

THE USE OF ENZYME IMMUNO ASSAY METHOD FOR MEASUREMENT OF MILK PROGRESTERONE WITHOUT EXTRACTION FOR EARLY PREGNANCY DIAGNOSIS IN COWS

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RINGKASAN

Metode *Enzyme Immuno Assay* (EIA) untuk Progesteron dengan penggunaan horse radish peroxidase (HRP) sebagai label, telah digunakan untuk pengukuran langsung progesteron dalam air susu. Metode ini digunakan untuk mendeteksi kebuntingan dini.

Penelitian telah dilakukan dengan menggunakan enam puluh ekor sapi laktasi dibagi dalam tiga kelompok, yaitu kelompok kontrol dan dua kelompok lainnya sebagai kelompok perlakuan. Pada kelompok I (kelompok kontrol), sampel susu diambil pada saat inseminasi sampai hari ke-24 setelah inseminasi dengan selang waktu pengambilan tiga hari. Untuk kelompok II, sampel susu diambil pada hari ke-18, 21 dan 24 setelah inseminasi dan untuk kelompok III sampel susu diambil pada hari ke-21 dan 24 setelah inseminasi.

Sampel-sampel susu tersebut diperoleh dari sapi-sapi laktasi pada pemerahan siang dari keempat kwartir dan diawetkan dengan kalium dikromat (Merck 4858), disimpan pada suhu -20°C sebelum digunakan.

Diagnosis kebuntingan dengan EIA dikonformasikan dengan palpasi perrektal pada hari ke-60 sampai hari ke-90 setelah inseminasi. Diagnosa kebuntingan dini pada sapi kelompok II dibandingkan dengan kelompok III menunjukkan bahwa perpanjangan waktu pengambilan sampel tidak dapat diperkirakan mampu meningkatkan ketepatan pemeriksaan.

ABSTRACT

An Enzyme Immuno Assay for Progesterone, using horse radish peroxidase as the label, was adapted for direct measurement of progesterone in milk. This was carried out to detect early pregnancy in cows.

The experiment used sixty milking cows divided into three groups, one as a control and two others as treatment groups. In group I (control) milk samples were collected on the day of insemination to 24 days afterward by quartan collection. In group

II, milk samples were collected on days 18, 21 and 24 after insemination. In group III, milk samples were collected on days 21 and 24 after insemination.

Milk samples were collected from lactating cows at noon from four quarters and preserved with Potassium dichromate (Merck 4858), stored at -20 degree C until analysed.

Pregnancy diagnosis by EIA was confirmed by rectal palpation at 60 to 90 days after insemination. The early pregnancy diagnosis in Group II compared with Group III showed that prolonged the time of sampling indeclinable improved the accuracy.

INTRODUCTION

The ideal calving interval for milk production in the dairy cows is 12 months. To achieve this target the management situation must be good and, furthermore, estrus detection and artificial insemination techniques need to be very well developed. It is, therefore, necessary to identify, as early as possible, cows that remain open after breeding to permit early rebreeding or may be culled.

The possibility of monitoring reproductive function of cows and conducting early pregnancy diagnosis is very important in dairy management. This may partly be done through careful estrus detection and recording as well as clinical examination of cows before artificial insemination. Analysis of progesterone in milk offers a possibility of closely monitoring cyclic ovarian activity (Robertson and Sarda, 1971).

Progesterone secreted mainly from corpus luteum plays an important role in human and mammalian reproduction, especially in preparing for nidation for maintaining pregnancy (Hafez, 1987). Therefore progesterone levels provide a very useful information for the diagnosis of pregnancy in some species (Heap *et al.*, 1973).

In dairy cows, milk sampling can be easily performed and progesterone in milk is much more stable than in blood plasma (Marcus and Hacket, 1986).

Thus, milk is one of the best fluid choices for progesterone assay.

MATERIAL AND METHODS

I. Animal

The experiment used sixty milking cows divided into three groups, one as a control and two others as treatment groups. In group I (control), milk samples were collected on the day of insemination to 24 days afterward by quartan collection. In group II, milk samples were collected on days 18, 21 and 24 after insemination. In group III, milk samples were collected on days 21 and 24 after insemination.

2. Enzyme Tracer

Horse radish peroxidase (HRP - Boehringer 814407) was conjugated with 4 Pregnane 6 B - 01 - dione hemisuccinate (Steraloid Q 3225).

3. Antibody

Antibody monoclonal Progesterone (Ig - M_{Ca} Prog.) was made by Monoclonal Antibody Techniques (Booman *et al.*, 1984).

Enzyme Tracer, Horse Radish Peroxidase coupled to Progesterone (HRP - P) and Antibody Monoclonal to Progesterone (Ig - M_{Ca} P) were obtained from

Dr Van de Wiel (IVO Schoonoord), The Netherlands under similar laboratory cooperation. All materials used have been previously described by Van de Wiel and others as method that developed at IVO Schoonoord (Van de Wiel and Koops, 1986).

4. Assay Procedures

A Microtitration plate (Costar 3590) was coated with 100 μl /well of Ig - M₁ Prog., diluted 1 : 20.000 in 0.05 M bicarbonate buffer (coating buffer), pH 9.6, containing Thimerosal (0.02%), Na₂CO₃ · 10 H₂O (0.29%) and NaHCO₃ (0.293%). The plate was sealed with accompanying lid, incubated overnight (15 hours) at 4°C, then drained, coated with BSA coating solution and washed with Tween 80 (0.05%). Then the plate was drained and dried by inversion on absorbent paper. It can be directly used or stored into aluminium bag in drying material for further analysis.

Samples of milk were 0.5 μl /well, diluted 1 : 200 in assay buffer (BSA 1%, Thimerosal 0.02% in 0.04 M PBS + 0.15 M NaCl, pH 7.2 ± 0.02), added 100 μl to the wells except the first column.

Progesterone dissolved in ethanol (62.9 nmol) was used for standard, diluted from 0, 1,625, 3,125, 6,25, 12,5 and 25 $\mu\text{g}/100 \mu\text{l}$ in assay buffer + 0.05% Progesterone free milk and then put into designated wells.

Enzyme tracer (15 ng/ml) was added and the plate shaken on plate shaker 500 shakes/min for 2 minutes, incubated at 37,5 degree C for 1 hour, emptied, washed five times with 0.05% Tween 20 (Merck 922187) and inverted on absorbent paper.

Substrate solution (Aquabidest 15 ml, TMB 200 μl , and PO buffer 1.5 ml : 1 M acetate + 0.5 M citric acid + 0.05% H₂O₂, pH 5.6) was added 150 μl to each well and the plate was incubated at room temperature for 40 minutes in the dark. The reaction was stopped by adding 4 N H₂SO₄ and shaken on plate shaker.

The absorbance was determined at 450 nm, in a CLS (Commonwealth Scientific Laboratory - UK) - 962 Spectrophotometer.

Progesterone levels of pregnant and non pregnant cows of Group I (control) are presented in Figure 1. Based on discriminant values of Group I, the detected pregnancy in Groups II and III, is shown in Table 1.

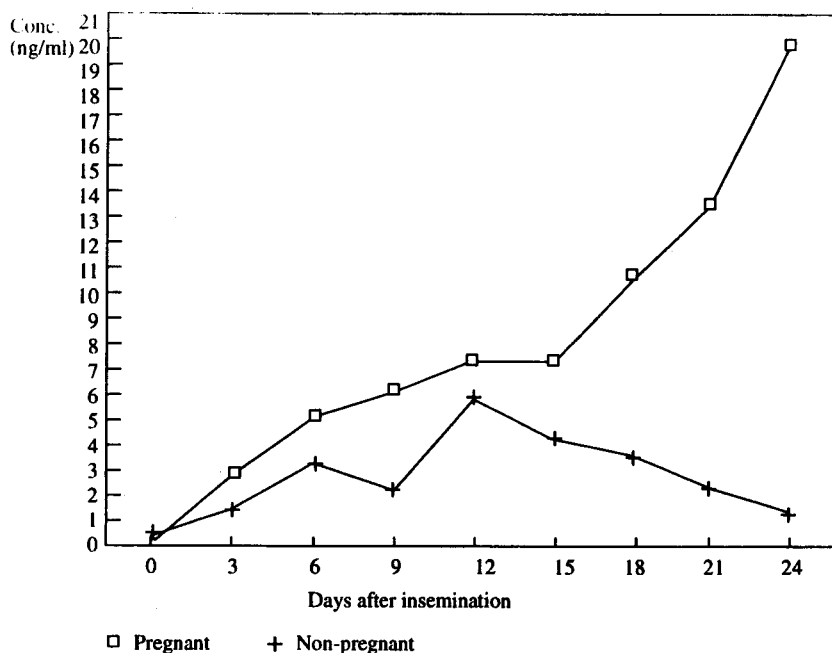


Figure 1. Progesterone levels of pregnant and non-pregnant cows of Group I (Control)

Of 20 cows tested in Group II, pregnancy was indicated by high milk progesterone levels in 9 cows, only 3 cows were described as dubious. Twelve cows were confirmed to be pregnant by rectal palpation 60 – 90 days after insemination (Table 2). Eight cows with low progesterone levels were considered to be non pregnant. Confirmation of non preg-

nant by rectal palpation was in agreement with EIA (100%).

In Group III, of 20 cows tested, pregnant was indicated by high milk progesterone levels in 10 cows and 2 cows were dubious (Table 3). Twelve cows were confirmed pregnant by rectal palpation. Eight cows with low milk progesterone levels were confirmed non pregnant by rectal palpation (100%).

Table 1. Discriminant values of progesterone on days 18, 21 and 24 after insemination based on Group I (Control)

Diagnosis	days after insemination		
	18	21	24
Pregnant	10.7 ± 4.5	13.6 ± 4.0	19.9 ± 11.1
Non-pregnant	3.6 ± 3.2	2.3 ± 2.5	1.5 ± 3.1

Tabel 2. Early pregnancy diagnosis by EIA of milk progesterone on Group II

Diagnosis	Milk progesterone assay 18, 21 and 24 days*	Rectal palpation 60 to 90 days*
Pregnant	9	12
Dubious	3	—
Non-pregnant	8	8
Total	20	20

* days after insemination

Tabel 3. Early pregnancy diagnosis by EIA of milk progesterone on Group III

Diagnosis	Milk progesterone assay 18, 21 and 24 days*	Rectal palpation 60 to 90 days*
Pregnant	10	12
Dubious	2	—
Non-pregnant	8	8
Total	20	20

* days after insemination

DISCUSSION

Application of EIA in this experiment was done to detect pregnancy by monitoring milk progesterone level, based on the approach of Robertson and Sarda (1971).

An elevated level of progesterone indicates that prolonged luteal function is always the case during pregnancy (Laing *et al.*, 1987). It was initially considered necessary to establish a basal progesterone level characteristic of pregnancy. Twenty cows in Group I (Control) were monitored for 24 days following breeding. Profile of milk progesterone determined for group I is presented in Figure 1.

The present results suggest that pregnant and nonpregnant cows were distinguished better by testing milk samples from days 21 and 24 (Group III) than samples from day 18. Results of the test on day 21 showed that milk progesterone levels values above 13.6 ng/ml were indicative for pregnant and values below 2.3 ng/ml for non pregnant. Determination of milk progesterone level on day 24 after breeding was considered useful for the confirmation of pregnancy if milk progesterone value on day 21 was dubious (2.3 – 13.6 ng/ml).

The early pregnancy diagnosis in Group II compared with Group III showed that prolonged time of sampling

indeclinable improved the accuracy (Chang and Estergreen, 1983).

Dubious diagnosis of pregnancy probably due to individual variation in progesterone level (Marcus and Hacket, 1986). Progesterone is a lipophilic substance (Estergreen *et al.*, 1977; Kassa, 1986; Hafez, 1987) therefore different milk fat content and different stage of lactation could alter hormone concentration in the milk (Naka *et al.*, 1982; Kon and Cowie, 1961).

In conclusion, EIA technique for progesterone determination in milk is a useful tool and was demonstrated successfully to detect early pregnancy. Although the values of progesterone are not absolute, they may be used conveniently to assess the reproductive status of the cows rapidly and accurately (Marcus and Hacket, 1986).

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