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IMPROVED AGAR PLATE ASSAYS OF BOVINE LYSOZYME AND HAEMOLYTIC COMPLEMENT ACTIVITY

By

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LIE, Ø., M. SYED and H. SOLBU: *Improved agar plate assays of bovine lysozyme and haemolytic complement activity.* Acta vet. scand. 1986, 27, 23—32. — Bovine serum and colostral whey samples were examined for lysozyme and haemolytic complement activity, employing agar plate techniques. The tests were carried out in agarose gel containing Micrococcus lysodeikticus (for lysozyme), and antibody sensitized rabbit erythrocytes (for complement), respectively. The confirmation of lysozyme (E.C.3.2.1.17) — dependent lysis has been presented elsewhere (*Lie & Syed* 1986), while heat-inactivation and antibody to C3 were used in the present study to confirm that the haemolytic activity was attributable to the complement cascade. Repeatability and sensitivity of the described tests were found to be superior to those of photometric procedures. Staining and preservation techniques were developed which extended the applicability of the assay, as they made reading of results independent of time and resulted in the plates being very suitable for photography and storage.

c a t t l e; n o n-s p e c i f i c i m m u n i t y; a s s a y s; e v a l u a t i o n.

According to the literature, photometric methods have been predomonantly employed in studies on lysozyme activity (see *Parry et al.* 1965, *Bratlid* 1977, *Lie* 1980, *Lie & Solbu* 1983), as well as on haemolytic complement (HC) levels (see *Mayer* 1961, *Barta & Barta* 1972, *Rurangirwa et al.* 1980, *Lie et al.* 1983). Such methods are, however, cumbersome, laborious and suffer from inaccuracy and poor sensitivity. Therefore they have been progressively replaced by the far more reliable agar plate techniques, the so-called lysoplate assay for lysozyme (*Osserman &*

Lawlor 1966, *Prieur & Camara* 1979), and HC assay in agarose
plate for complement (see review by *Lachmann & Hobart* 1979).

Another general disadvantage of photometric methods is that
one has to continuously monitor the enzymatic action, or at
least, the test has to be read shortly after the reaction has been
accomplished. This is also to some extent the case for the con-
ventional agar plate assays lacking built-in preservation or
staining techniques.

This report describes two highly repeatable and sensitive agar
plate methods each designed to assay one of the two lytic systems
i.e. lysozyme and the complement cascade. An improvement that
may be of particular interest is the successful staining and pre-
servation technique developed for each plate.

MATERIAL AND METHODS

Source of material

Blood and colostrum samples were collected from cows kept
at the research station of the Department of Animal Genetics
and Breeding. Serum and colostral whey were prepared as
described elsewhere (*Lie et al.* 1986).

Assays

L y s o p l a t e. Lysozyme activity was determined as describ-
ed by *Osserman & Lawlor* (1966) with some modification: 15 ml
of a 1 % agarose gel in 0.07 mol/l NaH_2PO_4/Na_2HPO_4 buffer,
pH $=$ 6.2, containing a photometrically determined amount of
50 µg/ml of Micrococcus lysodeikticus, was prepared on 10×10
cm defatted and agarose coated glass slides. Two-fold dilutions
of hen egg white lysozyme (E.W.L.)[*] with an original concentra-
tion of 1.6 µg/ml were employed as standards. The standards
were stabilized in 1 % bovine serum albumin (BSA)[*] dissolved
in phosphate buffered saline (PBS). Sample wells of 3 mm in
diameter were punched in 5 rows of 5, and the lysozyme sources
were applied in 9 µl volumes. The plates were incubated at 38°C
in a humidity chamber for 17 h (overnight), after which they
were washed in distilled water for about ½ h, pressed under
filter paper, completely air dried and flame fixed. For staining,
the following modified Gram procedure was used: The plates

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[*] Sigma Chemical Company, USA.

were placed in a 1.25 % methylviolet solution for 1 min after
which they were transferred to a lugol solution for 15 s. They
were then intensively destained with ethyl alcohol until stain-
less lysed zones appeared, and then left to dry. The diameters of
the cleared zones were determined employing a so-called Meas-
uring - Viewer*.

Within working levels of lysozyme, i.e. 1.6 μg/ml—0.1 μg/
ml the diameters of the zones were proportional to the $\log_2$ of
the lysozyme activity.

To confirm that lysis was due to a true lysozyme (i.e. mura-
midase), serum and colostrum samples of different lysozyme
activity were subjected to biochemical characterization in a
parallel study (*Lie & Syed* 1986).

H C a s s a y i n a g a r o s e p l a t e. Total haemolytic capa-
city was determined by a plate method according to *Lachmann &
Hobart* (1979) with modification as follows: A photometrically
standardized 1 % suspension of rabbit red blood cells (RRBC)
was prepared in 0.094 mol/l veronal/sucrose-veronal buffer,
pH = 7.3, Ca^{2+} and Mg^{2+} conc. 3×10^{-4} mol/l and 1×10^{-3} mol/l,
respectively (*Rapp & Borsos* 1963, *Barta & Barta* 1972, *Lie et al.*
1983). The RRBC were optimally sensitized with sheep antiserum
(haemolysin) as described previously (*Lie et al.* 1983), mixed
at 56°C with an equal volume (8 ml) of 2 % agarose in the same
buffer, and poured into defatted and agarose-coated prewarmed
10×10 cm glass slides. The final concentration of RRBC and
haemolysin in the agarose was 0.25 % and 1:600 respectively.
Two-fold dilutions of a bovine serum sample with high haemo-
lytic activity served as standards. Sample wells of 3 mm in dia-
meter were punched in 6 rows of 6 and filled with a standardized
volume, 9 μl, of haemolytic complement source (serum or whey).
Samples and standards, stored at —70°C, were thawed once on
ice, and plates were kept refrigerated during application. The
plates were left at 4°C for 20 h (for diffusion) after which they
were incubated at 38°C in a humidity chamber (CO_2-incubator)
for 90 min (to perform lysis). For preservation, the following
procedure was developed: The plates were left to soak in a 4 %
phosphate buffered formaline solution for about 30 min, after
which they were pressed under filter paper and allowed to dry.
The diameters of the lysed zones were measured as for the lyso-

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* Behringwerke, W. Germany.

zyme assay. Within working levels of haemolytic complement,
the zone diameters were proportional to the log of the concentra-
tion, employing average serum activity as 100 % (working range:
200 % — 6.25 % of normal serum). To correct for possible in-
fluence of naturally occurring haemolysins, absorption with
RRBC was performed on samples with different activity as
decribed by *Lie et al.* (1983). To confirm that the lysis was
elicited by the complement cascade, some representative samples
were subjected to heat-inactivation (56°C) and some to pre-
incubation with rabbit antibody to bovine C3 component* at
38°C for 1 h before plating. Some samples were also subjected
to double diffusion against anti C3 to confirm lysis-zone inhibi-
tion.

Statistical methods

A one-way analysis of variance was used to estimate the intra
class correlation or the repeatability (t) of the described assays.
To estimate the standard error of the estimated t, the method
described by *Becker* (1967) was used.

RESULTS AND DISCUSSION

Evaluation of the assays

S e n s i t i v i t y. Lysozyme activity could be detected at an
average dilution of 1 in approx. 800 corresponding to levels of
about 2×10^{-3} µg E.W.L. per ml. This is partly reflected in Fig.
1, where lysis can be seen at 1 in 512 and is a 20 fold improve-
ment over the photometric procedure which showed a sensitivity
of about 4×10^{-2} µg/ml (*Lie & Solbu* 1983). If desired, the
sensitivity can be further improved by multiple application or
by employing lysoracket (*Virella* 1977, *Lie & Syed* 1986).

Haemolytic activity was detected at a dilution of 1:256 on
average, or about 0.4 % normal serum corresponding to about
0.036 µl of neat serum (see Fig. 2). This is about 20—30 times
the sensitivity achieved by the photometric procedure. An
amount of whey of 220 µl had to be employed in the photometric
test to detect the lowest colostral haemolytic activity (unpublish-
ed data), whereas 9 µl whey was sufficient for the plate assay
in this study (see Fig. 2).

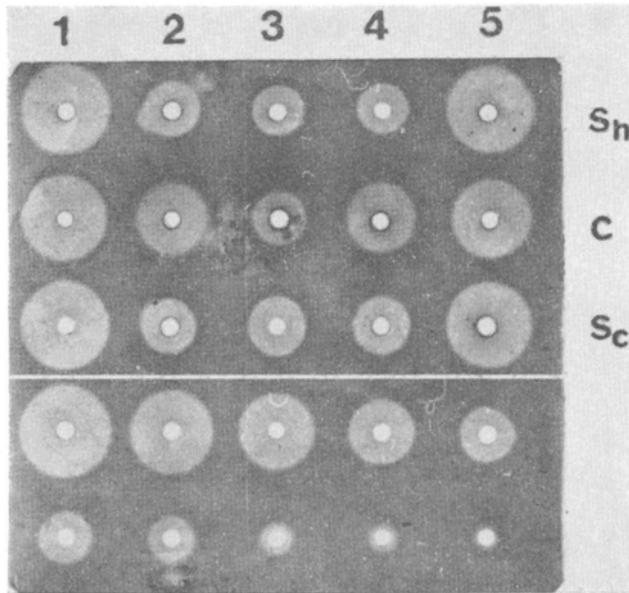---

* Cappel Laboratories, USA.

Figure 1. Lysosyme assay in agarose gel containing Micrococcus lysodeikticus.
Upper part: Serum 1 year prior to parturition (S_h), Colostrum (C) and serum (S_c) at the time of calving for 5 different animals of which no. 1 and no. 5 are high level individuals.
Lower part:
Two-fold dilutions of hen egg white lysozyme (E.W.L.) standard with an initial concentration of 1.6 μg/ml.

Repeatability and specificity. Zone diameters of different doubling dilutions of the standards (5 from E.W.L., 5 from a bovine serum standard for lysozyme, and 6 from a bovine serum standard for haemolytic complement) as well as a series of unknown samples, were collected from 20 plates in each assay. Variation between and within dilution, as well as between and within unknown samples, and the corresponding repeatability (t), was estimated for both assays. Findings were 0.98 ± 0.03 and 0.95 ± 0.03 for lysozyme and complement, respectively. The superiority over photometric procedures (*Lie & Solbu* 1983, *Lie et al.* 1983) is probably due to the many volumetric errors eliminated with the plate methods. The dilution step is automatically undertaken by the agar diffusion and is very reproducible. Moreover, the greater test capacity eliminates day to day variation.
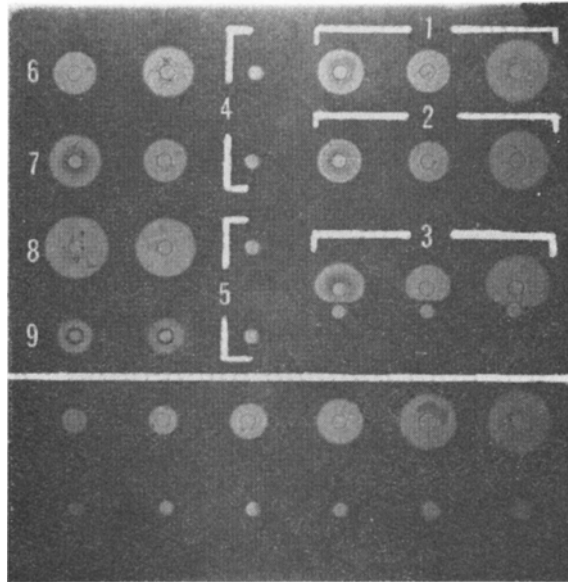
Figure 2. Haemolytic complement assay in agarose gel containing haemolysin-sensitized rabbit erythrocytes.

Upper part:
1. Normal serum from 3 different cows.
2. Sera from the same animals absorbed for natural haemolysins.
3. Sera from the same animals subjected to double diffusion against specific rabbit anti bovine C3.
4. Heat inactivated (56°C for ½ h) serum and colostrum.
5. Serum and colostrum preincubated (1 h at 37°C) with anti C3 before application.
6, 7, 8. Serum from heifers, primiparous cows and multiparous cows, respectively.
9. Colostrum from primiparous cows.

Lower part:
Two-fold dilutions of a bovine serum standard (high activity serum).

That lysozyme activity is constant within the same animal is clearly illustrated in Fig. 1, upper part. This is a reflection of the strong genetic influence and simple inheritance of this trait, i.e. either high or low activity (*Lie & Solbu* 1983). At the same time, it shows the reliability of this method in easily detecting this pattern, when may be overlooked when employing mehods with poor repeatability and sensitivity.

A parallel study (*Lie & Syed* 1986) has confirmed that the lytic power against M. lysodeikticus assayed in the present study

exhibits properties which are in accordance with those defined for a lysozyme (E.C.3.2.1.17), i.e. 1.4-β-N acetylmuramidase cleaving the glycosidic bond between C-1 of N-acetylmuramic acid and C-4 of N-acetylglucosamine (see also *Jollès & Jollès* 1984).

Confirmation that the haemolytic activity studied is attributable to the complement cascade can be clearly seen from Fig. 2. Parts 3 and 5 show the inhibition of specific antibody to complement component C3 of cattle, while part 4 shows the heat sensitivity of the system. Parts 1 and 2 are controls for the influence of naturally occurring cattle antibody to RRBC. A slight reduction in HC titer was observed after absorption (part 2) though there was no rearrangement between animals.

Parts 6, 7 and 8 show the effect of age on HC levels, the older cows having the highest levels, whereas part 9 indicates the very low HC of colostrum (about 5 to 10 % of normal serum).

The 50 % lysis end point in photometric procedures may probably be a more relevant measure of haemolytic capacity. This is especially the case when considering the possible limiting effect of certain C-factors with low rate agar diffusion. However, the prediffusion step before the lysis in the plate assay may have eliminated this problem, since with tube and plate methods running in parallel, a correlation of 0.90 was found.

Also with regard to lysozyme, one should be aware that different methods might be measuring different things (i.e. total lytic capacity or enzyme concentration). For instance, two individuals, one possessing high enzyme concentration and low specific activity and the other having the opposite profile, might appear to have the same lytic capacity in a tube assay, whilst in the plate assay, the one with the high concentration will be the superior. However, when comparing the two lysozyme assays, the overall correlation was high (r > 0.90). This indicates that the difference in lysozyme activity between individuals is probably attributable to different enzyme concentrations rather than to different specific activities of different types of enzymes (*Lie & Syed* 1986).

The above correlations between tube and plate assays, both for the HC and lysozyme, might be even higher with improved repeatability of the tube assays.

Economy and capacity. The amount of serum needed for photometric assays was 200 µl and 60 µl for lysozyme and haemolytic complement, respectively (1200 µl whey was needed for photometric H.C. assay), whereas 9 µl was sufficient for both plate methods. This is mainly due to the reagent-expensive dilution procedures of photometric methods. Taking also into account the superior sensitivity, the plate methods could be regarded as being more than one hundred times more efficient. In addition come the test capacity (10—20 times more samples per day) and the flexibility in reading the plates (non-restrictive in time). This means that the plate method is a more rational choice for screening purposes than conventional (non-automated) photometric procedures.

Applicability. One possible drawback of the plate methods is that they are practically impossible to automate. Optimalized arrangements of modern photometric equipment such as microtiter-plate readers might thus compete with the agar plate assays with regard to mass analysis of routine samples, provided sufficient controls are included. However, we have found the microtiter test to be quite labour intensive (especially for complement), and much less reliable than the agar plate test (especially for lysozyme). The high purchase and running costs of the equipment required for automated photometric methods is also a disadvantage to be taken into account.

The described agar plate methods have proved to be rational and inexpensive tools with a wide range of possible applications. In particular, the methods seem convenient for screening large numbers of samples in epidemiological, clinical or genetical studies. Also when dealing with purification procedures (chromatography, isoelectric focusing etc.), large numbers of fractions can be dealt with readily, due to the high capacity of the test. The methods also seem convenient for qualitative studies (components, types of enzymes, polymorphisms etc.). In such cases, the plates may be employed as detector tools such as overlayers after different electrophoretical procedures. In this context, the staining and preservation techniques may also be an extra benefit, extending the practical applicability of the plates and making them available for almost unlimited storage.

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SAMMENDRAG

Forbedrede agarplatemetoder til bestemmelse av bovint lysozym
og hemolytisk complement aktivitet.

Bovint serum og kolostrum ble undersøkt for lysozym- og hemolytisk complement-aktivitet ved hjelp av lysoplateteknikker. Testene ble utført i agaorse-gel inneholdende Micrococcus lysodeikticus (for lysozym) og antistoff — sensiterte kaninerytrocytter (for complement). Bekreftelsen på lysozym — spesifikk lysis er utført ved biokjemiske analyser i et annet studium (*Lie et al.* 1986) mens varmeinaktivering og spesifikke anti-C3-antistoffer ble anvendt i dette arbeidet for å bekrefte complement-avhengig hemolyse. Reproduserbarhet, sensitivitet og kapasitet på de beskrevne platemetoder ble funnet å være betydelig bedre enn for fotometriske tester. Dessuten ble fargings-/preserveringsteknikker utviklet, noe som øker anvendbarheten av testene i det avlesninger blir tidsuavhengige og platene velegnet for fotografering og lagring.