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AN AGAR DIFFUSION METHOD FOR THE DETERMINATION OF ANTIBODIES AGAINST STAPHYLOCOCCUS AUREUS DEOXYRIBONUCLEASE

By
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GUDDING, ROAR: *An agar diffusion method for the determination of antibodies against Staphylococcus aureus deoxyribonuclease.* Acta vet. scand. 1977, *18*, 480—493. — On the addition of small concentrations of deoxyribonuclease, produced by Staphylococcus aureus, to Toluidine Blue DNA agar, a medium is produced on which antibodies against S. aureus deoxyribonuclease may be detected. When samples of milk, or blood serum, containing antibodies against S. aureus are applied into wells in the agar, the deoxyribonuclease activity is inhibited by the antibodies diffusing into the agar. As a result of this inhibition, blue zones are produced around the wells in the otherwise bluish-red agar. The diameters of the zones correspond to the concentrations of antibodies, and the method may consequently be used for qualitative and quantitative examinations of antibodies against S. aureus deoxyribonuclease in milk and serum. The procedure and certain limitations of the method are described.

deoxyribonuclease; Staphylococcus aureus; antibodies.

Deoxyribonuclease (nuclease, DNase) is an enzyme produced by most, or all, strains of Staphylococcus aureus. The enzyme is antigenic, and cows respond to infections caused by S. aureus with the production of antibodies which give a specific inhibition of the enzyme activity. These antibodies (antinucleases) are present in, and may be detected in, samples of blood serum (*Sandvik* 1974) and milk (*Sandvik* 1975).

Antibodies against staphylococcal nuclease may be demonstrated using Ouchterlony double diffusion analysis (*Sachs et al.* 1972). The principles of the crosswise casein precipitating inhibition test for titration of antiproteinases, as described by *Fossum* (1970), may also be used for the detection and titra-

tion of antinucleases. *Sandvik* (1974, 1975) used the crosswise nuclease inhibition test using TDA (Toluidine Blue DNA agar) (*Lachica et al.* 1971) as substrate for the demonstration of antibodies against staphylococcal nuclease in serum and milk from cows. This method has the advantage that crude enzyme-containing material, as well as untreated antisera, can be used, as the resulting changes in the TDA reflect a specific activity. For the titration of antinucleases in series of samples of milk, blood serum and other biological fluids, this method may, however, be too laborious.

The present investigation was carried out in order to develop a method for the titration of antienzymes against S. aureus nuclease, which is more suitable when large numbers of samples have to be examined.

MATERIALS AND METHODS

Enzyme

The nuclease was prepared from Staphylococcus aureus ATCC 10832*. The bacterium was cultivated in beef-extract broth at 37°C. At an optical density of the broth of 0.75 at 600 nm, corresponding to a cell concentration of about $5 \cdot 10^8$ per ml, the liquid culture was centrifuged. The supernatant was heated on a boiling water bath for 15 min. and preserved by the addition of merthiolate to a final concentration of 1:10 000. This enzyme-containing solution could be stored at room temperature for at least 6 months without any significant change in nuclease activity.

The concentration of the enzyme was determined by an agar diffusion method according to the principles of *Sandvik* (1962), using TDA as test medium, and by a turbidimetric method described by *Erickson & Deibel* (1973). The latter method was slightly modified, as Tris-HCl pH 9.0 was used as buffer instead or boric acid-borax buffer. This method was also used for the determination of the nuclease activity at different temperatures.

Agar diffusion method

The Toluidine Blue DNA agar described by *Lachica et al.* (1971) was used as basic substrate. The pH of the substrate was 9.0 in all experiments. Heating of the substrate was performed

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

with constant stirring in order to dissolve the DNA completely. The TDA was cooled to 45—47°C and the enzyme-containing solution was added to a final concentration of 1:1000. The medium was poured into glass trays or Petri dishes to a depth of 2 mm. The TDA plates containing nuclease, which are referred to as antinuclease plates, were stored at 4°C for up to 6 hrs. Circular wells, with a diameter of 10 mm, were punched in the solid agar with a cork borer, and aliquots of 0.1 ml of the material to be examined for the presence of antinucleases were applied into the wells.

Incubation and storage of the antinuclease plates

After sample application, newly prepared antinuclease plates were preincubated at 4°C for 18 and 42 hrs., respectively, before further incubation at 37°C. Plates incubated at 37°C without any preincubation, were also included in the experiment. The suitability of antinuclease plates, which had been stored at 4°C one and two days respectively, before the application of the samples, was also tested using the same preincubation/incubation procedure as described for newly prepared plates.

After incubation, the diameters of the zones resulting from the antinucleases were measured to the nearest 0.5 mm. A series of typical antinuclease zones based on two-fold dilutions of a milk sample are shown in Fig. 1.

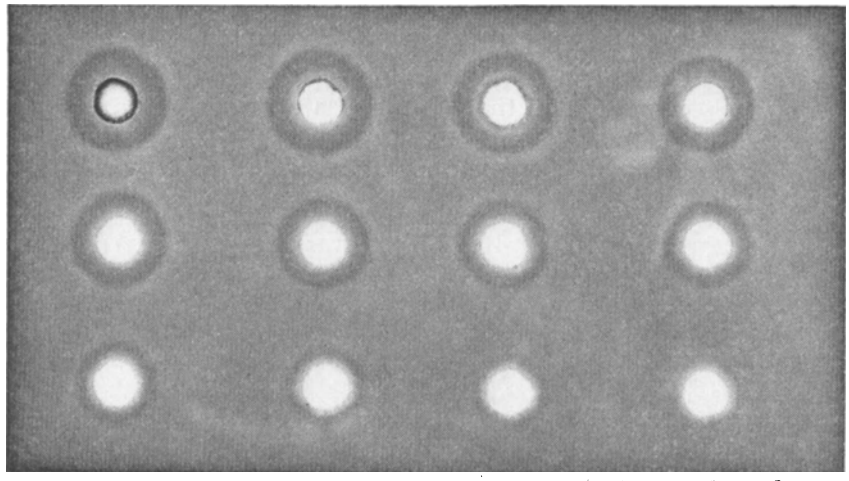

Figure 1. Antinuclease zones produced by a milk sample and two-fold serial dilutions (1:2, 1:4, ..., 1:1024, 1:2048) of this sample. Upper left: Undiluted sample.

Standard curve

The results of the titrations on the antinuclease plates were used for the determination of the titres of antinuclease according to the principles of *Sandvik* (1962), who studied proteolytic enzymes. Regression coefficients were calculated on the basis of the results of two-fold dilutions of 25 milk samples and 16 serum samples, respectively. The data for milk and serum samples were each divided into three groups. These included 1) all dilutions including the undiluted samples, 2) all dilutions except the undiluted samples and 3) the results of dilutions including undiluted samples with a zone diameter of 10 mm or less. The curve shown in Fig. 2 is drawn on the basis of the

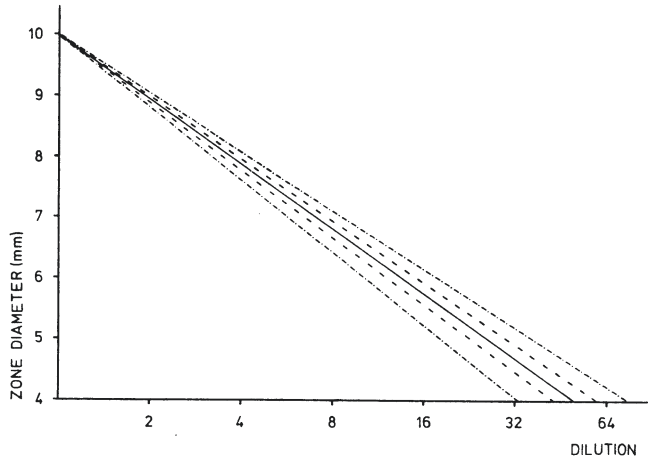

Figure 2. Line of regression (————) of zone diameter on log. sample dilution. Standard deviations (—·—·—) and 95 % confidence intervals (- - - - - - -) are indicated. Based on measurements of two-fold dilutions of 25 milk samples. Preincubation at 4°C for 18 hrs. and incubation at 37°C for 4 hrs.

results for the milk samples in the latter group. As shown in Fig. 2, the abscissa has been displaced 4 mm along the ordinate. The dilution of antinuclease corresponding to the point of intersection between the abscissa and the regression line for zone diameter as a function of dilution, is defined as one antinuclease diffusion unit per 0.1 ml liquor. The curves calculated and drawn on the basis of replicate titrations of milk samples were used for preparing a logarithmic standard curve for antinucleases (Fig. 3).

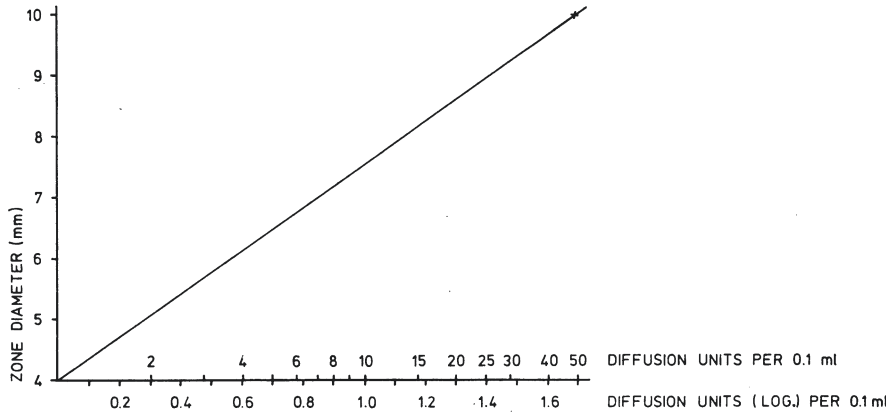

Figure 3. Standard curve for antinucleases in milk based on the results of Fig. 2.

Preservation and fixation of the milk samples

Fifty milk samples were mixed with potassium dichromate and formalin to final concentrations of 0.3 % and 0.02 %, respectively. The samples were stored overnight at 25°C before examination of the antinuclease titres.

Comparison of the antinuclease agar diffusion test and the crosswise inhibition test

The titres of antinuclease in samples of serum and milk were determined by the antinuclease agar diffusion test and the crosswise nuclease inhibition test (*Sandvik* 1974). The antinuclease agar diffusion test was performed by preincubating the plates at 4°C for 18 hrs. and subsequent incubation at 37°C for 4 hrs. The curve presented in Fig. 3 was used for the determination of the antinuclease titres.

RESULTS

The method

When the antinuclease plates, with samples of milk or serum applied into the wells, were incubated at 37°C after a previous incubation at 4°C for 18 hrs., a bluish-red colour developed in the agar after 3—4 hrs. When the plates were inspected against a white background and with lighting from below, zones with

the blue colour remaining unchanged could be observed around wells with samples containing antienzymes against S. aureus nuclease.

The diameters of the zones should be measured after about 4 hrs.' incubation when the plates have been preincubated for 18 hrs. at 4°C. Prolonged incubation at 37°C gave a reduction in the distinctness of the zones. When stored at 20°C after the incubation period, the changes in the zones were moderate during the first 24 hrs.

Both the distinctness and the diameters of the zones in the TDA were influenced by the concentration of the enzyme in the medium. Consequently, a standardization of the enzyme concentration was necessary in order to obtain satisfactory reproducibility. The addition of 0.1 ml of an enzyme-containing solution with a concentration of $2 \cdot 10^5$ diffusion units per 0.1 ml (agar diffusion method) or 650 units per ml (turbidimetric method) to 100 ml TDA represented the final enzyme concentration in the agar which produced the most distinct zones when the plates were preincubated at 4°C for 18 hrs. and finally incubated at 37°C for 4 hrs. If the enzyme concentration was increased, the distinctness and the diameters of the zones were reduced. A reduction of the enzyme concentration required a longer incubation time in order to obtain satisfactory colour contrast. For rapid and preliminary examinations or for demonstration of the principle of the method, 0.1 ml of S. aureus broth incubated overnight, added to 100 ml TDA may be a convenient procedure.

Practical applications

The titres of antinuclease could be determined in samples of bulk milk, foremilk and blood serum. The intensity of the blue coloured zones was generally uniform. However, with undiluted milk and serum samples, central colourless zones, 1 to 2 mm broad, were observed in most cases. These zones were not seen, or they were less pronounced when dilutions of milk and blood serum were examined. Foremilk samples with high concentrations of staphylococcal nuclease (*Gudding* 1976) generally produced pink zones around the wells when the antinuclease plates were preincubated/incubated for the prescribed times. When examining samples of bulk milk, pink zones as produced

by staphylococcal nuclease, could be observed in a few cases (approx. 0.1 % of the samples). The borders of these zones were diffuse and the diameters were generally in the range of 5—10 mm. The determination of the antinuclease titre could not be performed in samples producing these zones.

Regression coefficients and statistics

The regression coefficients with standard deviations and 95 % confidence intervals for the data of the titrations of milk and serum samples are given in Table 1. A separate calculation of all dilutions, including the undiluted samples of milk, showed a regression coefficient of -3.61 ± 0.35 for samples ($n = 9$) with a zone diameter of the undiluted sample of 10 mm or less, and -3.23 ± 0.30 for samples ($n = 16$) with a larger zone diameter.

Table 1. Regression coefficients for zone diameters on log. sample dilutions, calculated as averages for 25 milk samples and 16 samples of blood serum, with standard deviations (s) and 95 % confidence intervals (C.I.)

	Milk samples (n = 25)			Serum samples (n = 16)		
Group	1*	2**	3***	1*	2**	3***
Coefficient	—3.31	—3.64	—3.56	—3.63	—3.60	—3.63
s	±0.37	±0.32	±0.37	±0.51	±0.50	±0.43
95 % C.I.	±0.15	±0.13	±0.15	±0.27	±0.27	±0.23

* All dilutions (including undiluted samples).
** Undiluted samples deleted.
*** Dilutions including undiluted samples, giving a zone diameter of 10 mm or less.

The coefficient for milk samples, with the exclusion of dilutions with zone diameters larger than 10 mm (-3.56 ± 0.37), was used as the basis for the calculation of the regression line with its standard deviation (Fig. 2). The data from the calculation of the regression line were also used for the estimation of the standard curve, as shown in Fig. 3.

For the direct calculation of the number of diffusion units in an unknown sample the following equation may be used, in which dil_1 ($dil_1 = 1$) and dil_2 are dilutions of a sample and

d_1 ($d_1 = 10$) and d_2 ($d_2 = 4$) the zone diameters produced by these dilutions

$$dil_2 = \frac{dil_1}{\text{antilog}\,\frac{(d_1 - d_2)}{k}} = \frac{1}{\text{antilog}\,\frac{(10-4)}{-3.56}} = 48.42$$

The value thus obtained for dil_2 provides a measure of the number of times the sample (dil_1) must be diluted in order to give the same zone diameter as dil_2.

Enzyme activity at different temperatures

The influence of temperature on the activity of S. aureus nuclease is shown in Fig. 4. The increase in enzyme activity

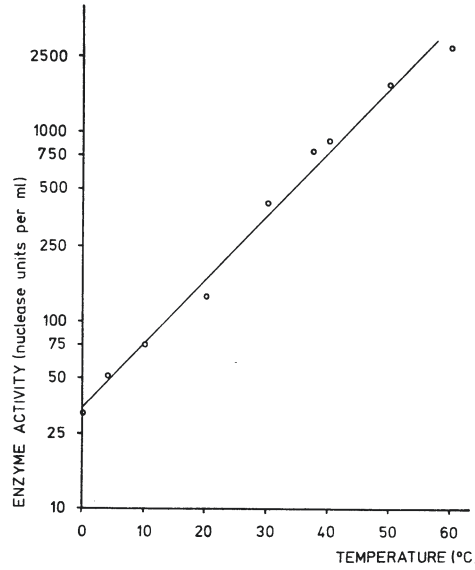

Figure 4. Enzyme activity as a function of temperature. Semi-logarithmic plot. Incubation time 30 min.

when the temperature was increased 10°C, was found to be 2.16 (Q_{10}). Within the temperature range tested in this experiment, the enzyme activity seemed to have a linear relationship to the incubation temperature. The enzymatic activity at an incubation temperature of 37°C was 13.0 times higher than that at the pre-incubation temperature (4°C).

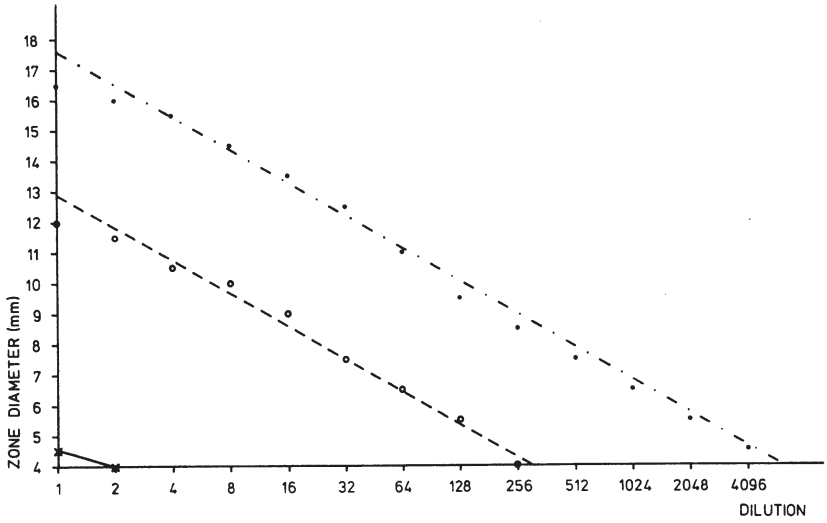

Figure 5. Zone diameters of the dilutions of a milk sample incubated with and without preincubation.
×———× No preincubation, incubation 5 hrs. at 37°C.
o----o Preincubation 18 hrs. at 4°C, incubation 4 hrs. at 37°C.
●–.–.–● Preincubation 42 hrs. at 4°C, incubation 3 hrs. at 37°C.

Effect of preincubation/incubation conditions and of storage of the plates

A representative example of the effect of preincubation on the diameters of the antinuclease zones and, consequently, the antinuclease titres is shown in Fig. 5. As can be seen, a preincubation for 18 or 42 hrs. gives a significant increase in zone diameters after incubation compared with plates incubated without preincubation. The storage of the plates at 4°C for 24 hrs. before sample application resulted in a reduction in the distinctness of the zones compared with the zones produced in plates used immediately after preparation. In plates stored at 4°C for 48 hrs. the zones were weak and indistinct, and the measurement of zone diameters was possible only in samples with high antinuclease titres.

Influence of potassium dichromate preservation or formalin fixation

The 50 milk samples, with potassium dichromate added, produced antinuclease zones of the same size as samples without

the potassium dichromate addition. A correlation test of the results of the antinuclease agar diffusion test performed on samples with and without potassium dichromate conservation gave a correlation coefficient of 0.91. For formalin preserved milk, the antinuclease zones were much smaller and less distinct than the zones of the same milk without formalin.

Comparison of the antinuclease agar diffusion test and the crosswise inhibition test

A scatter diagram of the titre of antinuclease determined by the antinuclease agar diffusion test and the crosswise nuclease inhibition test is shown in Fig. 6. The correlation coefficient based on the examination of 52 milk samples was calculated to 0.81.

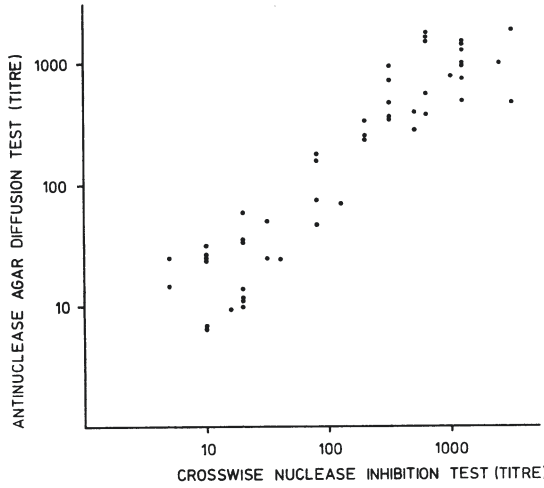

Figure 6. Scatter diagram of the titres of antinuclease determined by the antinuclease agar diffusion test and the crosswise nuclease inhibition test.

DISCUSSION

The method described for detecting and titrating antibodies against S. aureus nuclease is made possible by converting the whole TDA medium to a metachromatic bluish-red medium. Antinucleases in biological fluids applied into wells in the agar diffuse into the solid medium and neutralize the effect of the nuclease and develop circular zones around the wells. It soon

became clear that an important assumption for the zone development is the diffusion of antinucleases into the agar before the enzyme activity has caused a metachromatic change of the agar medium.

The concentration of the enzyme in the agar has to be a compromise between two opposite conditions. First, the concentration should be sufficiently high to produce a colour contrast between the neutralized zones and the rest of the agar. Second, the activity of the enzyme mixed in the medium should be neutralized by the relatively small amounts of antienzymes diffusing into the agar, and this presupposes the smallest possible enzyme concentration. Thus, too large amounts of nuclease added to the medium give diminished and undistinct zones. Too low enzyme concentrations also reduce the distinctness of the zones when read after the ordinary preincubation/incubation procedure. A moderate reduction of the enzyme concentration may, however, be compensated by a prolongation of the incubation time. The control of the enzyme concentration does not, however, represent any problem for routine laboratory procedures as the standardization of the enzyme is a simple process and the stability of the enzyme is good even when stored at room temperature.

The inner decoloured zones, which are most predominant when undiluted samples are tested, have the consequence that diameters below 4 mm cannot be measured accurately in undiluted samples. The reason for the development of these zones is unknown. One theory may be that the zones are produced by normally occurring nucleases in the samples. These nucleases have a pH-optimum below pH 9, but they may produce zones in TDA at pH 9 which differ from staphylococcal nuclease zones (TDA pH 9.0), or zones produced by non-bacterial, normally occurring nucleases (TDA pH 5.5), by lacking the pink colour. The observation that the zones disappear, or are less predominant, in diluted samples strengthens the theory of normally occurring nucleases as the concentrations of these in bovine milk and serum are generally low (*Gudding* 1978).

The appearance of pink nuclease zones in the antinuclease plates when foremilk samples are examined, seems to be related to the nuclease concentrations. As a consequence of the short incubation time, nuclease zones generally develop in the antinuclease plates only when the concentration of nuclease is fairly

high. The pink zones, which are rarely observed in samples of bulk milk, are also supposed to be caused by nucleases of bacterial origin, probably staphylococcal nuclease as a result of the delivery of mastitic milk. The activity of staphylococcal nuclease in milk is generally neutralized by the antibodies present, and too low content of homologous antinucleases may be an explanation for this observation.

As shown in Table 1, the regression coefficient is lower when all dilutions, including the undiluted samples, are tested compared to the test where the undiluted samples are deleted. This means that the zone diameters of undiluted milk samples, as seen in Fig. 5, are relatively smaller than zones produced by dilutions of milk samples. An inhibitory factor against the antinuclease does not seem to be a reasonable explanation for this observation. A more probable explanation seems to be that the fat of the milk hinders the diffusion of the fairly large immunoglobulin molecules.

When the material is divided on the basis of zone diameters of the undiluted samples, it may be seen that the regression coefficient for samples with zone diameters of the undiluted samples of 10 mm or less (—3.61), is within the 95 % confidence interval of the coefficient from which the semilogarithmic standard curve (Fig. 3) is estimated (—3.56 ± 0.15). This standard curve should be used primarily for milk samples with a zone diameter of the undiluted sample of 10 mm or less. Milk samples with a higher titre of antinuclease should be diluted. On the basis of the regression coefficients, it seems that the error associated with the use of the standard curve (Fig. 3), also for the estimation of the titres of serum samples, is insignificant.

In this experiment, the abscissa has been displaced 4 mm along the ordinate, as zones less than 4 mm will not be measured in practical work with undiluted samples. By means of the equation ($dil_2 = \frac{dil_1}{\text{antilog}\,\frac{(d_1 - d_2)}{k}}$) the number of diffusion units can be calculated for any other diameter (d_2) which may be found convenient.

The Q_{10}-value of S. aureus nuclease was found to be 2.16 and this value is of the same order as the Q_{10} for most enzymes (*Whitaker* 1972).

As seen from Fig. 5, a preincubation of the antinuclease plates at a low temperature before the incubation period, is necessary to allow the antienzymes to diffuse into the agar. Although a preincubation for 42 hrs. gives a further increase in the zone diameters, an overnight preincubation (18 hrs.) is preferred for the practical use of the method. The suitability of the antinuclease plates is acceptable even after 24 hrs. storage at 4°C before sample application. As the daily preparation of the plates is not very time-consuming and the accuracy of the method is increased when newly prepared plates are used, it is generally advisable to prepare the antinuclease plates the same day as they are used.

Conservation of the samples with potassium dichromate does not seem to influence the results of the antinuclease agar diffusion method. However, samples preserved for electronic cell counting by the addition of formalin, are less suitable for the examination of the antinuclease titre, probably because the proteins may be denatured by the formalin.

The comparison between the antinuclease agar diffusion test and the crosswise nuclease inhibition test showed that the titres determined by the two methods were of the same order. The procedures of both methods, and the reading of the plates in particular, represent possible sources of error and, on this basis, the correlation coefficient of 0.81 may be characterized as acceptable. The great difference in rapidity in favour of the antinuclease agar diffusion test makes this method a more suitable one for serial titrations of antinucleases than the crosswise nuclease inhibition test.

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SAMMENDRAG

En agardiffusjonsmetode for bestemmelse av antistoffer mot Staphylococcus aureus deoxyribonuklease.

Et medium for påvisning av antistoff mot deoxyribonuklease produsert av Staphylococcus aureus kan lages ved å tilsette lave konsentrasjoner av stafylokokk-deoxyribonuklease til toluidinblått-DNA-agar. Når prøver av melk eller blodserum dryppes ut i brønner i agaren, vil antistoffene som diffunderer ut i mediet, hemme virkningen av deoxyribonuklease. Som en følge af dette vil det utvikles blå soner rundt brønnene i det ellers blå-røde mediet. Diameteren på sonene vil avhenge av konsentrasjonen av antistoff og metoden kan derfor benyttes ved kvalitative og kvantitative undersøkelser av antistoff mot stafylokokk-deoxyribonuklease i melk og serum. Metoden og enkelte begrensninger ved den er beskrevet.

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