ANALYSIS OF FACTORS LIMITING ON-FARM CLINICAL USE OF RAPID PROGESTERONE TESTING

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SUMMARY

To evaluate four commercial enzyme immunoassay progesterone test kits, milk samples (25 ml each of foremilk, bulkmilk and after milk) were collected at various intervals after milking from animals with various reproductive status. Effects of protein and fat content, high somatic cell counts and quarters were evaluated by comparison between diseased and clinically normal quarters (n=33 pairs) and between normal quarter (n=8). Accuracy of enzyme immunoassay was determined by calculating the percentages of similarity with radioimmunoassay progesterone values. Results obtained showed that neither composition type nor time of milk sampling had effect on progesterone concentration. Each of kits obtained an accuracy acceptable for clinical use. Data suggest that on farm progesterone tests are potentially useful management aids to confirm oestrus and cyclicity.

INTRODUCTION

Progesterone in either blood or milk is a reliable indicator of reproductive activity in cattle (Hoffmann and Hamburger, 1973; Foote, 1987). As an indicator of the cow's reproductive status, progesterone can play an important role in reproductive management. Determination of progesterone in milk has been possible since the early sixties. But until recently, the technique required the use of radioisotopes, and was effectively confined to specialized laboratories. Practical application of the technique to the dairy industry was therefore limited and results were not available quickly to be acted upon on the farm (Hoedemaker and Grunert, 1984). However, the advent of rapid on farm enzyme immunoassay test kits has removed many of the constraints imposed by radioisotopes. Now the farmer and veterinarian have access to results within minutes (Drew, 1986; Elmore, 1986; Arnstadt *et al.*, 1987; Nebel *et al.*, 1989). As these enzyme test kits are further refined, there has been increasing awareness that milk sampling is a constraint, because progesterone concentration in milk depend on type of milk. Though principles of progesterone assay are similar, procedures for performing rapid on farm enzyme immunoassay are specific for each company producing a test kit, particularly the type of milk to use (Eddy and Clark, 1987). Disadvantages of sampling aftermilk, for example, lies in the need for the cow to be milked out before sampling is carried out. This slows availability of results (Schiano *et al.*, 1975; Hoedemaker and Grunert, 1984). Apparently no concrete evaluation has been carried out to find out the effect of using any type of milk on accuracy of rapid on farm test kits. The objectives of this study were to evaluate the use of four rapid enzyme immunoassay test kit for ease of use, accuracy in determining progesterone concentration in milk and to determine

if the percentage of milk fat affects the results of these test kits.

MATERIALS AND METHODS

Experimental plan and analytical methods

Experiment 1:

In order to evaluate the effects of time interval between milking and sampling as well as type of milk on progesterone concentration, three pregnant (three month pregnancy established by hand palpation), three mid-luteal phase (Day 12 - 15 after observation of oestrus) and two non-cycling Germany Simmental cows aged 2 to 6 years, housed at the University of Munich Animal Clinic were assigned to this experiment. Milk samples were collected into polypropylene tubes for 3 consecutive days during the morning and afternoon milking. First, 25 ml of foremilk was hand drawn before the foremilk was hand drawn before the milking process into a sterile 30 ml tube. Following the completion of milking, a further 25 ml sample of post milk strippings was collected into a second sterile 30 ml tube. A representative sample of bulk milk (25 ml) was taken after thorough agitation from the bucket containing the complete milking (total bucket yield) into polypropylene tubes. Additionally, 25 ml of foremilk was collected from each cow at 3, 6 and 9 hour post morning milking. All milk samples were stored without preservatives at -18^0C. blood was collected by venipuncture after morning milking into heparinised receptacle centrifuged at 1500g, 40^0C for 15 min, plasma was obtained and stored at -18^0C. In this experiment, concentrations of progesterone were determined by using radioimmunoassays described by Hoffmann *et al.* (1973) for blood and by Hoffmann and Haburger (1973) for milk. Four commercial rapid enzyme immunoassay progesterone test kit Enzygnost(R) serum test kit, Hoechst Co., Munich, F.R. Germany; Progestassay(R) milk test kit. Pitman Co., USA; Hormonost(R) kit, Biolab Co., Munich, F.R. Germany; Reprostrip(R), Epignost Co., Linz, Austria) were performed according to the directions provided with each kit.

Experiment 2:

In order to evaluate the effects of high somatic cell counts, milk fat percentage and type of quarter on progesterone concentration in milk, six hundred Germany black and white cows of all ages were screened during veterinary ambulatory clinics for mastitis using California Mastitis Test (CMT; Schalm, 1960). A quarter with a CMT of $<1.5 \times 10^6$ cells/ml was characterized as normal (control) and that with a CMT of $>5 \times 10^6$ cells/ml was characterised as mastitic. 50 ml each of foremilk from the left forequarter of 5 cows were collected in sterile polpropylene tubes daily for 30 consecutive days. 50 ml each of foremilk from one mastitic and one normal quarter (paired milk samples) from 33 mid-luteal phase cows and also 50 ml each of foremilk from normal left forequarter and right hindquarter of 8 mid-luteal phase cows were collected into tubes containing sodium azide tablets (Merck Art 6687/100 mg in 35 ml milk; Darmstadt, F.R. Germany) as a preservative. All milk samples were vortex mixed and aliquots of 10 to 15 ml were immediately dispensed into 3 separate tubes. Two aliquots were stored overnight at 4^0C for milk fat and protein analysis and submitted to a government of Bayern laboratory (Milchprufring Bayern e.V., Untersuchungsstelle, Troppauerstrasse 10, Munich) where they were analyzed similar to samples submitted from

commercial herds for milk fat analysis using a Gerber method and for protein Kjeldahl technique (Hauke, 1965). All aliquotes for progesterone determination were kept frozen at -18°C. Progesterone analysis in milk was carried out by use of radioimmunoassay (Hoffmann and Hamburger, 1973) and by Enzygnost® rapid enzyme immunoassay test kit.

Experiment 3

The use of the on-farm progesterone test kits for prevent insemination errors was also evaluated using both determination of progesterone in milk by radioimmunoassay and Enzygnost® enzyme immunoassay test kit. The protocol was to test cows for progesterone not later than 12 hours from when service was done. A total of 1044 cows presented for insemination to the University of Munich veterinary clinic were used in this study. Cows were inseminated on basis of determination of vaginal mucus electrical resistance according to the method described by Metzger *et al.* (1972). Milk samples were taken prior to insemination; All samples were foremilk. After the udder was washed, disinfected and dried, first milk was thrown away and foremilk collected. Progesterone analysis was carried out within 12 hours of sampling. If the progesterone test indicated high progesterone, additional milk sampling was done every other two days until low progesterone was obtained. Relative concentrations of progesterone were classified as low or high by comparison with standard progesterone samples, which were supplied with each kit. Pregnancy diagnosis for all 1044 cows was done by rectal palpation of the genital organs 8 to 10 weeks after service.

Progesterone determination by rapid test kit

Milk samples were thawed at 25°C, mixed thoroughly by homogenization for 6 sec and progesterone determine. Four commercial progesterone tests were performed immediately after thawing. Progesterone concentrations were classified visually by comparison with standard progesterone samples, which were supplied with each kit. The progesterone concentration was characterized as low, intermediate or high at the recommended processing time only on the basis of comparison of colour (no colour, light, dark colour) development. For Hormonost® and Enzygnost® tests, samples were determined photometrically at 492 nm. Enzygnost® samples initially were determined by a microtitre plate spectrophotometer (MR 700 - plate reader: Dynatech GmbH. Frankfurt, F.R. Germany). Then 300ul of sample was transferred from each well of the microtitre plate into curvettes. 400 microliter distilled water was then added and samples read by a standard spectrophotometer (PCP 6121, Eppendorf Co., Hamburg, F.R. Germany). In each of the assays mentioned above high and low progesterone control samples were included for the determination of intraassay and interassay coefficient of variations.

Statistical Analyses

An objective of this study was to determine the accuracy of results obtained from four commercial rapid milk progesterone kits. To measure accuracy of classification of progesterone concentration into low and high levels by commercial tests, the percentage of similarity with radioimmunoassay progesterone values was determined by using discriminant analysis, chi-square and student t-test. To determine the effect of the

content of milk fat or content of protein on progesterone classification by four commercial progesterone tests, progesterone classification by each test was regressed (linear and quadratic) and was assessed by correlation analysis (Weber, 1972).

RESULTS

Results of effects of time interval between milking and sampling as well as type of milk progesterone concentration are shown in Figure 1 and Table 1. Assessment of reproductive status (pregnancy, cycling mid-luteal or non cycling) of 8 cows by radioimmunoassay was assigned scores of + ve (correct) when test assessment agreed with true reproductive status established by rectal palpation of genital organs and negative when false. Total assessment was 100% correct (all + ve scores) for classification of concentration of progesterone for each type of milk. Estimation of progesterone based on colour development and visual interpretation by enzyme immuno test kits was also assigned scores of +ve (correct) when test kit interpretation based on visual interpretation agreed with progesterone concentration determined by radioimmunoassay -ve when false. These scores were tested for an association between kit and type of milk. The high incidence of misclassification was observed by Enzygnost(R) for postmilk strippings (83.3% +ve scores). Further analysis showed that effects of type of milk, progesterone concentrations were less strong than those due to the method of interpretation of the results (Visually vs spectrophotometric). In test kits where both visual and photometric interpretations were made photometric interpretation were superior and in most cases agreed 100% to classification by radioimmunoassays; whereas visual interpretation agreed 90% to the classification. Concentration of progesterone in foremilk, bulkmilk, aftermilk and in - between milking samples collected at various time intervals after milking (Figure 1) were not significantly different ($P > 0.05$) from each other when Enzygnost(R) test kit was used, though a tendency towards increased progesterone values with increase in the interval from milking was marked. Radiommunoassay values showed significant differences between foremilk and and aftermilk, especially during the same milking. Radioimmunoassay values were two to three fold lower than those of Enzygnost(R) test kit; but the two were highly correlated ($r = 0.9$; $P < 0.01$). The same pattern was observed by high and low progesterone concentration.

Results of effects of milk fat percentage, high somatic cell counts and type of quarter on progesterone concentration are shown in Figure 2 and Table 2. Fat content varied between 2.5g/ 100 ml and 8.5g/100 ml for foremilk. The content of fat in milk or an interaction of the percentage of milk fat by day of the oestrous cycle did not influence progesterone based on colour development and visual interpretation by enzyme immuno test kits was also assigned scores of +ve (correct) when test kit interpretation based on visual interpretation agreed with progesterone concentration determined by radioimmunoassay and +ve when false. These scores were tested for an association between kit and type of milk. The high incidence of misclassification was observed by Enzygnost(R) forpostmilk strippings (83.3%) +ve scores). Further analysis showed that effects of type of milk, progesterone concentrations were less strong than those due to the method of interpretation of the results (visually vs spectrophotometric). In

Table 1: Accuracy of Progesterone Classification into Different types of milk using four commercial enzyme immunoassay tests.

Progesterone	Interpretation	Type of Milk Fore-milk N	A	Bulk-milk N	A	After-milk N	A
Radio-immunoassay	Photometric	36	100	36	100	36	100
Enzygnost	visual	36	97.2	36	88.9	36	83.3
	Photometric	36	100	36	88.9	36	100
	Minireader	36	100	36	100	36	100
Hormonost	Photometric	21	95.2	21	100	21	100
Reprostrip	visual	18	100	18	100	18	100
Progestassay	visual	18	72.2	18	94.4	18	94.4

(A) Accuracy was determined according to the percentage of similarity in progesterone concentrations determined quantitatively with radiommunoassay and qualitatively with four enzyme immunoassay tests.

(N) total number of samples.

Photometric = interpretation was done by use of a standard photometer

Minireader = interpretation was done by use of a microtiter plate photometer.test kits where both visual and photometric interpretations were made, photometric interpretations were superior and in most cases agreed 100% to classification by radioimmunoassay; whereas visual interpretation agreed 90% to the classification. Concentration of progesterone in foremilk, bulkmilk, aftermilk and in between milking samples collected at various time intervals after milking (Figure 1) were not significantly different ($P>0.05$) from each other when Enzygnost® test kit was used, though a tendency towards increased progesterone values with increase in the interval from milking was marked. Radioimmunoassay values showed significant differences between foremilk and aftermilk, especially during the same milking. Radioimmunoassay values were two to three fold lower than those of Enzygnost® test kit; but the two were highly correlated ($r=0.9$; $P<0.01$). The same pattern was observed by high and low progesterone concentration.

Results of effects of milk fat percentage, high somatic cell counts and type of quarter on progesterone concentration are shown in Figure 2 and Table 2.

Fat content varied between 2.5g/100ml and 8.5g/100 ml for foremilk. The concentration of fat in milk or an interaction of the percentage of milk fat by day of the oestrous cycle did not influence progesterone concentration determined by

Figure 1

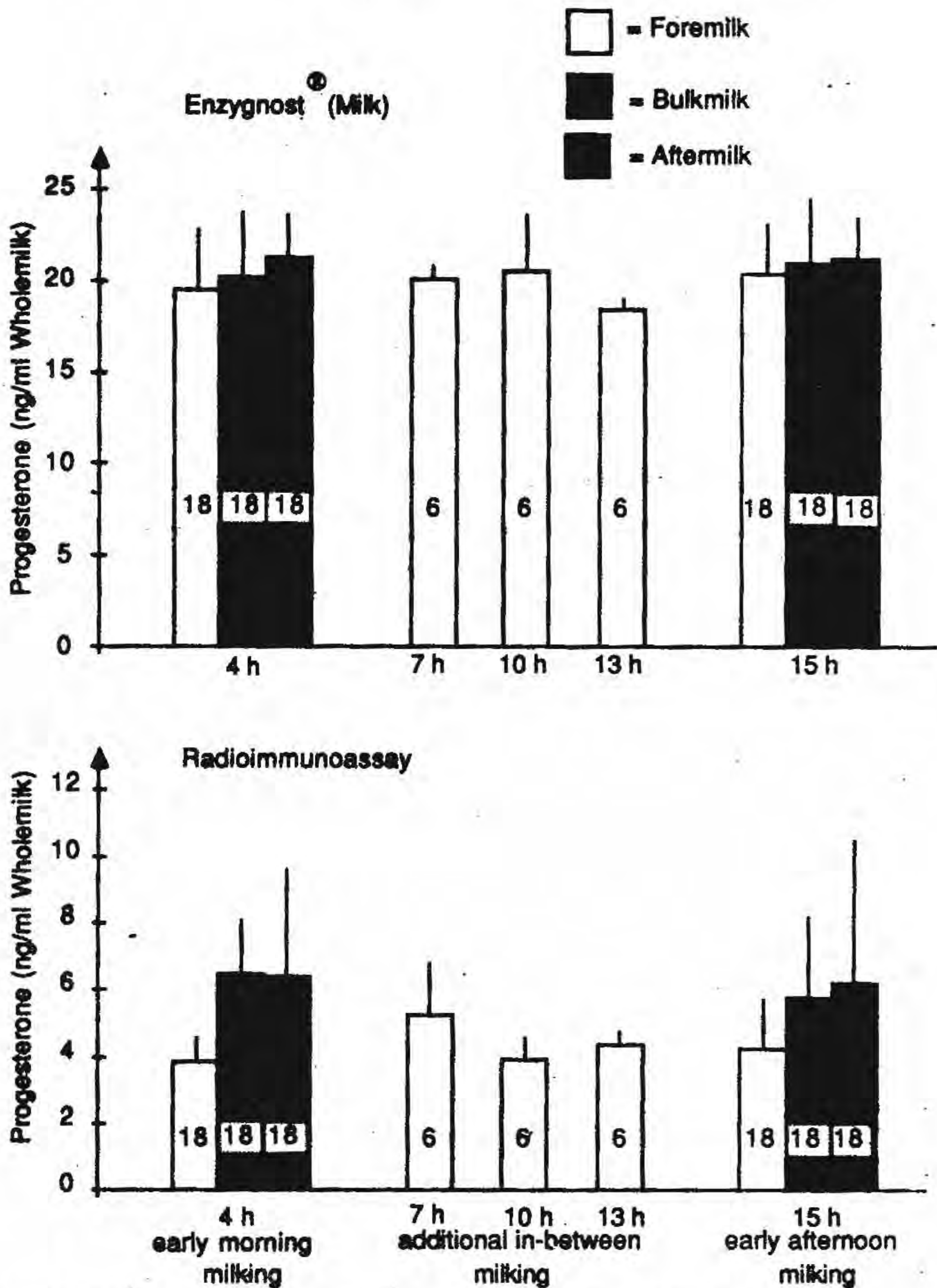


Fig. 1: Concentrations of progesterone in different types of milk obtained at different times of the day from six (6) cows with active Corpus luteum (Mean and standard deviation; Values in columns = number of samples).

Table 2: Comparison of progesterone concentration between two quarters determined by Enzygonost[I] milk test kit.

(a) Comparison between mastitic (high somatic cell count), diseased and clinically normal quarter.

		Foremilk quarter (n =33 pairs) mastitic	normal
Progesterone (ng/ml)	low (n=16)	3.1±2.1	2.9±1.8
	high(n=17)	21.1±3.8	21.4±3.3
Fat (g/100ml)		3.2±1.2	3.0±1.3
Protein (mg/100ml)		3.8±0.5	3.8±0.4

b) Comparison between two clinically normal quarters (n=8 pairs)

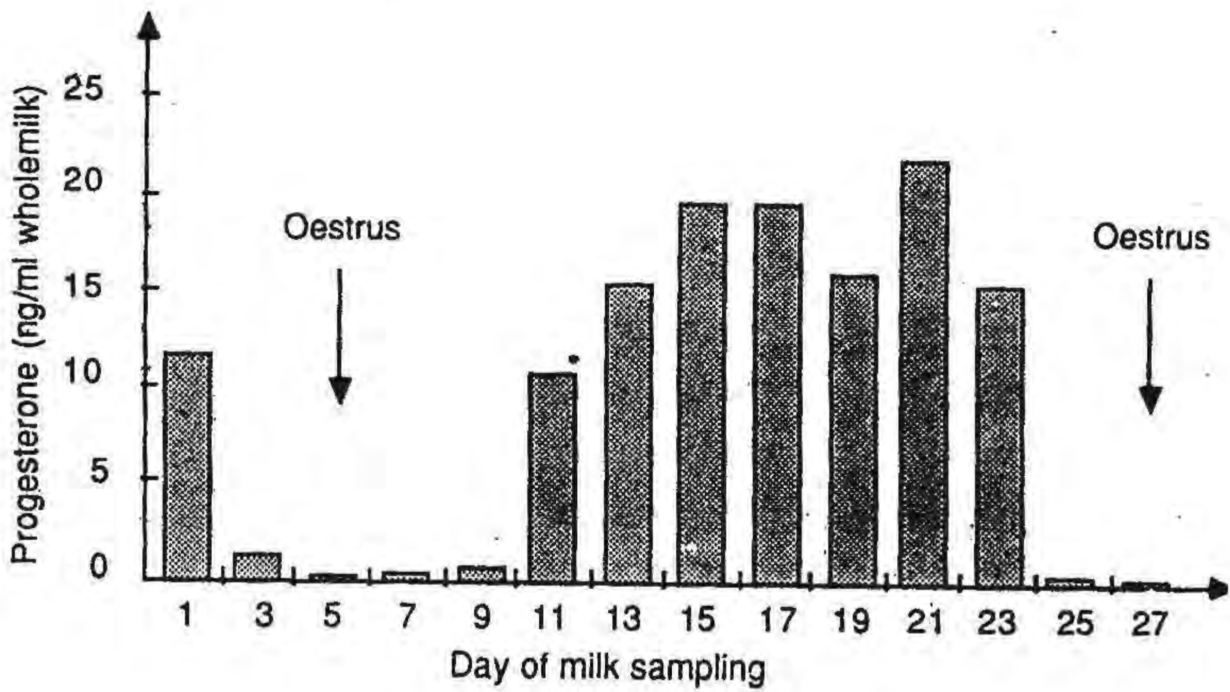
	Quarter left forequarter	right hindquarter
Progesterone (ng/ml) high (n=6)	20.8±3.2	20.9±2.9
Fat (g/100ml)	3.3±1.0	3.3±1.3
Protein (mg/100ml)	3.7±1.2	3.8±0.6

[I] In a and b, the level of somatic cell content was determined by the California Mastitis Test according to Schalm (1960).

Table 3: Results of rectal palpation, progesterone concentration and vaginal mucus electrical resistance in cattle wrongly inseminated at time of high progesterone concentration

Diagnosis	Progesterone (ng/ml)	Vaginal Mucus Resistance (Ohms)
Endometritis (n=6)	18.5±5.7	34.0±2.5
Ovarian Cysts (n=6)	17.3±6.7	30.8±8.9
Early pregnancy (n=7)	18.2±4.2	40.0±8.6
Normal (n=40)	17.2±5.9	35.1±6.3

Figure 2



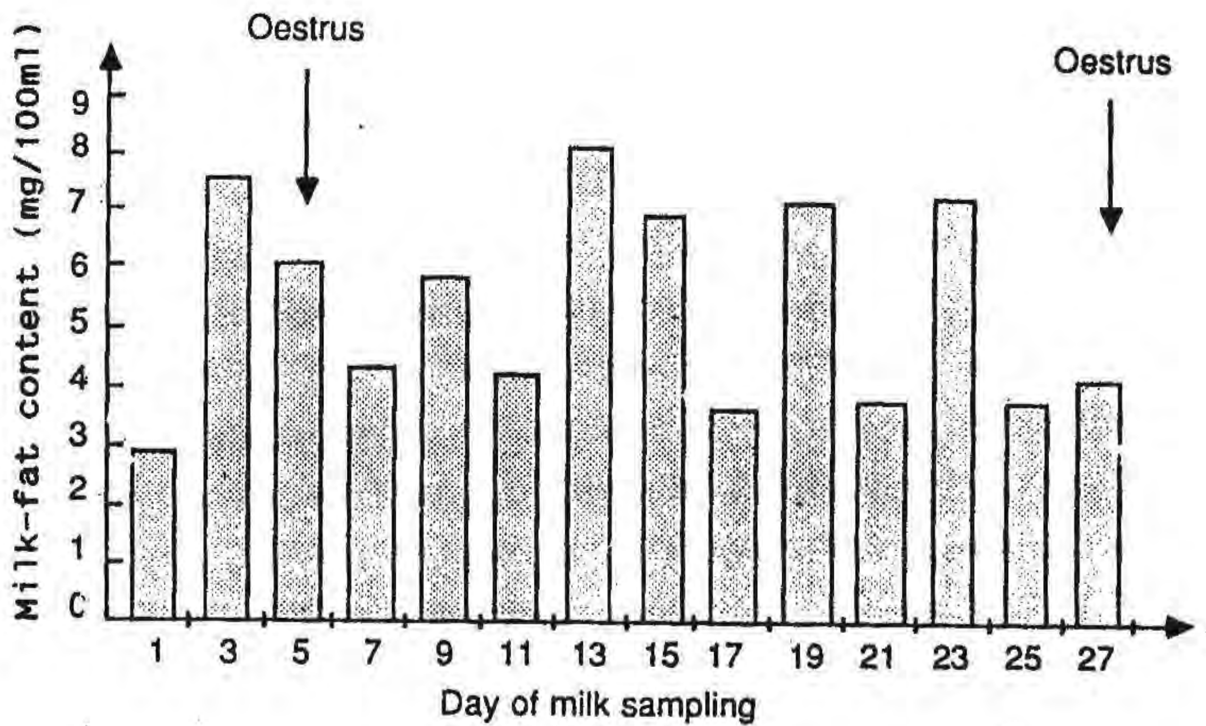


Fig. 2 Concentration of progesterone and milk-fat content in wholemilk for a complete oestrous cycle of a cow.

Figure 3

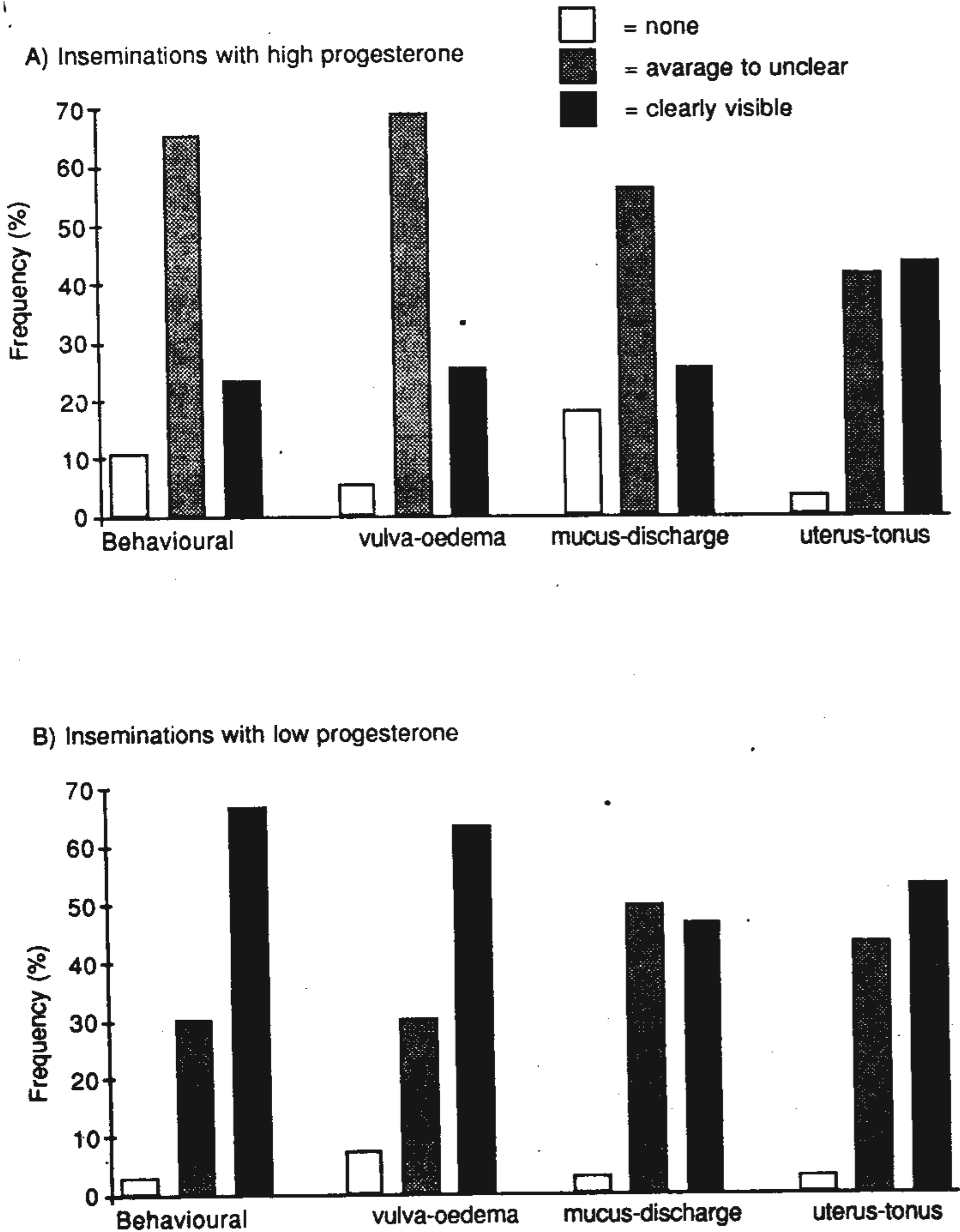


Fig 3: Comparisons for intensity of oestrous behavioural signs at times of insemination carried out after observation of oestrus mimicing signs but having high progesterone values and after low progesterone values were obtained.

Enzygnost[R]. No relationship was found between the percentage of milk fat and day of oestrous cycle, type of quarter of somatic cell content. progesterone values represented the stage of the reproductive cycle rather than composition of milk.

The use of radiommunoassay and on farm progesterone testing to prevent insemination errors showed that 59 cows (5.7%) were inseminated wrongly. Progesterone concentration determined by Enzygnost[R] test kit were closely associated with type of oestrous signs the cow exhibited. A progesterone concentration of <5.5 ng/ml showed clearest signs of oestrus with pregnancy rates of 57.7% (n=899; Figure 3 and Table 3). Those with progesterone concentrations of >10 ng/ml gave low pregnancy rates (16.7%), and cows exhibited poor signs of oestrus at insemination. Detailed analysis on causes of mid-luteal phase insemination of 59 cows revealed that, 6 cows had endometritis, 6 ovarian cysts, 7 were in early pregnancy and 40 had normal luteal function. In all these cases progesterone and vaginal mucus electrical resistance values were high.

DISCUSSION

In this study Enzygnost[R] test kit was chosen for use in several experiments because preliminary results showed that data could be analyzed both visually and photometrically. The intraassay coefficient of variation for milk and serum Enzygnost[R] test kits was less than 6.8% and therefore similar to results obtained by Stanley *et al.* (1986), Theissen (1986) and Worsfold *et al.* (1987). The interassay coefficient of variation was below 16.8% and also within the range of 1.4% to 17.9% given in literature for enzyme immunoassay (Ginther *et al.*, 1976; Fantly *et al.*, 1981; (Yasuhara and Kleeberg-Ruppert, 1984; Edward-Allen and Porter, 1987; Oikawa *et al.*, 1987). For most practical applications, on farm enzyme immunoassay milk progesterone testing is recommended for assessment of endocrine status to estimate stage of oestrous cycle. In this study, there were no differences between kits in their ability to predict progesterone status, a finding also reported by Nebel *et al.* (1989). However, accuracy of prediction varies for all kits according to type of milk (Table 1). Most kits that utilized aftermilk were slightly more accurate than those using foremilk. However, for practical purposes, no differences were found between test kits in terms of accuracy of predicting the reproductive status of the cow. Different methods of interpretation, visual against spectrophotometric, contributed to and were responsible for the differences in the progesterone values. The values obtained by visual interpretation were 10% lower than those obtained by photometric determination. This can be explained in that experience is required in the separation of different shades of colour. The high incidence of misclassification probably results from colour responses which overlap with those produced by standard samples. Similar results have been report by Sauer *et al.* (1986).

Progesterone concentrations in milk were measured using enzyme immunoassays and compared with radioimmunoassay. In general the assays showed similar trends in progesterone concentration changes which occur during various reproductive states, but absolute values varied considerably. In agreement with many other authors measurement of progesterone by enzyme immunoassay gave higher concentrations that those by radioimmunoassay (Hoffmann *et al.*, 1976; Gunzler *et al.*, 1979; Arnstadt

and Schmidt-Adamopoulou, 1982; Holtz *et al.*, 1986; Edward Allen and Porter, 1987).

The percentage of fat in milk or the interaction of high somatic cell content or protein by day of oestrous cycle did not influence results of Enzygnost[R] test. This effect of fat emphasizes the necessity and advantage to sample foremilk for rapid progesterone determination.

The use of on farm milk progesterone tests to predict insemination errors reduced the number of cows wrongly inseminated, which was at 5.7% in this study. The high incidence of misclassification of cows in oestrus probably resulted from conditions which looked similar to oestrus, but which could not be clearly identified. This was a small number of cattle. To make use of the enzyme immunoassay tests economically and profitably need must exist either because of a higher incidence rate of errors in detection of oestrus or higher semen costs. Otherwise, the test can only be used in problem cows, and not to the whole herd as earlier suggested by Heap *èt al.* (1976).

In conclusion, all commercial test kits evaluated in this study obtained accurate values for use in bovine fertility clinics. The small differences observed between the kits due to fat or type of interpretation may not necessary affect the overall interpretation of the results.

ACKNOWLEDGEMENTS

We are grateful to Danish Agency for International Development (DANIDA) and the Sokoine University of Agriculture for the financial support. This work was partly funded and carried out at Professor W. Leidls' Laboratory in Munich, Federal Republic of Germany.

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