# Milk progesterone concentrations: an accurate early pregnancy diagnostic aid in dairy cattle

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The optimal day of milk sampling for pregnancy diagnosis by milk progesterone quantitation was determined as well as the diagnostic efficiency of the test for days 14 – 24 post insemination in dairy cattle. The results show that on days 22 and 23 after insemination diagnostic efficiencies of approximately 100% can be obtained. Enzymeimmunoassay (ELISA) has numerous advantages over the radioimmunoassay in terms of capital outlay, suitably trained staff, and radioactivity-handling facilities when establishing large-scale milk progesterone testing schemes. A positive and negative predictive value of 93,8% and 94,2% respectively was obtained for this test, both of which compared well with similar parameters for the radioimmunoassay test. With regard to correct diagnosis, prolonged exposure of samples preserved with potassium dichromate to elevated temperatures and sunlight did not affect the progesterone concentration.
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Die optimale dag van melkmonsterneming vir die bepaling van dragtigheid met behulp van die melkprogesteroon-bepaling, sowel as die diagnostiese doeltreffendheid van die toets vanaf dag 14 tot dag 24 na inseminasie, is in melkbeeste bepaal. Uit die resultate blyk dit dat vir dag 22 en dag 23 na inseminasie 'n diagnostiese betroubaarheid van ongeveer 100% verkry kan word van melkmonsters wat in kaliumdichromaat bewaar is. Die ensiemimmunologiese-bepalingstegniek het talryke voordele bo die radioimmunologiese-bepalingstegniek in terme van aanvangskapitaal, opgeleide personeel en fasiliteite vir die gebruik van radioaktiewe stowwe in die vesting van grootskaalse melkprogesteroon-toetsskemas. Verlengde blootstelling aan verhoogde temperature en sonlig het geen effek nie.
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## Introduction

Progesterone is a hormone secreted by the corpus luteum and is responsible for the maintenance of pregnancy in dairy cattle. A raised serum progesterone concentration during the first 3 or 4 days of the oestrus cycle is indicative of a prolonged functional corpus luteum and therefore, in the absence of any pathological condition, of pregnancy. Equilibration of progesterone in serum with that in other body fluids, such as milk, has led to the quantitation of progesterone in this medium for the diagnosis of pregnancy by Booth, Davies & Holdsworth (1979), and has subsequently been used extensively for pregnancy testing by the Milk Marketing Board in the United Kingdom. This technique has two main advantages, namely the early stage at which pregnancy can be detected and the high degree of accuracy of the diagnosis. Two techniques are described in the literature, namely radioimmunoassay (Holdsworth, Chaplin & Booth, 1979) and enzymeimmunoassay (Arnstadt & Cleere, 1981; Sauer, Foulkes & Cookson, 1981). The aim of the present study was firstly to emphasize the importance of the correct day of sampling to give maximum diagnostic efficiency, secondly, to compare the methodology and diagnostic efficiency of the radioimmunoassay (RIA) and the enzymeimmunoassay (EIA), and thirdly, to verify the stability of potassium dichromate-preserved samples, stored under extreme conditions, simulating those that may be found in hot climates.

## Materials and methods

### Milk samples

Samples of whole milk from the afternoon milking were collected on days 14 through 24 post-insemination from 46 Friesland cows, eight of which were inseminated on two different heats. Milk samples (40 ml) were preserved with 20 mg potassium dichromate as recommended by the National Milk Recording Scheme and stored at −20°C until assayed. For the study of the effect of temperature and sunlight on the stability of preserved milk, a single sample of 1 litre was collected on day 22 after insemination from two cows and 40 ml aliquots pipetted into sample bottles containing potassium dichromate as above.

### Radioimmunoassay of progesterone

Progesterone was quantitated utilizing a single antibody non-extraction iodine − 125 radioimmunoassay employing dextran-coated charcoal as separating agent (Nordiclab, Finland). Milk samples were defatted by centrifugation at 1 000 rpm at 4°C for 10 minutes. Aliquots of the lower aqueous phase were assayed.

All samples were assayed in duplicate, and the precision

of the assay was expressed in the form of a dose precision profile according to Ekins, Newman, Piyasena, Banks & Slater (1972).

### Enzymeimmunoassay of progesterone

Progesterone concentrations were estimated using a commercial (Noctech, Ireland) non-extraction solid phase microtitre plate immunoassay employing progesterone labelled with horseradish peroxidase as tracer. The antiserum against the bovine serum albumin conjugate of progesterone-11α - hemisuccinate was raised in rabbits and showed a cross reactivity of 4,6% with 17α-hydroxy-progesterone and < 0,20% with pregnenolone. The end-point was determined spectrophotometrically at 492 nm. At the time of assay, the samples were thawed and well mixed after which aliquots of the whole milk were used for the assay.

### Reference pregnancy test

All cows were subjected to rectal palpation for confirmation of pregnancy from 2 months after insemination by a veterinarian experienced in this technique. Examinations were repeated until a conclusive diagnosis could be made. Animals were classified as pregnant and non-pregnant according to the rectal examination results and returns to oestrus. These classifications served as a standard against which diagnosis by milk progesterone concentration was compared.

### Diagnostic efficiency of milk progesterone test

The diagnostic efficiency of the milk progesterone test was calculated according to the method of Kiesling & Watson (1980), which takes into account the effect of false positive and false negative diagnoses. A milk progesterone value of 1 ng/ml or greater was regarded as being indicative of pregnancy when using the RIA and 2 ng/ml and greater for the EIA.

### Effect of temperature and sunlight on the stability of milk progesterone concentrations

Aliquots of milk samples from both cows were exposed to temperatures of 25°C and 37°C continuously for 0 – 8 days and thereafter the milk was stored at −20°C until assayed. Aliquots from the same two cows were exposed to direct sunlight for 11 hours per day for 0 – 16 days and thereafter stored at −20°C until assayed.

## Results

### Radioimmunoassay of progesterone

The precision dose profile based on 140 random duplicate determinations is shown in Figure 1.

The profile shows a relative error of 18%, equivalent to 0,18 ng/ml, at the decision-making point of 1 ng/ml.

### Reference pregnancy test

Of 54 inseminations, 34 were classified as successful and resulting in pregnancy, while 20 were classified as unsuccessful. The mean milk progesterone values (RIA) for these two groups for days 14 through 24 are shown in Figure 2.

No significant difference was found between the two groups for days 14, 15 or 16. On day 17 the values were different ($P < 0,05$) whereas for days 18 – 24 the values were significantly different ($P < 0,01$).

### Diagnostic efficiency (RIA)

Using a limit of 1 ng progesterone/ml, animals were diagnosed as pregnant or non-pregnant for days 14 – 24. These diagnoses

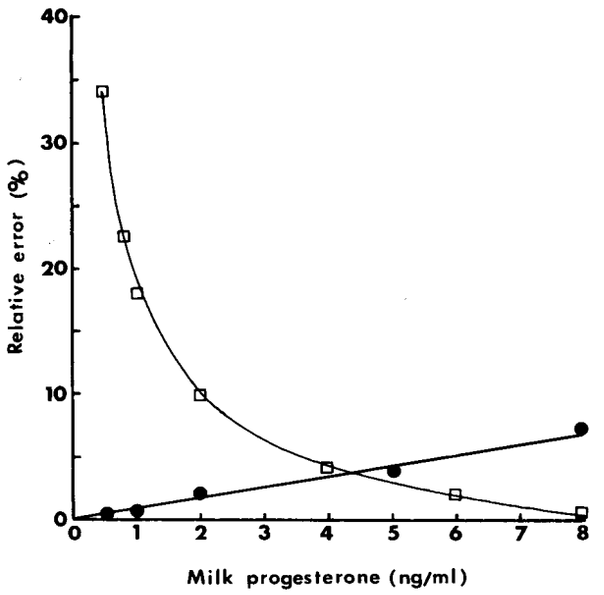


**Figure 1** Precision dose profile of the RIA (⊟–⊟) and EIA (–•–•–) milk progesterone assays.

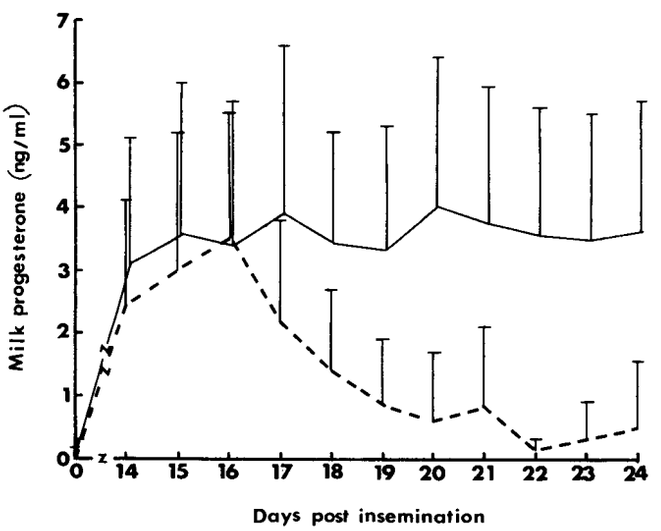


**Figure 2** Milk progesterone concentration (+ 1 *sd*) profile for pregnant (_____) and non-pregnant (--------) animals.

were compared to the reference pregnancy test diagnosis, and the efficiency of the milk test expressed in terms of the positive predictive value (ability to successfully identify a pregnant animal) and the negative predictive value (ability to successfully identify a non-pregnant animal). The predicitive values for days 14 – 24 are shown in Figure 3.

The positive predictive values rise slowly reaching a maximum of 100% on day 22 post insemination, whereas the negative predictive values rise sharply reaching values of 90% on day 18 and reaching a maximum of 100% on day 23 (95,6% on day 22).

### Enzymeimmunoassay of progesterone

The precision dose profile of the assay is shown in Figure 1. At the decision-making point of 2 ng/ml, the profile shows a relative error of 2% equivalent to 0,04 ng/ml.

### Diagnostic efficiency (EIA)

Using the progesterone values obtained from day 22 samples, the animals were classified as before, as pregnant or non-

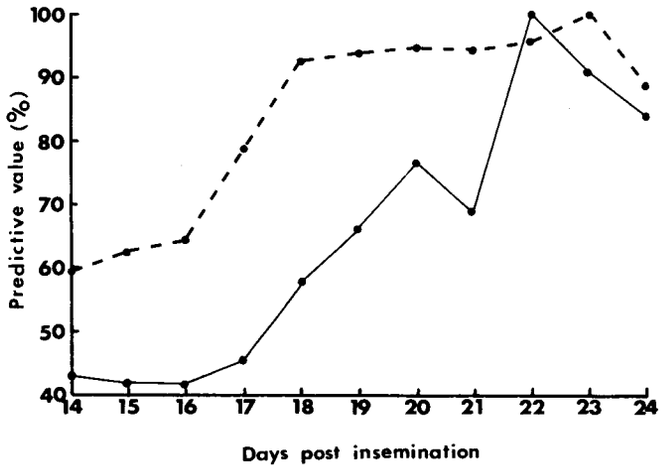


**Figure 3** Positive (_____) and negative (---------) predictive values for the milk progesterone test during early pregnancy.

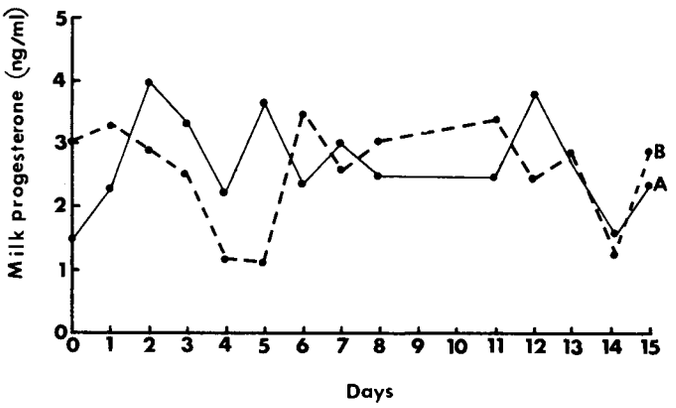


**Figure 5** Stability of series A (____) and B (--------) milk samples exposed to sunlight.

pregnant and then related to the reference pregnancy test diagnoses. A positive predictive value of 93,8% and a negative predictive value of 94,2% for the test was obtained for day 22 after insemination.

### Sample stability

The milk progesterone values obtained (RIA) for the two series (A and B) of aliquots of milk samples preserved with potassium dichromate and maintained at 25° or 37°C for 0 – 8 days are shown in Figure 4.

Despite coefficients of variation (CV) of 31,8% and 39,3% for series A at 37°C and 25°C, and 15,2% and 37,1% for series B at 37°C and 25°C respectively, the mean progesterone values remained constant.

The effect of sunlight on preserved milk samples is shown in Figure 5. The coefficients of variation were 30,6% and 32,8% for series A and B respectively, but at no stage did the initial diagnosis alter.

## Discussion

The results of this study indicate that the concentration of progesterone in milk of inseminated dairy cattle can act as an accurate pregnancy test, confirming the results of Booth, *et al.* (1979); Holdsworth, Booth, Sharman & Rattray (1980); Stupnicki & Kula (1980). Milk progesterone concentrations were highly significantly different between the pregnant and non-pregnant animals for days 18 – 24 after insemination, allowing accurate diagnosis. The diagnostic efficiencies on day 22 of the RIA method were 100% (positive) and 95,6% (negative) whilst those for the EIA were 93,8% and 94,2% respectively. In general, of the two possible results of insemination, the correction diagnosis of the non-pregnant animal is of more interest, there is little to choose from between the two diagnostic efficiencies (95,6% — RIA; 94,2% — EIA) and the choice of method would then be based on cost, capital equipment outlay, complexity, and requirement of special expertise or facilities. If a service laboratory is equipped for RIA work, then probably the RIA method would be the one of choice. However, if the laboratory is equipped for neither RIA nor EIA, the method of choice would be the EIA as the equipment outlay is small, the technique is robust and simple, requiring no special expertise and special facilities for the handling of radioactivity are not required.

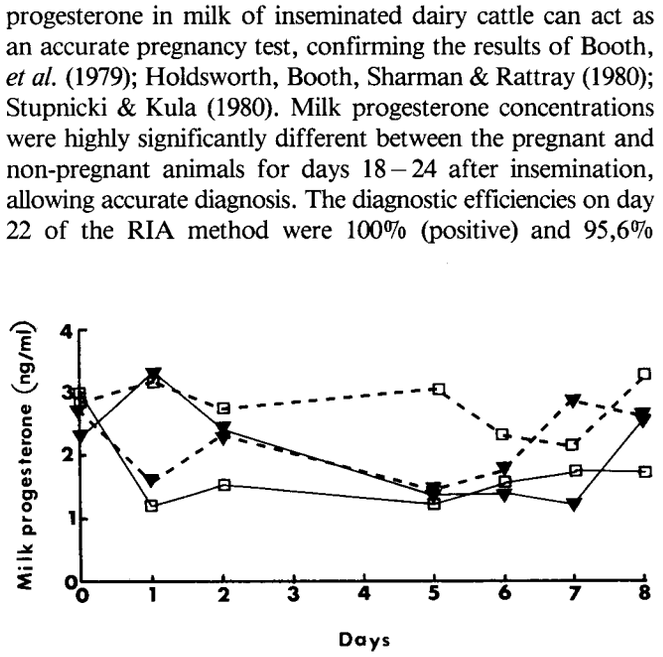


**Figure 4** Stability of milk progesterone concentration for series A at 37°C (⊟⊟), A at 25°C (▼▼), B at 37°C (⊟⊟), and B at 25°C (▼▼)

A number of authors have indicated the difficulty of motivating farmers to collect milk samples on a specific day after service (Bishop, Bond & Roberts, 1976; Booth & Holdsworth, 1976), however, little information is available indicating the degree to which the diagnostic efficiency of the test is affected. Arora, Batra, Pahwa, Jain & Pandey (1980) have shown that the percentage of correct diagnoses reached a maximum on day 23 for both pregnancy and non-pregnancy, however, this parameter is misleading in that false positives and false negatives are not taken into consideration. In this article the predictive value (which takes into account the sensitivity and specificity of the test which is essential in the context of making a diagnosis on a single laboratory result) has been calculated for the results of samples collected on days 14 – 24 after insemination. From Figure 3 it can be seen that the predictive value of the test for correctly diagnosing pregnancy from a single result is unacceptably low for days 14 – 20 (42% – 77%), reaching a short-lived maximum of 100% on day 22 after insemination. The profile for correctly diagnosing non-pregnancy from the result of a single milk sample reaches an acceptable level as early as day 18 after insemination (92%), increasing constantly to 100% on day 23 after insemination. These data clearly indicate that the success of correct diagnosis using the milk progesterone test is very dependent on the day of sampling.

Sample stability at various temperatures has been investigated by Bishop, Bond & Roberts (1976) (– 20°C); Heap, Holdsworth, Gadsby, Laing & Walters (1976) (4°C), and Pennington, Spahr & Lodge (1981) (– 10°C, 4°C, 10°C, 22 and 37°C), all of whom found that progesterone values remain unaffected. This study confirms that temperatures of 25°C and 37°C do not adversely affect the progesterone values although there were markedly high coefficients of variation

on determinations made on the same sample stored for different times, probably due to physical deterioration of the sample. Similarly exposure of the preserved samples to direct sunlight caused a deterioration in the physical condition (large CV's) of the sample but not the progesterone content.

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