or mastitis. For a valid assessment of the reliability of the test for nuclease in mastitic milk, measurements of bovine serum albumin in milk and the in vitro nuclease production of S. aureus in milk have been included in the presentation.

MATERIALS AND METHODS

Samples

The material comprised quarter milk samples from 1533 cows examined at the National Veterinary Institute during a period of about 2 years. A great majority of the samples originated from cows with a clinical mastitis. Milk samples from the affected quarters of 151 cows with a subclinical mastitis, 74 of which had S. aureus mastitis, were also included. Information concerning the udder health status of 188 cows before and/or after clinical mastitis was also available.

Bacteriological and cytological examinations

Routine examination of milk samples and interpretation of the results was carried out as described by *Klastrup & Schmidt Madsen* (1974).

Examination of nuclease and antinucleases

The concentration of nuclease and heated nuclease (nuclease activity determined after heating the milk at 100°C) in the quarter milk samples was determined in TDA (*Gudding* 1980 a). The antinuclease test was used for the quantification of antibodies against S. aureus nuclease in the milk samples (*Gudding* 1977).

The excretion of nuclease during growth on a solid medium was tested by subinoculation from blood agar onto DNase Test Agar (Difco)* and on an "enriched" TDA (*Gudding* 1980 c). Quantitative examination of nuclease production was performed by cultivation in nutrient broth for 24 h. The amount of nuclease

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

was determined in TDA before and after heating the broth culture on a boiling water-bath for 10 min.

Determination of bovine serum albumin (BSA) in milk

The concentration of BSA in the quarter samples of 140 mastitic cows was tested using a Mancini technique (*Giesecke & Viljoen* 1974). Based on preliminary tests, the threshold value for mastitis was changed from a zone diameter of 8 mm (*Giesecke & Viljoen*) to 9 mm. The results of the examination of samples from 321 quarters taken during the period 10 to 250 days after parturition are included in the presentation.

In vitro nuclease production at various temperatures

Strains of S. aureus were inoculated into tubes containing nutrient broth, autoclaved skimmed milk and untreated milk originating from 3 healthy cows. The titre of antinucleases in the milk from 2 of these cows was 7 and 35 diffusion units per 0.1 ml, respectively. Antibodies against S. aureus nuclease could not be demonstrated in milk from the third cow. The milk was collected after disinfection of the teat orifice and after discarding the foremilk. It was immediately cooled to 4°C and inoculated, within 3 h after sampling, with S. aureus to a final concentration of approx. 10,000 colony forming units per ml. Milk from 1 additional cow with subclinical S. aureus mastitis was also included, but with no supplementary inoculation. The tubes were incubated at 4°C, 10°C, 15°C, 20°C, 25°C, 30°C and 37°C for up to 72 h.

Data analyses

The levels of nuclease, heated nuclease and antinucleases in milk were converted to square roots before computer calculation of averages. As regards nuclease and heated nuclease, quarters showing the highest levels were used for calculations of the average concentrations. For antinucleases, those showing the lowest levels were used for the calculation of the average titre.

RESULTS

Prevalence of S. aureus

S. aureus was isolated from samples from 1191 quarters collected from 709 cows, 833 of these quarters (from 643 cows)

being given the diagnosis mastitis (S. aureus mastitis) on the basis of the CMT-test. After the exclusion of cows with mixed infections, data from 539 cows with S. aureus mastitis in 1 or more quarters (CMT $\geq$ 3) and 21 cows with S. aureus latent infection (CMT $\leq$ 2) were used for further calculations.

Occurrence of nuclease

As seen in Table 1, nuclease activity could be detected in the heated samples from 85 % of the cows with S. aureus mastitis. The concentration of heated nuclease and the percentage of positive samples increased with the severity of the mastitis. The concentration of heated nuclease given in diffusion units per 0.1 ml and the percentage of cows with positive samples were as follows: Mild or chronic mastitis, 1184/82; subacute mastitis, 3582/85; acute mastitis, 5526/92 and peracute mastitis, 24149/100.

Table 1. Nuclease, heated nuclease and antinucleases in quarter samples.

Diagnosis	Number of cows	Nuclease		Heated nuclease		Anti-nucleases*
		positive samples (percentage)	average concentration**	positive samples (percentage)	average concentration**	average titre**
Staphylococcus aureus mastitis	539	29	186	85	2481	5.3
Staphylococcus aureus latent infection	21	13	7	28	153	4.8

* Cows in the period 10 to 250 days after parturition.
** Diffusion units per 0.1 ml. The figures were converted to square roots before calculation of averages.

The enzyme was also demonstrated in 58 % of the quarter samples from cows with subclinical S. aureus mastitis. However, the concentration of the enzyme in these samples was generally low, being on average 75 diffusion units per 0.1 ml.

Testing for heated nuclease in samples from 28 cows with S. aureus mastitis in the dry period or shortly after parturition was partly impeded due to coagulation of the udder secretions.

However, nuclease activity was detected in clots from 16 of the 20 cows which were examined.

Samples from 11 of 198 cows with a non-specific mastitis (CMT $\geq$ 3, and no microorganisms isolated) were positive for heated nuclease. In 4 of these samples, antibacterial substances identified as penicillin were demonstrated. The nuclease of some of these samples, with and without penicillin, was identified enzymo-serologically as S. aureus nuclease.

Levels of heated nuclease found in cows with clinical mastitis are presented in Table 2, according to previous and subsequent udder health status. Heated nuclease was found in the highest concentrations and most frequently in quarter samples from cows which proved to have S. aureus mastitis in the identical quarter upon laboratory examination 30 to 240 days after the clinical mastitis. The concentration of heated nuclease in these cows was significantly higher than in cows which had recovered completely. The average concentration of heated nuclease was also found to be high at the time of the clinical mastitis, 5461 diffusion units per 0.1 ml, in quarter samples from 15 cows with non-specific mastitis following clinical mastitis.

Level of antinucleases

The average titres of antinucleases in cows with S. aureus mastitis or latent infection are presented in Table 1. Cows with a previous S. aureus mastitis were generally found to have a higher titre of antinucleases than cows with no recorded mastitis before the clinical mastitis in question (Table 2).

Altogether 69 % of the cows with clinical S. aureus mastitis had a higher antinuclease titre in the udder secretions from the affected quarter than in those from the healthy quarters. However, 9 % of the cows had the lowest antinuclease titre in milk from the affected quarter. The concentration of heated nuclease in the milk from these cows was high in most cases.

Bovine serum albumin

When subdividing the samples into 4 groups according to the CMT-score (<3 versus ≥ 3) and the level of BSA (<9 mm versus ≥ 9 mm), 65 % of the samples were negative (low CMT and BSA), 11 % had an irrelevant or relevant teat canal infection (high CMT and low BSA) and 24 % had mastitis (high BSA) of

Table 2. Heated nuclease and antinucleases in cows with clinical Staphylococcus aureus mastitis in relation to former and subsequent mastitis history*.

Laboratory diagnosis of identical quarter 0 to 240 days before the clinical mastitis	Clinical mastitis				Laboratory diagnosis of identical quarter 30 to 240 days after the clinical mastitis
	number of cows	heated nuclease		antinucleases titre**	
		positive samples (percentage)	concentration**		
S. aureus mastitis	32	81	1266	8.1	Unknown
S. aureus mastitis	14	93	1102	9.5	S. aureus mastitis
S. aureus mastitis	5	60	125	0.5	No mastitis (all quarters)
No mastitis (all quarters)	79	77	2052	3.2	Unknown
No mastitis (all quarters)	19	89	4870	3.6	S. aureus mastitis
No mastitis (all quarters)	29	66	289	4.8	No mastitis (all quarters)
Unknown	58	91	3214	4.6	S. aureus mastitis
Unknown	50	70	262	4.2	No mastitis (all quarters)

* Cows with diagnoses other than "S. aureus mastitis of identical quarter" and "no mastitis" are excluded.

** Concentrations of heated nuclease and antinuclease titres are given in diffusion units per 0.1 ml.
The figures were converted to square roots before calculation of averages.

which 4 % with a low CMT-value. Heated nuclease was demonstrated in 30 of the samples, all of which were mastitis positive according to the criteria of *Giesecke & Viljoen* (1974).

S. aureus was isolated from 50 quarter milk samples which were distributed as shown in Table 3. In the samples from quarters with a relevant teat canal infection (CMT $\geq$ 3 and BSA

Table 3. Distribution of heated nuclease in relation to the CMT-score and the level of bovine serum albumin in 50 quarter samples from cows with a Staphylococcus aureus mastitis or latent infection*.

CMT-score	Concentration of bovine serum albumin (zone diameter)			
	< 9 mm		$\geq$ 9 mm	
	number of quarters	number with heated nuclease	number of quarters	number with heated nuclease
1	9	0	0	0
2	1	0	2	2
3	4	0	5	4
4	4	0	11	10
5	0	0	14	13

* Cows in the period 10 to 250 days after parturition.

< 9 mm) no heated nuclease was detected. However, nuclease activity was measured in 29 of 32 samples which were considered to be mastitic according to the BSA-level. The amount of BSA in the quarter milk samples was found to be correlated ($P < 0.001$) to the CMT-values ($r = 0.45$), the concentration of heated nuclease ($r = 0.28$) and the titre of antinucleases ($r = 0.24$), respectively.

In vitro nuclease production

All 222 strains of coagulase positive staphylococci tested produced a nuclease when grown in nutrient broth, on DNase Test Agar, or on "enriched" TDA. However, successful demonstration using the latter medium presupposed that a large colony mass was streaked on the agar. The nuclease of all S. aureus strains was heat stable, and in vitro nuclease production was generally abundant. The nuclease activity of S. aureus was generally seen in the "enriched" TDA after incubation at 37°C for 5 to 6 h.

Growth and nuclease production of S. aureus at different temperatures

The number of colony forming units remained unchanged in the tubes incubated at 4°C for 72 h. During incubation at 10°C a very slight growth was observed and at 15°C an increase in the number of organisms of approx. 10 times was recorded after 24 h. The growth at all temperatures was less vigorous in the tubes with unsterilized milk as compared with the autoclaved substrates.

Nuclease production at different temperatures is shown in Fig. 1. Nuclease was not demonstrated in tubes incubated at 4°C and 10°C. The nuclease concentrations in laboratory media were generally higher than those in the milk samples. There was no difference in the production of nuclease in milk with different antinuclease titres. However, in milk with antibodies against S. aureus nuclease, the enzyme was neutralized by these antibodies and heating was necessary for successful demonstration.

Neutralization of heated nuclease

The heated nuclease in milk samples was neutralized by the addition of milk containing S. aureus antinucleases from identical or different quarters or cows, or by the addition of rabbit serum

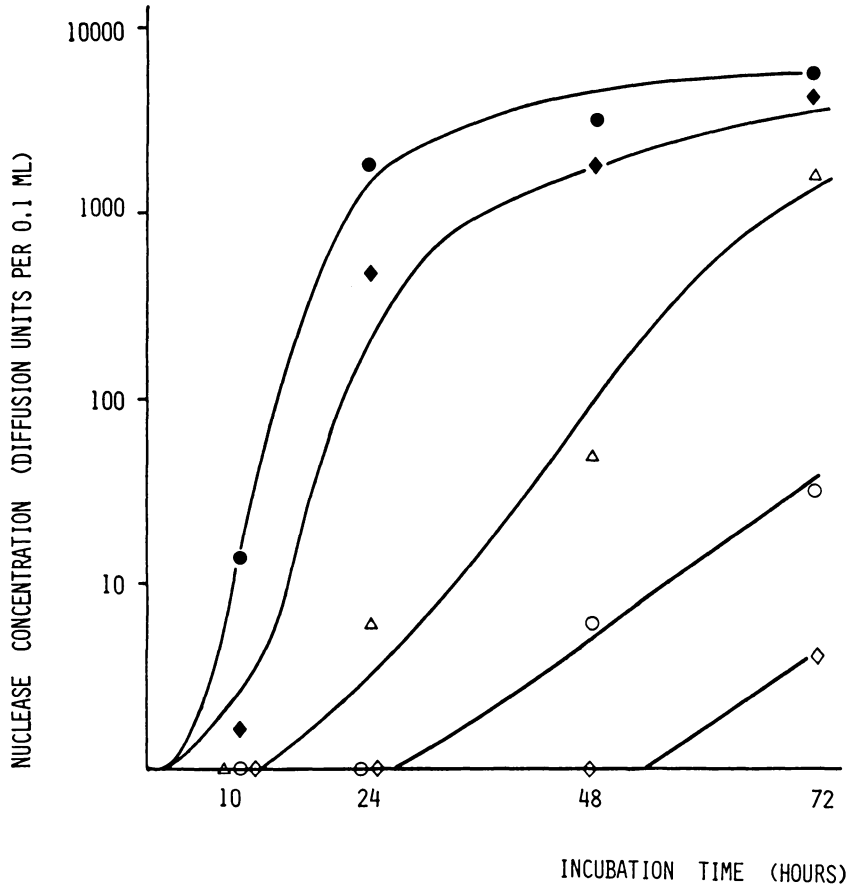

Figure 1. Production of Staphylococcus aureus nuclease in unsterilized milk samples incubated at 37°C ●——●, 30°C ◆——◆, 25°C △——△, 20°C ○——○ and 15°C ◊——◊.

containing specific antibodies against the S. aureus nuclease. After subsequent heating, the nuclease activity of the samples reappeared.

Alternative to heat treatment of the milk

A successful disruption of the linkage between the nuclease and its neutralizing antibodies was performed by heating the milk to minimum 80°C. Addition of various concentrations of sodium chloride, urea, mercaptoethanol and acids did not release the nuclease in such a way that it could be demonstrated by a subsequent examination in the TDA.

DISCUSSION

A bacteriological mastitis diagnosis is generally based on the isolation of a causative organism in samples from infected quarters, a final diagnosis being based on biochemical and/or serological characteristics. When diagnosing S. aureus, the nuclease, in addition to the coagulase, has been assigned importance as a diagnostic criterion. Using TDA, the demonstration of the staphylococcal nuclease in broth cultures has been simplified.

In the present study, the staphylococcal nuclease was demonstrated in quarter samples from cows with mastitis caused by S. aureus. The basis for a general detection of the enzyme in mastitic milk is the heat stability of the enzyme. In most milk samples antibodies neutralize the activity of the nuclease. However, due to the resistance of the nuclease, these antibodies can be inactivated by heat without affecting the enzyme activity.

The presence of detectable amounts of the enzyme in udder secretions presupposes a heavy growth of the microorganism, and even a certain distribution of the microbial activity in the udder tissues. The demonstration of the heated nuclease should consequently indicate an inflammation of the quarter or a large part of it. There seems to be some disagreement between this statement and the data presented in Table 1 showing 85 % and 28 % positive samples of heated nuclease in mastitic and normal milk with S. aureus, respectively. However, the methods used for diagnosing mastitis seem to provide some explanation for this discrepancy. A diagnosis of mastitis is generally based on the cytological reaction seen when applying the CMT-test. By means of the additional BSA-examinations as presented in Table 3, the heated nuclease was merely demonstrated in milk samples considered to be mastitic according to the BSA-level, including 2 samples with a latent infection according to the CMT-test. Nuclease activity was not recorded in any of the 8 samples diagnosed as relevant teat canal infections based on the CMT-score and BSA-level. In addition to information about the validity of the test for heated nuclease in quarter milk samples, these observations support the conclusion of *Giesecke & Van den Heever* (1974) concerning the limitations of cell count in the accurate detection of inflammation in the bovine udder.

The enzyme was present in detectable amounts in subclinical as well as clinical udder inflammations. However, both the con-

centration of the enzyme and the frequency of positive samples increased with increasing severity of the mastitis.

The amount of nuclease in quarter milk samples and the frequency of its demonstration were also related to the previous and subsequent udder health status, as positive samples were found in the highest concentrations and most frequently in udder secretions from cows with a subsequent S. aureus mastitis or non-specific mastitis of the identical quarter. In contrast, approximately every third cow in which the nuclease could not be demonstrated recovered completely after the clinical mastitis. Thus, the presence or absence of heated nuclease in quarter samples also provides some prognostic information. The results shown in Table 2 are also in conformity with the conclusion of *Gudding* (1980a) regarding the significance of S. aureus nuclease for the pathogenicity of the organism.

The test for heated nuclease in mastitic milk may be especially useful in certain situations. Due to antibacterial activity in the milk, the isolation of the causative microorganism may be unsuccessful and what is in fact S. aureus mastitis may therefore sometimes be erroneously diagnosed as non-specific mastitis. Such misdiagnosis can be prevented by the demonstration and identification of metabolites such as the nuclease.

The CMT-test and the BSA-test are only applicable for the examination of milk from cows in the main period of the lactation. This is not so as regards the detection of heated nuclease which can be used in the diagnosis of S. aureus mastitis in cows outside the main period of lactation. Despite clot formation, it is possible to detect the heated nuclease of S. aureus even in samples of colostrum or udder secretions from the dry period.

In the present study, nuclease production was found to be an invariable property of S. aureus. Though nuclease negative strains of S. aureus certainly do exist, they seem to be exceptions which constitute no problem in the practical bacteriological diagnosis of mastitis.

The transport of samples intended for microbiological examination should preferably take place under refrigeration. However, presupposing a transport time of less than 24 h and temperatures of 15°C (maximum 20°C) the excretion of the nuclease is so insignificant that "false positive" results do not occur.

Antibodies in the bovine milk seem generally to result from an "overflow" from the circulatory system into the udder tissue

alveoles. The size of the molecules and the permeability of the membranes thus determine the level of antibodies in the milk. In the mastitic quarter of a clinically affected cow, there is an elevated transfer of immunoglobulins into the udder secretions which can usually be demonstrated by the antinuclease test. However, in a subclinical mastitis the change of permeability due to tissue damage is insignificant and the slightly increased transfer of immunoglobulins from the blood into the milk is difficult to detect, in contrast to the situation with bovine serum albumin. Consequently, the antinuclease test, unlike the BSA-test of *Giesecke & Viljoen* (1974), is not applicable for the diagnosis of subclinical mastitis.

In nearly one third of the cows, antinuclease levels in the mastitic quarters were the same or lower than those in the healthy quarters. However, as some of the antinuclease molecules are bound to the S. aureus nuclease in the milk, the measureable amount of antinucleases in milk from cows with S. aureus mastitis actually represents the excess of "free" unbound antibodies.

The level of antinucleases in quarter milk samples from cows with clinical or subclinical mastitis may reflect former udder health status, and consequently give information which may be used prognostically. A high antinuclease titre is likely to be the result of a previous subclinical S. aureus mastitis (*Gudding* 1980 b). The data of the present work indicate that prognosis for these cows regarding complete recovery is dubious. Table 2 shows that the few cows with a former subclinical S. aureus mastitis, and which recovered after the clinical mastitis, had a low level of antinucleases in the milk.

The antinuclease titre in cows with mastitis differed only moderately from levels found in cows with latent infection (Table 1). There may be several explanations for the relatively high titres in cows with latent infection. The diagnosis is based on the CMT-test, and the possibility exists of misdiagnosing mastitis as a latent infection (*Gudding* 1980 b). Secondly, although the cytological picture after a previous S. aureus mastitis might have been restored, microorganisms may still continue to be shed. Finally, even non-mastitic cows may to some extent be exposed to various microbial antigens such as the S. aureus nuclease, and respond to this exposure with the production of antibodies.

The biological inhibitors which neutralize the nuclease in

milk have been found to be humoral antibodies, belonging to IgG (*Gudding* 1980 b). In the laboratory, heating the milk in order to inactivate the neutralizing antibodies is a convenient procedure. For a possible field application of the principle, a disruption of the nuclease-antinuclease linkage by chemical agents would be useful. However, attempts to achieve an alternative separation procedure were unsuccessful. The results of the present study are generally based on laboratory examinations and consequently give no answer to the question as to whether or not the neutralization of the nuclease occurs inside the mammary gland.

The demonstration of S. aureus nuclease in the TDA has several advantages as compared with conventional methods. The procedure is simple and the lapse of time before a preliminary or final result is available is shorter than with bacteriological cultivation. Due to the lack of nutrients and the inhibitory activity of Toluidine Blue, the demonstration of the microorganism is performed without any bacterial growth. All this implies that requirements for laboratory equipment are small and that the method, perhaps modified, may be suitable for field application by practising veterinarians. As regards laboratory diagnosis of mastitis the tests for nuclease, heated nuclease and antinucleases may represent a supplement to accepted microbiological methods.

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SAMMENDRAG

Bestemmelse av Staphylococcus aureus nuklease og antinukleaser. Anvendbarhet ved vurdering av mastittsekret.

Konsentrasjonen av nuklease produsert av Staphylococcus aureus og titeret av antinukleaser er blitt bestemt i jursekret fra 539 kyr, de fleste med klinisk mastitt forårsaket av Staphylococcus aureus. Nuklease ble i gjennomsnitt påvist i 85 % av prøvene av mastittsekret. I melk fra kyr vil det ofte være antinukleaser som nøytraliserer aktiviteten av nukleasen og en vellykket påvisning av det varmestabile enzymet forutsetter at antistoffer inaktiveres ved oppvarming på kokende vannbad. Nukleasen kan påvises oftest og i høyest konsentrasjoner i prøver fra kyr med alvorlig mastitt og fra kyr som ikke blir fullstendig helbredet.

Nukleasetesten utført på mastittprøver direkte og etter oppvarming, og antinukleasetesten kan være et supplement til vanlige laboratorieundersøkelser. Beskrivelsen omfatter også en omtale av fordelene og enkelte begrensninger ved metodene.

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Reprints may be requested from: Roar Gudding, the National Veterinary Institute, P. O. Box 8156, Dep., Oslo 1, Norway.