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Evaluation of a micro method for the routine determination of serum pepsinogen in cattle

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SUMMARY

Estimation of serum pepsinogen concentration in cattle is used to aid the detection of clinical or subclinical infections with the abomasal nematode *Ostertagia ostertagi*. An inexpensive, simple micro method for the routine determination of pepsinogen concentration in bovine serum samples is described which is based on the hydrolysing effect of serum on buffered bovine albumin substrate. Comparison of this assay with a macro method, based on the same principle, gave almost identical results in the range of 0 to 10 Units tyrosine. The reproducibility of the assay was shown to be very satisfactory, intra-assay and day-to-day coefficients of variations were less than 4·7 and 8 per cent, respectively.

THE measurement of serum pepsinogen concentration in cattle has been recommended as an aid in the diagnosis of ostertagiosis (Berghen et al 1993) and a number of different methods are available for this purpose. Most of these estimate pepsinogen by measuring the total acid-stable proteolytic activity of serum (eg Edwards et al 1960, Jennings et al 1966, Berghen et al 1987); other methods are based on radioimmunoassay (Wang et al 1979), ELISA (Turner and Shanks 1982) or radial gel diffusion techniques (Thode-Jensen 1977, Mostofa 1989). However, the existing assays are generally laborious and cumbersome and there is little standardisation, making comparison of results from different laboratories difficult (Berghen et al 1993, Scott et al 1995). Therefore, there is a need for a simple and inexpensive assay that would be applicable for routine screening while giving reliable and reproducible results.

A simple, direct method based on the hydrolysing effect of serum on buffered bovine albumin substrate was described by Berghen et al (1987). This method was shown to be very precise and reproducible: intra-assay coefficient of variation was less than 3 per cent and day-to-day variation less than 4 per cent, irrespective of pepsinogen concentration (Berghen et al 1993). This method was consequently successfully adopted for research purposes by several laboratories and the value of pepsinogen concentration, as a predictor for outbreaks of parasitic gastroenteritis and for the diagnosis of clinical or subclinical disease was demonstrated (Shaw et al 1997). However, this assay needs a large volume of serum (1 ml) and serum blanks for the calculation of pepsinogen concentration, making it impractical and expensive for routine analysis.

The aim of the present study was to evaluate a micro method for the determination of serum pepsinogen concentration that was based on the simplified test described by Berghen et al (1987).

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MATERIALS AND METHODS

Techniques

Both the Berghen et al (1987) method and the proposed micro method were performed on all serum samples tested. The Berghen et al (1987) method (hereafter referred to as the macro method) was used as the standard for the validation of the micro method. The same chemical solutions were used in both methods and they were all prepared using double-distilled water and analytical grade chemicals (Sigma, Chemical Co., St Louis, U.S.A., unless stated otherwise).

The macro method was performed as described by Berghen et al (1987). The micro method was performed as follows: in a 1·5 ml conical Eppendorf tube 50 µl of serum was added to 250 µl of a 2·0 per cent bovine serum albumin (fraction V) solution in a pre-adjusted glycine-NaCl-HCl buffer (0·1 M) at pH 2·5. The tube was closed, vortexed and incubated at 37°C for 24 hours. After incubation the peptic digestion was arrested and the undigested substrate precipitated by the addition of 500 µl of trichloroacetic acid (4 g dl^{-1}). Following vortexing and standing for 10 minutes, the tube was centrifuged for five minutes at 10,000 *g* in a bench top microcentrifuge (Eppendorf 5402, Hamburg, Germany). Three separate 20 µl aliquots of the supernatant were then transferred to wells of a flat bottomed microtitre plate (polystyrene plate, Falcon, Becton Dickinson, New Jersey, U.S.A.) and 200 µl of a 0·25 N NaOH solution was added to each well. After mixing for two minutes, 30 µl of diluted (1:3 water v/v) Folin and Ciocalteu's colour reagent (Merck Chemical Co., Darmstadt, Germany) were added to each well. The microtitre plate was agitated for two minutes and incubated at room temperature (range 20-25°C) for 30 minutes. The optical density was then measured at 680 nm with an ELISA-reader (Labsystems Multiskan RC, Helsinki, Finland) connected to a PC.

To calculate the amount of tyrosine produced, a set of tyrosine standard solutions of 0·1 µmol ml^{-1}, 0·2 µmol ml^{-1} and 0·3 µmol ml^{-1} was freshly prepared from a sterile stock solution (0·01 M L-tyrosine in 0·1 N HCl) and measured on the microtitre plate together with the supernatant

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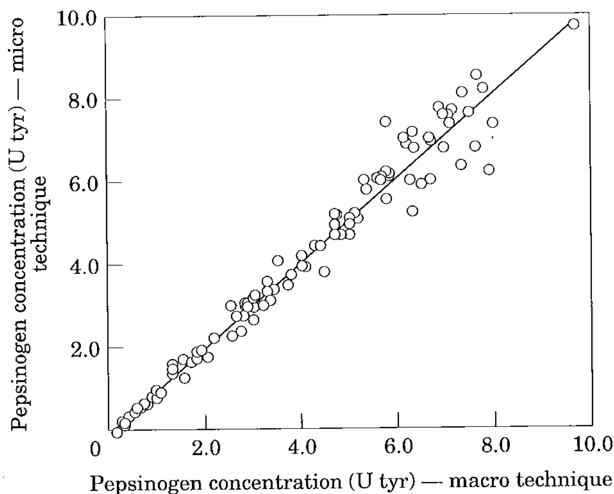

FIG 1: Comparison of macro and micro technique for serum pepsinogen determination. The relationship of pepsinogen concentrations measured by the macro to micro methods (expressed as U tyr) was described by the following linear regression equation: $y = -0.09 + 1.02x$ ($r^2 = 0.97$)

of the processed samples. A substrate blank (double-distilled water instead of serum sample) and a serum sample with known pepsinogen content (5·1 U tyr, measured with the macro method) (serum standard) were also run concurrently in the assay.

Unincubated bovine serum contains a small amount of tyrosine. In the macro method the tyrosine content of each unincubated sample is measured (serum blanks) and taken into account when calculating the pepsinogen content; in the micro method a fixed factor was used to correct for tyrosine-like substances, originally present in the mixture. This factor was obtained from the measurement of the tyrosine content of 200 unincubated mixtures assayed by the same method. The tyrosine concentration in unincubated bovine serum samples was found to be remarkably constant (see further results section) therefore obviating the need for this determination on each sample.

The mean of the three optical density measurements for each serum sample was converted to Units tyrosine (U tyr) (ie micromoles of tyrosine released per litre of serum per minute), using a standard curve prepared from the tyrosine standards (see Appendix).

Samples and experimental design

Three series of bovine serum samples were used which had been stored at –20°C.
S1: 100 serum samples taken on five occasions during the 1996 grazing season from a herd of 48 first-grazing season calves that had been exposed to natural infections for six months of a mixed population of nematode species, predominantly *Ostertagia ostertagi* and *Cooperia oncorphora*;
S2: 100 serum samples obtained from grazing cattle from six commercial farms;
S3: serum samples from three, four-month-old calves that had been experimentally infected with 100,000 infective L3 larvae of *O ostertagi*. The serum samples were taken twice a week for six weeks post-infection.

For the validation of the micro test the following measurements were made:
(1) the amount of tyrosine present in unincubated serum was measured with the micro method of S1 and S2 samples to enable determination of the fixed factor referred to above.
(2) The micro and macro methods were compared by estimating the pepsinogen concentration in sera of S1 with both techniques.
(3) The reproducibility of the technique was tested by repeating the micro method three times on S1 sera for calculation of day-to-day variations. From the same series of samples a serum with, respectively, low (0·9 U tyr), medium (2·1 U tyr) and high (5·1 U tyr) pepsinogen concentrations were selected and each sample was analysed 10 times in the same assay for calculation of intra-assay variation.
(4) Finally it was investigated whether the micro method showed the same pattern in change of pepsinogen concentration as the macro method during the course of an experimental infection with *O ostertagi* (S3 sera).

Statistical analysis

The effect of farming on the amount of tyrosine present in unincubated serum was assessed by analysis of variance (ANOVA). Linear regression was calculated between the two methods (S1 samples). Significance of regression coefficient was tested by ANOVA. Coefficients of variation were calculated for intra-assay and day-to-day variations. Spearman Rank-correlation coefficient was calculated for assessing correlation of the measurements obtained by the two assays on the S3 serum samples.

RESULTS

Measurement of tyrosine-like substances in unincubated samples

Very little variation in the amount of tyrosine originally present in the mixture was measured between samples. For the 100 serum samples from one herd (S1), examined by the micro method without incubation, a mean optical density at 680 nm of 0·020 (99 per cent confidence limits: lower 0·0193; higher 0·0208) was recorded. For the 100 serum samples from various farms (S2) this was 0·020 (99 per cent confidence limits: lower 0·0186; higher 0·0206). There was no effect of farming on the amount of tyrosine detected in unincubated serum. Consequently, a fixed factor of 0·020 was included in the calculation of the pepsinogen concentration to correct for the presence of tyrosine.

Comparison between the micro and macro techniques

Fig 1 presents the linear regression of the pepsinogen determination on S1 serum samples using the macro and micro methods. Very similar pepsinogen concentrations were calculated by both methods in the range of 0 to 10·0 U tyr. The coefficient of determination was 0·97, the slope was almost 1 and the intercept near zero. The regression coefficient was highly significant ($F = 14,902$; $P<0.001$).

Reproducibility of the assays

The precision and reproducibility of the micro method were slightly lower than that of the macro method (Berghen et al 1993); intra-assay coefficient of variation was less than 4·7 per cent and day-to-day variation less than 8 per cent, independent of pepsinogen concentration.

Serum pepsinogen during experimental infections

Fig 2 shows the pepsinogen levels of the three calves

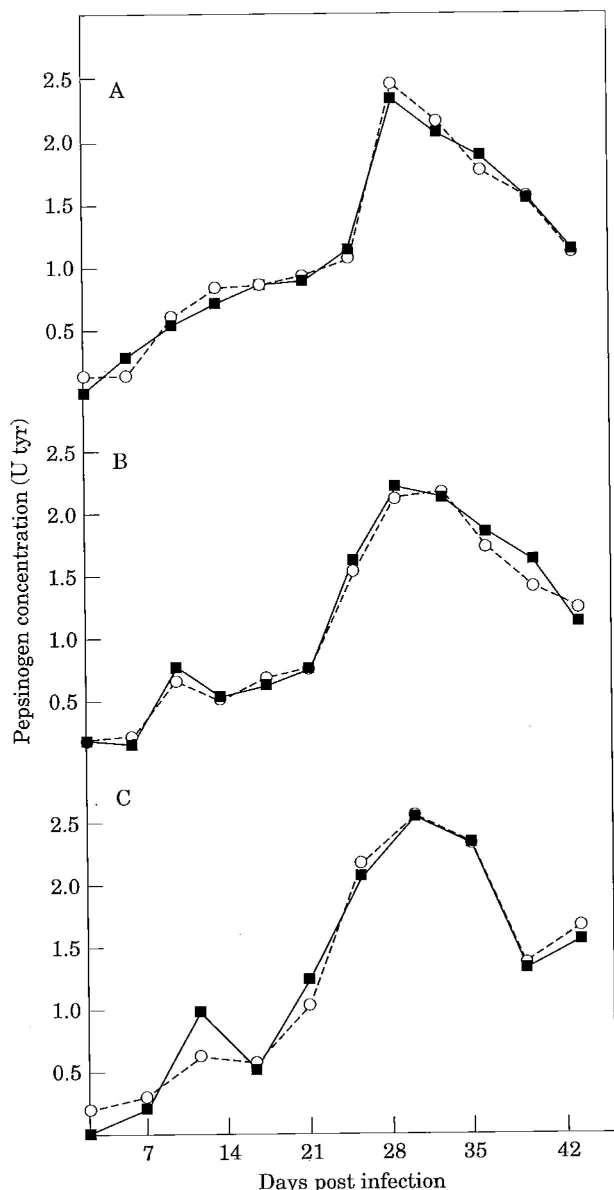

FIG 2: Serum pepsinogen concentrations in three calves (A, B, C) during an experimental infection with 100,000 L3 larvae of *Ostertagia ostertagi* measured by the micro and macro methods.
Key: -■-, micro method; --□--, macro method

during the course of an artificial *O ostertagi* infection, assayed by the two methods (S3 serum samples). It was demonstrated that the micro method successfully showed the change in pepsinogen during infection (Spearman Rank-correlation coefficient $r_s = 0{\cdot}96\text{-}1{\cdot}00$; $P<0{\cdot}001$).

DISCUSSION

Comparison of both techniques on bovine serum samples showed that the simplified micro method gave almost identical results to the macro method in the range of 0 to 10 U tyrosine. By using the mean of three measurements for each serum, variations due to the use of small volumes were compensated. Precision and reproducibility of the micro technique were almost as high as for the macro method. The use of a buffered substrate may be responsible for the low day-to-day variation (Berghen et al 1987, Scott et al 1995).

The micro method of the present study avoids some of the main disadvantages of the original Berghen et al (1987) method. The separation of undigested substrate by centrifugation and not by filtration allowed a considerable reduction of serum volume (20-fold). Consequently the amount of each reagent required was considerably reduced, thereby reducing the cost of the assay. The determination of tyrosine concentration in unincubated samples (serum blanks) was also omitted which represents half of the work in the macro method. It was shown that there is very little variation between samples in the amount of tyrosine, originally present in the mixture, and consequently that measurement of this level for each sample was not justified. Retrospective data analysis of 1473 serum samples examined by the macro method also showed a low variation in optical densities obtained with unincubated sera (unpublished results) (99 per cent confidence limits: lower 0·0159; higher 0·0162). A fixed factor (0·020) to compensate for the presence of these tyrosine substances in the calculation of the pepsinogen concentration can therefore be proposed for the micro method.

Another major disadvantage of the macro method is that samples have to be read spectrophotometrically one by one. In the micro method the use of a flat bottomed microtitre plate for the colour reaction step enables reading of a whole plate at the same time (32 samples), by means of an ELISA reader. A further advantage is that by connecting the reader to a PC the obtained measurements can be automatically converted to Units tyrosine.

The main role for the estimation of serum pepsinogen concentration in cattle is for the detection of clinical or subclinical infection with *O ostertagi*. Serum pepsinogen, measured in first-grazing season calves at housing appears to be a valuable parameter for estimating the level of infection to which the animals have been exposed during the grazing season (Ploeger et al 1994). However, there is considerable variation in pepsinogen levels among animals that were grazing on the same pasture. Therefore, a representative number of animals from a herd should be screened.

In conclusion, the micro method is inexpensive, simple and can be used for routine screening of bovine serum samples. The method has very low and acceptable levels of variation and the results obtained were identical to those obtained using the method of Berghen et al (1987).

APPENDIX

Calculation of pepsinogen concentration with the micro method

$$\text{U tyr} = (\text{OD sample} - 0{\cdot}020) * F * 11{\cdot}11$$

where

U tyr denotes Units tyrosine: micromoles of tyrosine released per litre of serum per minute; OD denotes the arithmetic mean optical density of the three wells; 0·020 is the correction factor for the presence of tyrosine in unincubated samples; F is the calculation factor derived from the standard curve prepared from the tyrosine standards:

$$F = \left(\frac{0{\cdot}1}{\text{OD tyr } 0{\cdot}1\ \mu\text{mol ml}^{-1}} + \frac{0{\cdot}2}{\text{OD tyr } 0{\cdot}2\ \mu\text{mol ml}^{-1}} + \frac{0{\cdot}3}{\text{OD tyr } 0{\cdot}3\ \mu\text{mol ml}^{-1}}\right)\Big/3$$

and 11·11 is the conversion factor for the serum dilution and the incubation time.

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