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ANTIBODIES AGAINST STAPHYLOCOCCAL AND STREPTOCOCCAL NUCLEASES IN BOVINE BLOOD SERUM AND MILK

By

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GUDDING, R.: *Antibodies against staphylococcal and streptococcal nucleases in bovine blood serum and milk*. Acta vet. scand. 1980, 21, 242—255. — Antibodies against the nuclease of *Staphylococcus aureus* were demonstrated in 99 % and 60 % of samples of bovine serum and milk, respectively. The titres of *S. aureus* antinucleases in milk and serum of cows with *S. aureus* mastitis were significantly higher than those in cows with no mastitis. The prevalence rate of *S. aureus* mastitis in the second lactation of cows which had not suffered mastitis during the first lactation period, was examined. Cows with high antinuclease titres in the milk during the first lactation had lower prevalence rate than those with low titres.

Antibodies against the nuclease of 8 streptococcal species were demonstrated in the serum samples. However, the frequency of occurrence and the levels varied. Antinucleases against *S. agalactiae* were, with one exception, exclusively found in samples from cows or herds with a recorded mastitis caused by this particular organism.

antinucleases; staphylococci; streptococci; milk; serum.

The production of antibodies against *Staphylococcus aureus* is induced by cell bound antigens as well as by extracellular products with antigenic properties. In relation to mastitis, the staphylococcal antitoxins, especially those neutralizing the α -toxin, have been studied most thoroughly (*Schalm et al.* 1971).

Using a cross-wise inhibition test, antienzymes against *S. aureus* nuclease were demonstrated in blood serum from different animal species and in bovine milk (*Sandvik* 1974, 1975).

Antibodies against extracellular antigens of *Streptococcus pyogenes*, among these the anti-DNase B, have been used diagnostically in human medicine (*Stollerman* 1972). In the veterinary field the presence of antibodies against somatic antigens of

mastitis streptococci following a natural infection or an artificial stimulation has been thoroughly documented (*Derbyshire 1962, Schalm et al.*). However, limited attention seems to have been paid to the immunological response to enzymes produced by these organisms.

The present study deals with the examination of antienzymes against the nucleases (DNases) of different staphylococcal and streptococcal species in bovine serum and milk. However, due to the significance of *S. aureus* as an aetiological factor of bovine mastitis and the availability of a convenient method for testing *S. aureus* antinucleases in a series of samples, most attention was paid to the antinucleases of this organism.

MATERIALS AND METHODS

Samples

The material was selected from quarter milk samples collected as part of a mastitis research programme in which approx. 300 herds were examined 4 times at intervals of 6 months. The titres of *S. aureus* antinucleases were determined in approx. 21,000 milk samples. Data concerning contemporary udder health and udder health status at the antecedent and/or subsequent examination were available. Samples of blood serum from 1316 of the cows were also included.

Bacteriological examinations

Routine bacteriological examination of the milk samples was performed as described by *Klastrup & Schmidt Madsen (1974)*. The terminology applied in mastitis diagnosis has been described by *Gudding (1980 a)*.

Antinuclease investigations

The titre of antibodies neutralizing the nuclease of *S. aureus* (ATCC* 10832) in milk and serum was examined using the antinuclease test (*Gudding 1977*). The cross-wise inhibition test (*Sandvik 1974*) was used for monitoring the level of antibodies in serum against the nucleases of *S. aureus* (ATCC 10832, NVH** 3341, NVH 3342, NVH 3344, NVH 3346), *S. epidermidis* (NVH

* American Type Culture Collection, Rockville, Maryland, USA.

** The culture collection at the Department of Microbiology and Immunology, Veterinary College of Norway, Oslo.

2359, NVH 3343, NVH 3345), *S. pyogenes* (NVH 3144), *S. agalactiae* (NVH 3148, NVH 3215), *S. dysgalactiae* (NVH 3153, NVH 3216), *S. equi* (NVH 3150), *S. equisimilis* (NVH 3159), *S. zooepidemicus* (NVH 3161), Streptococcus group G (NVH 3155) and Streptococcus group L (NVH 3157). Inhibitory activity was demonstrated visually in Toluidine Blue DNA Agar (TDA (*Lachica et al.* 1971) or in a TDA modified for the demonstration of streptococcal nucleases (*Gudding* 1979). The titres obtained in the cross-wise inhibition test were given value from 0 to 4, based on a visual judgement of the enzyme inhibition produced by the antibodies.

Electrophoresis

The inhibitors against *S. aureus* nuclease in milk, colostrum and serum were separated by paper electrophoresis as described by *Fossum* (1970). However, the caseinate medium and the proteolytic enzymes were replaced by TDA and *S. aureus* nuclease.

Pure IgG was isolated from serum by sodium sulphate precipitation (*Heide & Schwick* 1973).

Data analyses

Values for antinucleases were converted into square roots before computer calculation of averages.

RESULTS

Antinuclease test

Antibodies against the nuclease of *S. aureus* ATCC 10832 were recorded in samples of milk and serum from 60 % and 99 % of the cows examined, respectively. The overall average titre of these antibodies, expressed in diffusion units per 0.1 ml, was 3.8 in milk ($n = 5022$) and 96 in serum ($n = 1316$). However, the average titre in milk from cows in the main stage of lactation (10 to 250 days after parturition) was 1.9 diffusion units per 0.1 ml.

The amount of *S. aureus* antinucleases in milk in relation to different levels of antinucleases in serum is presented in Table 1. When these 2 parameters were subjected to correlation testing, a correlation coefficient of $r = 0.43$ ($P < 0.001$) was found.

The variation of the *S. aureus* antinuclease titre in milk with increasing age of the cows, time after parturition and daily milk

Table 1. Distribution of cows according to the amount of antibodies against *Staphylococcus aureus* nuclease in milk* and serum.

Amount of anti-nucleases in serum (zone diameter in mm)	Amount of antinucleases in milk (zone diameter in mm)					
	< 4	4 and 5	6 and 7	8 and 9	10 and 11	≥ 12
< 4	14	0	1	0	0	0
4 and 5	17	2	0	0	0	0
6 and 7	47	1	3	1	2	0
8 and 9	110	24	12	6	5	3
10 and 11	115	60	70	18	8	8
≥ 12	39	50	66	44	20	9

* Milk from cows in the main period of lactation (10—250 days after parturition).

yield is shown in Fig. 1. Antibody levels were lower in young cows, especially those in their first lactation, than in older cows. The average antibody titre in serum from cows in their first lactation was 72 diffusion units per 0.1 ml, increasing to approx. 100 units in samples from cows in the following 4 lactations.

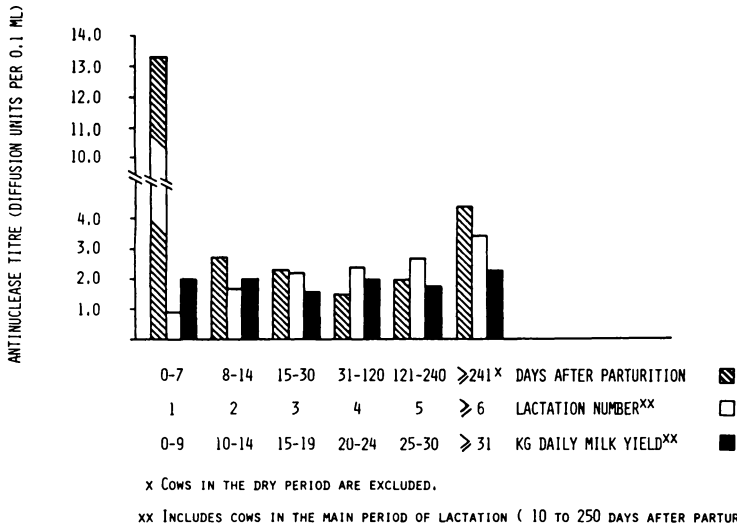


Figure 1. The titre of antibodies against *Staphylococcus aureus* nuclease in bovine milk in relation to time after parturition, lactation number and daily milk yield.

As seen from Fig. 1, the level of *S. aureus* antinucleases was comparatively high in milk and udder secretions from cows outside the main period of the lactation period. The titre in udder secretions from 268 cows in the dry period being found to be 78 diffusion units per 0.1 ml.

The titres of *S. aureus* antinucleases in milk and serum from cows with an infectious mastitis, and infectious mastitis and latent infection caused by *S. aureus* (Table 2) were higher than the overall average titre of these antibodies in the milk samples examined (3.8 diffusion units per 0.1 ml). The difference between titres in cows which had and which had not been so diagnosed was statistically significant ($P < 0.001$). This was not the case when titres in cows with and without non-specific mastitis were compared.

Table 2. Titres of antibodies against *Staphylococcus aureus* nuclease in bovine milk ($n = 5192$) and serum ($n = 1316$) in relation to contemporary udder health.

Diagnosis	Milk		Serum	
	percentage of cows	titre* (diffusion units per 0.1 ml)	percentage of cows	titre* (diffusion units per 0.1 ml)
Infectious mastitis** <i>Staphylococcus aureus</i> mastitis***	20.0	8.6	21.2	136.5
Non-specific mastitis**** <i>Staphylococcus aureus</i> latent infection*****	9.7	15.9	10.7	175.2
	12.1	3.6	11.9	105.7
	5.7	8.7	7.0	142.2

* Values for antinucleases were converted to square roots before calculation of averages. This applies also to Fig. 2.

** CMT ≥ 3 , udder pathogenic microorganisms demonstrated.

*** CMT ≥ 3 , *S. aureus* demonstrated.

**** CMT ≥ 3 , no udder pathogenic microorganisms demonstrated.

***** CMT ≤ 2 , *S. aureus* demonstrated.

Cows with *S. aureus* mastitis in a single quarter showed an antinuclease titre in milk of 13 diffusion units per 0.1 ml, the corresponding figures for cows with 2, 3 and 4 mastitic quarters being 28, 31 and 32. A corresponding increase, although slight, also statistically significant, was found for antinuclease titres in serum from cows with *S. aureus* mastitis and for those in serum

or milk from cows with infectious mastitis or *S. aureus* latent infection. Levels of *S. aureus* antinucleases in the mastitic quarters of the 529 cows with subclinical *S. aureus* mastitis were higher, equal or lower than those in the non-mastitic quarters in 53.7 %, 43.3 % and 3.0 % of cases, respectively. Corresponding percentages for 504 cows with an infectious mastitis caused by organisms other than *S. aureus* were 21.8 %, 75.4 % and 2.8 %.

Cows with neither mastitis nor latent infection in the main part of the first lactation period (10 to 250 days after parturition) were grouped according to antinuclease levels in milk and serum, a "high" group (zone diameter ≥ 5.0 mm) consisting of 62 cows, and a "low" group (zone diameter < 5.0 mm) consisting of 145 cows. Of these, 2 of the cows in the "high" group and 19 of those in the "low" group proved to have developed *S. aureus* mastitis when examined in the main period of the second lactation. The difference was found to be statistically significant. A similar, but minor trend was observed for cows later shown to have developed an infectious mastitis, but the difference in prevalence rate between the groups was not statistically significant.

When the grouping of cows was based on the serum titre during the first lactation, no difference in udder health during the second lactation was observed. Nor was the prevalence of *S. aureus* mastitis and infectious mastitis in cows during the third lactation influenced by the amount of antibodies against the *S. aureus* nuclease in milk and serum during the second lactation.

Cross-wise inhibition test

Antibodies against the nucleases of all the examined staphylococcal and streptococcal strains were demonstrated in samples of bovine serum. Frequency of occurrence and amount varied (Fig. 2). Samples of serum from cows in the same herds exhibited similarities as regards the occurrence of antibodies against the microorganisms in question, both qualitatively and quantitatively. The antibodies seemed to be distributed according to a pattern which was characteristic for each herd.

The titres of antibodies against the nuclease of 5 strains of *S. aureus* were correlated by coefficients in the range of $r = 0.81$ to 0.96 . Though a similar correlation was found between the strains of *S. epidermidis*, there was no correlation ($r = 0.06$)

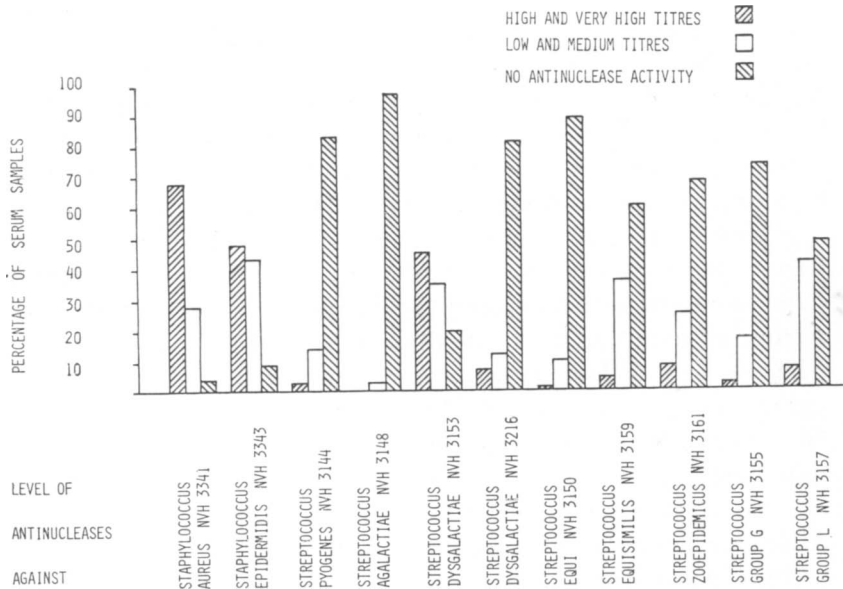


Figure 2. Distribution of staphylococcal and streptococcal antinucleases in serum samples from 354 cows.

between the amount of antibodies against the nucleases of *S. aureus* and *S. epidermidis*. The titres of antinucleases against the non-bovine strains of *S. aureus* (ATCC 10832) and *S. epidermidis* (NVH 2359) were slightly lower than those against the bovine strains of these same 2 species.

Antibodies against *S. agalactiae* nuclease were demonstrated in samples from 5 cows originating from 3 of the 66 herds. In 2 of these herds, 2 of the 3 tested cows in each herd had detectable amounts of antinucleases in serum. In each of these 2 herds, 1 of the tested cows had a mastitis caused by *S. agalactiae*. This organism was not isolated from milk samples from any other of the tested herds. In the third herd, the lowest detectable amount of antinucleases was measured in the serum sample of 1 of 11 tested cows.

Antinucleases against 1 of the *S. dysgalactiae* strains (NVH 3153) were found in 80 % of the serum samples, most of which had a high titre. In contrast, antinucleases against *S. dysgalactiae* NVH 3216 were demonstrated in only 19 % of the samples. However, in the herds with cows showing positive titres, these antibodies were found in samples from all or most of the cows. In

all these samples, antibodies against the nuclease of *S. dysgalactiae* NVH 3153 were demonstrated in amounts which were equal to or greater than the amounts of antibodies against the nuclease of *S. dysgalactiae* NVH 3216. *S. dysgalactiae* was isolated from 4 cows, all of which showed high levels of *S. dysgalactiae* NVH 3153 antinucleases. In none of these cows were antibodies against the nuclease of *S. dysgalactiae* NVH 3216 demonstrated.

The percentage of serum samples showing antinuclease activity against the other streptococci was as follows: *S. pyogenes* 17 %, *S. equi* 11 %, *S. equisimilis* 40 %, *S. zooepidemicus* 33 %, Streptococcus group G 19 % and Streptococcus group L 48 % (Fig. 2). Antinucleases against the streptococci of group A and G were exclusively demonstrated in samples with antinucleases against *S. dysgalactiae* NVH 3216, amounts present being generally equal to or less than those of *S. dysgalactiae* NVH 3216 antinucleases. Antibodies against *S. zooepidemicus* nuclease were also recorded in a limited number of herds. Titres were generally low, except in samples from cows from a few herds in which all tested animals were positive. The cows with an antinuclease titre against *S. equisimilis* and Streptococcus group L were generally identical. A slight inhibitory activity against the nuclease of *S. equi* was found in samples from some cows in different herds.

Streptococcus group L was isolated from quarter samples from cows in 1 herd. The titre of antinucleases against this particular organism was high in most samples from that herd. However, in a few other herds with comparatively high titres, Streptococcus group L could not be isolated from milk samples collected at the same time as the serum samples. None of the other streptococci (*S. pyogenes*, *S. equi*, *S. equisimilis*, *S. zooepidemicus* and Streptococcus group G) were isolated from quarter milk samples from any of the cows in the herds examined.

The occurrence of streptococcal antinucleases in bovine milk was not examined systematically, as preliminary examinations of quarter milk samples from cows with and without streptococcal mastitis were negative. Antinucleases against *S. dysgalactiae* NVH 3153 were demonstrated in a few samples.

Electrophoresis

The zones of nuclease inhibition after electrophoretic separation of bovine milk, colostrum and serum were located on the

anode side near the line of application. Only 1 inhibitor was demonstrated which migrated similarly to bovine IgG.

DISCUSSION

All or most strains of *S. aureus* excrete nuclease. The enzyme acts as an antigen and provokes the production of antibodies. Consequently, *S. aureus* antinucleases should be considered as being as reliable a diagnostic criterion as *S. aureus* antibodies in general.

The occurrence of antinucleases in 99 % and 60 % of the samples of bovine serum and milk, respectively, found in this study, and the corresponding figures for anti- α -toxin of 79 % (serum) and 6 % (milk) presented by *Edwards & Smith* (1959), indicate a wide distribution of the organism in dairy herds. The difference in the results of these studies, especially as regards the level of antibodies in milk, can most reasonably be attributed to the superiority of nuclease as an immunogenic marker of *S. aureus*.

The results as regards the variation of antinuclease titres according to age, time after parturition and health status are generally in accordance with the data from previous studies on anti- α -toxin and anti- β -toxin in bovine milk and serum, reviewed by *Brown* (1960).

As seen from Table 2, the titre of antinucleases in milk from cows with a subclinical *S. aureus* mastitis was approx. 4 times greater than the overall average (3.8 diffusion units per 0.1 ml). The excretion of nuclease during a subclinical *S. aureus* mastitis provides a long-lasting antigenic stimulation causing a vigorous antibody production. The relatively high antinuclease titres found in cows diagnosed as *S. aureus* latent infection are indicative of a state of some duration. Moreover, it is questionable whether the production of antigenic nuclease during an infection, in contrast to an inflammation, provides a sufficient stimulus for the observed rise in the milk and serum antinuclease titres. A reasonable assessment of the high titres is that cases of *S. aureus* mastitis have been included in the term *S. aureus* latent infection. The distinction between a latent infection and a mastitis is based on the CMT-test, which is actually a quantitative test for DNA from the nuclei of somatic cells. The cleavage of DNA from ruptured cells by the nuclease of *S. aureus* before performance of the CMT-test in the laboratory may thus explain

why a mild *S. aureus* mastitis is sometimes misdiagnosed as *S. aureus* latent infection in the laboratory.

The registration of antinuclease titres in cows with a non-specific mastitis, of about the same levels as in healthy cows is a strong indication that a majority of these cows have no contemporary or previous inflammation of the udder caused by *S. aureus*, the microorganism most frequently associated with mastitis in Norway. The present study thus supports the view that the current definition of mastitis in terms of elevated somatic cell numbers encompasses quarters with no real udder inflammation.

The higher antinuclease titres in samples from inflamed quarters, as compared with those found in samples from healthy ones, are probably not a result of any substantial antibody production in the mammary gland itself. However, as the epithelium breaks down during a mastitis there is an increased transfer of antibody molecules from blood to milk. Even though antinuclease titres in milk and serum show positive correlation, the data in Table 1 suggest individual variation in the selective transfer of immunoglobulins across the glandular epithelium. The present study thus seems to link up the work of *Edwards & Smith* who showed a definite relationship between blood and milk antitoxin titres, with that of *Sandvik* (1975) which indicated no regularity in the relationship between antinuclease levels in serum and milk.

The present paper gives no conclusive answer to the question of how antibody levels in milk or serum influence the development of a mastitis. However, the observation that there was a lower prevalence rate of *S. aureus* mastitis during the second lactation in cows with high antibody titres in milk during the first lactation needs some further comment. A high antibody level is a result of antigenic stimuli and indicates that the cows concerned had been exposed to udder pathogenic microorganisms to a greater extent than cows with low levels of antibodies in the milk. Furthermore, it is interesting to note that any preventive effect is a result of high antibody levels in the milk, not in the serum. This indicates that both antibody levels in general, resulting from antigenic stimulation, as well as selective transfer of immunoglobulins through the glandular epithelium, are of importance for the establishment of an adequate humoral immunity in the mammary gland.

The nucleases of the bovine *S. aureus* strains examined in the present work as well as that of *S. aureus* ATCC 10832 seemed to be serologically identical or closely related. According to *Scharmann & Blobel* (1968) the nucleases of bovine and human strains are serologically different from those of canine *S. aureus* strains. The lack of correlation between titres of antinucleases against *S. aureus* and *S. epidermidis* is in accordance with the findings of *Gudding* (1980 b) that there is no enzymo-serological relationship between the nucleases of bovine *S. aureus* and *S. epidermidis*.

The level of microbial antibodies in blood serum may give valuable information concerning the distribution of pathogenic organisms in a population. The fact that *S. aureus* antinucleases were found in samples from 99 % of the cows, indicates a wide distribution of the organism. In contrast, the presence of *S. agalactiae* antinucleases seems to be limited to cows or herds from which this particular organism can be isolated. This observation strongly indicates that *S. agalactiae* is an obligate parasite of the mammary gland, and not an ubiquitous organism in dairy herds. Furthermore, the present results indicate that serological surveillance might throw light on the occurrence of *S. agalactiae* among animals and human beings, and consequently provide information concerning the role of non-bovine sources as aetiological factors in bovine *S. agalactiae* mastitis. Antibodies against the nuclease seem to be applicable in sero-epidemiological examinations of the distribution of *S. agalactiae*.

The presence of *S. dysgalactiae* antibodies in 80 % of the cows indicates that also this organism is widely distributed among dairy cows. Antinucleases against *S. dysgalactiae* NVH 3216, which was shown in a previous study (*Gudding* 1979) to deviate enzymo-serologically from the majority of strains of the same species, were also found among the examined cows though less frequently than those of *S. dysgalactiae* NVH 3153.

Inhibitory activity against the nucleases of group A and G streptococci was exclusively demonstrated in cows with antibodies against *S. dysgalactiae* NVH 3216. This should be interpreted as being the result of serological cross-reactions (*Gudding* 1979).

The demonstration of inhibitory activity against the nucleases of *S. equi*, *S. equisimilis*, *S. zooepidemicus* and Streptococcus group L in samples from cows of some herds is not conclusive

evidence of the distribution of these organisms and their significance in bovine mastitis. The slight inhibition of the nuclease activity of *S. equi* is much more likely to be due to unidentified cross-reactions than to the presence of the organism in the herd environment.

With the exception of 1 herd with several cows infected with *Streptococcus* group L, the presence of *S. equisimilis*, *S. zooepidemicus* and *Streptococcus* group L was not demonstrated in quarter samples from the cows included in the serological examination nor from any of the other cows from these herds. According to a review by *Wilson & Salt (1978)*, *S. zooepidemicus* can be isolated from normal and pathological processes in a variety of animal species, especially the horse, while *S. equisimilis* is most often associated with infections in pigs. In a comprehensive study of group L streptococci, *Olsen (1964)* demonstrated the significance of infected sows in the aetiology of bovine mastitis due to these organisms. Thus group L streptococci may be present in the herd environment, either associated with infections of the cows themselves, or in association with non-bovine sources. However, the species composition of the domestic animal population was not recorded in the present study.

Biological inhibitors which neutralize the activity of nucleases may be of different origin. Normally occurring inhibitors of *S. aureus* nuclease have been demonstrated in the intestine of the pig (*Liven 1977*). In the present study, inhibitors of *S. aureus* nuclease in samples of bovine serum, colostrum and milk were considered to be humoral antibodies as they moved slowly, exactly as purified IgG, in the electrophoretic experiments. The heat sensitivity of the inhibitors, and the elevated inhibitory activity in cows with *S. aureus* mastitis, also confirm that the inhibitors are nuclease inhibiting antibodies.

In the present study, antibodies against staphylococci and streptococci were demonstrated in serum and udder secretions of both mastitic and non-mastitic cows. Apart from serological cross-reactions which may represent a source of error, antibody levels reflect the distribution of the organisms in the herd environment. The findings concerning occurrence and levels of antinucleases against staphylococci and streptococci may contribute to a better understanding of the epidemiology of some of these microorganisms. Similarities in the antinuclease titre in cows within the same herd, and qualitative and quantitative dif-

ferences between cows from different herds were found to be characteristic features of the antibody pattern, which itself seems to represent a kind of "immunological fingerprint" of the microflora of the herds.

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SAMMENDRAG

Antistoffer mot stafylokokknukleaser og streptokokknukleaser i blodserum og melk fra storfe.

Antistoffer mot nuklease produsert av *Staphylococcus aureus* ble påvist i 99 % og 60 % av prøvene av henholdsvis serum og melk fra storfe. Titeret av *S. aureus* antinukleaser i melk og serum fra kyr med *S. aureus* mastitt var signifikant høyere enn titeret fra kyr uten mastitt. Forekomsten av *S. aureus* mastitt i annen laktasjonsperiode ble undersøkt hos kyr uten mastitt i første laktasjonsperiode. Det var mindre forekomst av mastitt hos kyr med høyt antinukleasetiter i melken i første laktasjonsperiode sammenlignet med de med lavt titer.

Variierende mengder av antinukleaser mot 8 forskjellige streptokokker ble påvist med ulik frekvens i blodserum fra ku. Med ett unntak ble antinukleaser mot *Streptococcus agalactiae* utelukkende påvist i prøver fra kyr eller besetninger med diagnostisert mastitt forårsaket av denne bakterien.

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