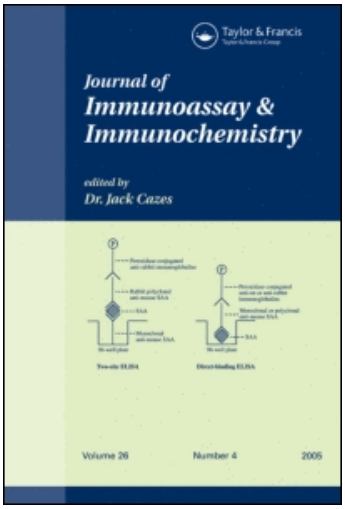

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ENZYME LINKED IMMUNOSORBENT ASSAY FOR MILK PROGESTERONE

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□ *A direct antigen heterologous enzyme linked immunosorbent assay (ELISA) for milk progesterone has been developed using progesterone-3-O-carboxymethyloxime-bovine serum albumin (P-3-O-CMO-BSA) antiserum and 17-α-hydroxy-progesterone-3-O-carboxymethyloxime-horseradish peroxidase (17-α-OH-P-3-O-CMO-HRP) enzyme conjugate. The data of the present study reveal that the homologous assay, which employed P-3-O-CMO-HRP as the label, showed no displacement. On the contrary, replacement of P-3-O-CMO-HRP with 17-α-OH-P-3-O-CMO-HRP as the label showed significant displacement and led to the development of a sensitive and specific assay. The recovery of the exogenously spiked progesterone from milk pools was in the range of 94.3–97.88% for toned milk and 97.6–101% for full-cream milk. The intra-assay and interassay coefficients of variation (CVs) ranged from 4.1–7.8% and 4.4–7.0%, respectively. A high ionic strength buffer was used to obtain released progesterone from binding protein/fat. The progesterone values measured in toned and full-cream milk ranged from 1.198–9.745 ng/mL and 6.949–14.923 ng/mL, respectively. The milk progesterone values obtained by this method correlated well with those obtained by radioimmunoassay; $r = 0.95$ ($n = 65$).*

Keywords 17-α-OH-progesterone, antigen heterologous, chaotropic agent, direct, immunoassay, progesterone

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INTRODUCTION

Progesterone, a C_{21} steroid hormone secreted by the corpus luteum, is associated with the establishment and maintenance of mammalian pregnancy and monitoring of the estrous cycle, and hence the reproductive status of animals.[1] There is high correlation between progesterone concentration in blood and milk (e.g., $r = 0.88$).[2] The estrous cycle in cattle is 21 days. The progesterone concentration increases to 18 ng/mL from day 4 to day 12 of the estrous cycle, decreases to 4.4 ng/mL two to four days before estrous and during pregnancy, then it again increases to 24.8 ng/mL.[3,4] The cow is pregnant for about seven out of 10 months lactation, which indicates that at least two-thirds of milk is extracted from pregnant cows. Therefore, there is a need to establish normal ranges of hormones present in dairy products.

Enzyme immunoassays (EIA) developed so far for milk progesterone estimation give high cross-reaction with 11-α-OH-progesterone, 19-OH-progesterone, 5β-pregnane-3,20-dione, 5-α-pregnane-3,20-dione, 17α-OH-progesterone, and pregnenolone.[1,5–11] In these assays, attempts were made to develop sensitive and specific ELISA for measurement of progesterone in milk that is still lacking. Attempts at automation and online application of quantitative EIAs for progesterone have been described.[12,13] A flow-through ELISA for online use that utilizes microinjection pumps for fluid handling has been reported to take 8 min for completion and can detect 0.2–20.0 ng/mL of milk progesterone.[14] Electrochemical immuno/biosensors have been developed. The main problems associated with their widespread use continue to be their stability and cost.[15,16] Surface plasmon resonance (SPR) technology has also been utilized for the development of highly sensitive immunoassays for progesterone in milk.[17,18] The SPR-based immunoassays reported so far have achieved sensitivity comparable with EIA, but they require sophisticated instruments, which increases the overall cost of the assay. Therefore, enzyme immunoassay are still a preferred choice over others because they are more cost effective and do not have radiation hazards. Moreover, they permit endpoint determination on comparatively simple, widely used pieces of equipment, and their intrinsic nature incorporates an amplification step, which is likely to increase the overall assay sensitivity.

In the present study, specific and sensitive ELISA for the direct measurement of progesterone in milk has been developed using P-3-O-CMO for immunogen preparation and 17-α-OH-P-3-O-CMO for enzyme conjugate preparation. The lack of an easy, reliable, cost-effective, and specific EIA system for milk progesterone analysis has prompted us to develop a rapid, sensitive, and viable antigen heterologous ELISA for the direct estimation of milk progesterone, which is specific and cost-effective.

EXPERIMENTAL

All procedures conform to the stipulation of the Institutional Animal Ethical Committee's norms.

Chemicals and Reagents

All solvents, chemicals, and salts used in the present study were of analytical grade and used without prior purification. All steroids used for the synthesis and cross-reactivity were obtained from Sterloids, Inc. (Newport, CT, USA). Other chemicals such as bovine serum albumin, N-hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC), complete Freund's adjuvant, gelatin, and thimerosal were purchased from Sigma Chemical Company (St. Louis, MO, USA). Horseradish peroxidase and tetra-methyl benzidine/hydrogen peroxide (TMB/H_2O_2) solution were purchased from Bangalore Genei (Bangalore, India), and Arista Biochemical (Allentown, PA, USA), respectively. Microtiter plates were procured from Greiner(Frickenhausen, Germany).

Buffers

Buffer A

The most frequently used buffer was 10 mM phosphate buffer (10 mM PB), pH 7.0, ($Na_2HPO_4.2H_2O$: 0.895 g/L and $NaH_2PO_4.2H_2O$: 0.39 g/L) containing 0.9% NaCl (10 mM PBS) and 0.1% NaN_3.

Buffer B

HRP conjugate dilution buffer consisted of 10 mM acetate buffer (10 mM AB), pH 5.6, (CH_3COONa: 0.84 g/L and 1 N CH_3COOH 1.5 mL/L), containing 0.1% thimerosal and dextran T-70, 0.3% BSA.

Buffer C

Microtiter well blocking and stabilizing buffer was 10 mM PB containing 0.9% NaCl, 0.2% BSA, 0.1% gelatin, thimerosal, dextran T-70, ethylene diamine tetra acetic acid:di-potassium salt (EDTA:K salt), and 0.01% gentamicin sulfate.

METHODOLOGY

Preparation of Progesterone-3-O-CMO-BSA

Progesterone-3-O-carboxymethyloxime (P-3-O-CMO) was coupled to BSA by an active ester method with some modification.[19] To 5 mg of

P-3-O-CMO, 200 μL of each of dioxan and dimethyl formamide were added. To this solution, 100 μL of water containing 10 mg NHS and 20 mg EDAC was added. The reaction mixture was activated for 24 h at 4°C. Activated P-3-O-CMO solution was added to the aqueous solution of BSA (1 mg/0.3 mL), then it was vortex-mixed and kept for 24 h at 4°C. The P-3-O-CMO-BSA conjugate was dialyzed against four changes of water. The dialysate was frozen, lyophilized, and kept at 4°C.

Immunization

The intramuscular injections were given to New Zealand white rabbits according to the method of Shrivastav et al.[20] In brief, P-3-O-CMO-BSA (1 mg) was dissolved in saline (0.5 mL) and emulsified with Freund's complete adjuvant (0.5 mL). The emulsion (250 μL) was injected intramuscularly into the limbs of the rabbits. The five primary injections, given weekly, were followed by the monthly booster doses. The booster doses were given in Freund's incomplete adjuvant, and the rabbits were bled 10 days after the booster injection. Antiserum was collected after centrifugation at 750 × g for 10 min and stored at −30°C.

Preparation of Progesterone-3-O-CMO-HRP

Progesterone-3-O-carboxymethyloxime (P-3-O-CMO) was coupled to HRP by an active ester method with some modification.[21] To 5 mg of P-3-O-CMO, 200 μL of each of dioxan and dimethyl formamide were added. To this solution, 100 μL of water containing 10 mg NHS and 20 mg EDAC was added. The reaction mixture was activated for 24 h at 4°C. Activated P-3-O-CMO solution was added to the aqueous solution of HRP (1 mg/mL) and kept for 24 h at 4°C. Then the reaction mixture was passed through a G-25 column that was previously equilibrated with 10 mM PBS containing 0.1% thimerosal. The brown-colored fractions containing enzyme activity were pooled, and to them 1% of sucrose, ammonium sulfate, BSA, and an equal volume of ethylene glycol were added. The solution was kept at −30°C in aliquots for future use.

Preparation of 17-α-OH-Progesterone-3-O-CMO-HRP Conjugate

17-α-OH-progesterone-3-O-CMO was directly conjugated to HRP by an activated ester method.[21] In brief, 5 mg of 17-α-OH-P-3-O-CMO was dissolved in 200 μL each of dimethyl formamide and dioxan. To this solution, 100 μL of water containing 10 mg NHS and 20 mg EDAC were added; the reaction mixture was activated for 24 h at 4°C. Activated 17-α-OH-P-3-O-CMO solution was added to the aqueous solution of HRP (1 mg/mL)

and kept for 24 h at 4°C. Then the reaction mixture was passed through a G-25 column, which was previously equilibrated with 10 mM PBS containing 0.1% thimerosal. The brown-colored fractions containing enzyme activity were pooled, and to them 1% of sucrose, ammonium sulfate, BSA, and an equal volume of ethylene glycol were added. The solution was kept at −30°C in aliquots for future use.

Coating of Antibody to Microtitre Plates

The 96-well microtiter plates were coated using Shrivastav et al.'s the immunobridge technique for primary antibody immobilization.[20] In brief, 250 μL of normal rabbit serum (NRS) diluted (1:250) in water was dispensed into each well and incubated at 37°C overnight. Following incubation, the plate was washed under running tap water 15–20 times. To the NRS-coated wells, 250 μL of 1:1000 diluted goats anti rabbit gamma globulin (ARGG) was added and incubated for 2 h at 37°C. Then the plate content was decanted and washed under running tap water. To the ARGG-coated microtiter plates, 200 μL of 1:30000 diluted P-3-CMO-BSA antiserum in buffer "A" was dispensed. The plate was kept at 37°C for 2 h. Unbound antibody was then washed off, and 250 μL of buffer "C" was then added to block the unoccupied sites of the plate.

Standardization of Assay: Preparation of Calibrators and Recovery Pools in Milk

Full-cream milk was used to prepare calibrators, and recovery pools were prepared in full-cream and toned milk.

Preparation of Calibrators and Recovery Pools

Unboiled full-cream milk (200 mL) was taken, and to it 5% charcoal was added and stirred for 2 h at 45°C. Then it was centrifuged at 3000 × g to remove the charcoal and was diluted five times with 3 M potassium chloride solution. Nine progesterone working standards (0, 0.125, 0.25, 0.5, 1, 2.5, 5, 10, and 20 ng/mL) were prepared in charcoal-treated milk. Five recovery pools for each were prepared in full-cream and toned milk by spiking with different concentrations of progesterone, viz., 1.0, 2.5, 5.0, and 10.0 ng/mL.

Dilution of Milk Samples

For estimation of progesterone in milk sample, unknown and recovery pools were diluted five times with 3 M potassium chloride solution.

Assay Procedure

To the P-3-O-CMO-BSA antiserum (1:30000)–coated microtitre wells, 50 μL of standards (0–20 ng/mL) and diluted milk samples were added in duplicate followed by the addition of 50 μL of 17-α-OH-P-3-O-CMO-HRP enzyme conjugate (1:4000) in all the wells. The wells were then kept for 1 h at rt. Then the contents of the wells were decanted to remove unbound enzyme conjugate, and the wells were washed 15–20 times under running tap water by filling, decanting, and flicking. TMB/H_2O_2 (100 μL) substrate was added to all the wells and kept for 20 min at rt. Then 100 μL of 0.5 M H_2SO_4 was added to all the wells. The developed yellow color was read at 450 nm in a Tecan Spectra micro-plate reader.

Radioimmunoassay (RIA) of the Milk Samples

RIA of milk samples was performed by utilizing a progesterone antibody–coated tube and I^{125} labeled progesterone of the Immunotech RIA kit (Immunotech, Cedex, France), according to the manufacturer's guideline.

Data Analysis

The standard curve and its logit-log transformation were created by MS Excel and MS PowerPoint. The statistical analyses, such as mean, standard deviation, coefficient of variations, and correlation coefficient were done by MS Excel.

RESULTS

Standard Curve

The homologous combination of P-3-O-CMO antiserum and P-3-O-CMO enzyme conjugate did not show displacement. The composite dose–response curves of eight assays for the antigen heterologous ELISA of progesterone are shown in Figure 1. The CVs for the A/A0 ratio of each standard ranged from 3.4 to 10.8% for the enzyme immunosorbent assay. Thus the standard curves obtained over several assays remained stable and precise. Upon logit-log transformation of the standard curve, the equation for the relationships for ELISA was $y = -1.896x + 6.42$ ng/mL.

Sensitivity

The assay sensitivity is usually expressed in terms of its lower detection dose (LDD) and the effective displacement at 50% (ED_{50}). The LDD is the

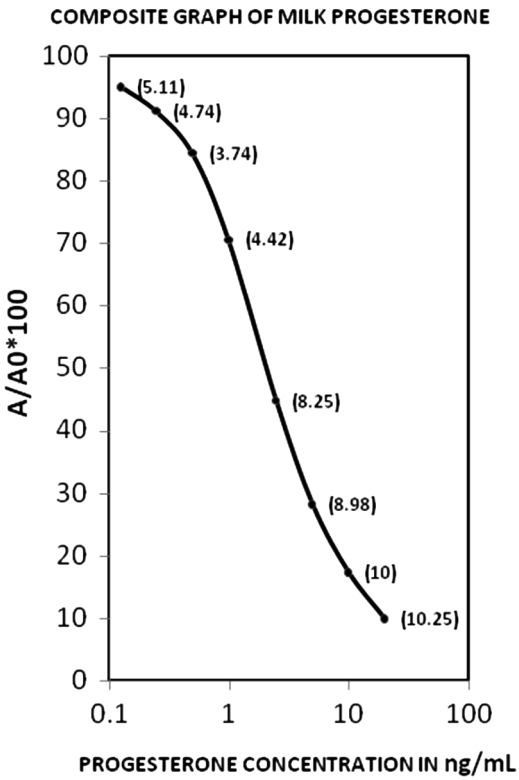

FIGURE 1 Composite standard curve for progesterone using P-3-CMO-BSA antibody and 17α-OH-P-3-CMO-HRP enzyme conjugate. Each value is a mean ± SD of eight assays (in duplicate). The coefficient of variation at each concentration is shown in parentheses.

lowest concentration of analyte (A) giving a response that is statistically different from that observed in the absence of analyte (A0). It is calculated as A0 − 2SD, after 32-fold determination of A0. The ED_{50} is the effective concentration at which 50% of inhibition in the binding of enzyme conjugates occurs in assays in the presence of analyte. It is calculated as $ED_{50} \pm SD$, after eight times determination of ED_{50}. The LDD and the ED_{50} of the present assay are 0.07 ng/mL and 2.0 ng/mL, respectively.

Specificity

The specificity of the P-3-O-CMO-BSA antibody was estimated as the percentage of cross-reaction with commercially available steroids with analogous structure. The P-3-O-CMO-BSA antibody showed 8.73%, 4.8%, and 1.58% cross-reaction with 17-α-OH-P, pregnenolone, and cortisone,

respectively, and less than 0.1% cross-reaction with other structurally related steroids, as shown in Table 1. The percent cross-reaction was calculated from the following formula:

$$\%\text{cross-reaction} = \frac{\text{Concentration of progesterone required to give 50\% inhibition}}{\text{Concentration of related steroid, required to give 50\% inhibition}} \times 100$$

TABLE 1 Cross-Reactivity of Steroid Compounds with Progesterone for Anti-Progesterone Antibody by ELISA

Steroid Measured	% Cross Reactions with 17α-OH-P-3-CMO-HRP Enzyme Conjugate
C-27 Steroid	
Cholesterol	<0.025
C-22 Steroid	
Danazol	0.1
C-21 Steroid	
Progesterone	100
Pregnenolone	4.8
17α-OH progesterone	8.7
17α-OH pregnenolone	0.1
11α-Hydroxyprogesterone	0.09
5α-Pregnane-3,20-dione	<0.025
5β-pregnane-3,20-dione	<0.025
Pregnanediol	0.09
Cortisol	0.09
Tetrahydrocortisol	0.05
Prednisolone	0.08
Aldosterone	0.066
Corticosterone	0.08
Cortisone	1.56
Tetrahydrocortisone	0.1
C-19 Steroid	
Testosterone	<0.025
Dihydrotestosterone	<0.025
Etiocholanolone	<0.025
Dehydroepiandrosterone	<0.025
Androstenedione	<0.025
11β-OH androstenedione	<0.025
C-18 Steroid	
Estrone	<0.025
Estradiol	<0.025
Estriol	<0.025
Nandrolone	<0.025

Recovery

The ability of an assay to accurately quantify progesterone in milk was tested. Table 2 details the % recoveries of known amounts of progesterone added to four 10-mL aliquots of full-cream and toned milk, and they ranged from 97.6–101% and 94.3–97.88%, respectively.

Precision

Milk specimens containing approximately the same concentrations of progesterone were combined to form four pools with different concentrations. We analyzed each pool six times within the assay and also in six separate assays. Table 3 depicts the intra- and interassay coefficient variations. The intra- and interassay coefficient variations ($n = 6$, replicate of each pool) were $<7.8\%$.

Estimation of Progesterone in Single Toned and Full-Cream Milk Samples

The range of progesterone in single toned and full-cream milk samples as determined by present ELISA is given in Table 4.

Correlation Coefficient

The correlation coefficient for values of progesterone in milk samples ($n = 65$) measured by both the RIA kit (Immunotech) and ELISA are found to be 0.95, i.e., $r = 0.95$.

TABLE 2 Recovery of Progesterone from Exogenously Spiked Milk Aliquots

Toned Milk Aliquots	Progesterone Added (ng/mL)	Progesterone Observed (ng/mL)	Progesterone Expected (ng/mL)	% Recovery
Aliquot A (Basal)	–	3.52	–	–
Aliquot B	1.0	4.35	4.52	96.3
Aliquot C	2.5	5.8	6.02	97.88
Aliquot D	5.0	8.34	8.52	94.3
Aliquot E	10.0	12.75	13.52	96.42
Full cream milk aliquots				
Aliquot A (Basal)	–	7.4	–	–
Aliquot B	1.0	8.2	8.4	97.6
Aliquot C	2.5	10.0	9.9	101
Aliquot D	5.0	12.0	12.4	96.7
Aliquot E	10.0	17.3	17.4	99.4

TABLE 3 Intra- and Interassay Coefficient of Variation for the Measurement of Progesterone in Four Milk Pools

Variation	Sample Value ng/mL (mean ± SD)	Coefficient of Variation (%)
Intraassay $n=6$	4.01 ± 0.2337	5.8
	6.59 ± 0.51	7.8
	9.87 ± 0.44	4.4
	17.43 ± 0.78	4.1
Interassay $N=6$	4.53 ± 0.32	7.0
	6.30 ± 0.36	5.7
	9.95 ± 0.66	6.6
	19.04 ± 0.84	4.4

n = Number of times same sample analyzed for intra-assay variation. N = number of times assays carried out for interassay variation.

TABLE 4 Reference Range of Progesterone in Single Toned and Full-Cream Milk Samples as Determined by the Developed ELISA

Milk Samples	Range of Progesterone (mean ± 2 SD)[a] (ng/mL)
Single toned milk (40)[b]	1.198–9.745 (5.471 ± 2.136)[a]
Full-cream milk (40)[b]	6.949–14.923 (10.936 ± 1.993)[a]

[a]Denotes mean ± 2 S.D.
[b]Data within brackets are sample number.

DISCUSSION

We have developed a sensitive and specific antigen heterologous ELISA for milk progesterone using HRP as a label that requires a low volume of milk sample, and the assay can be completed within 1.5 h. The present study is an extension of the previously developed antigen heterologous ELISA for progesterone estimation in serum using horse radish peroxide (HRP) as a label.[22] With the use of milk as the matrix, the sensitivity and ED_{50} achieved are 0.07 ng/mL and 2.0 ng/mL, respectively.

The incorporation of heterology at the level of bridge, site, or antigen in the hapten immunoassay system is one of the ways to achieve a highly sensitive assay. We have exploited antigen heterology to achieve sensitivity because of the reduced antigen-binding effects. Achieving specificity along with sensitivity is a well recognized problem in progesterone enzyme immunoassay development.[23] To this end, different combinations of antibodies (monoclonal and polyclonal) and enzyme conjugates have been tested for the measurement of milk progesterone. Homologous assays were developed using P-11-hemisuccinate for preparation of immunogen and enzyme conjugate. Arnstadt has developed a milk progesterone assay having sensitivity of 9.4 pg/tube, with 10% cross-reactions with 5α-pregnane-3,20-dione

and 19.1% cross-reactions with 5β-pregnane-3,20-dione.[7] Simersky et al. have developed a milk progesterone assay having sensitivity of 1.3 ng/mL, with 21.41% cross-reactions with 5α-pregnane-3,20-dione and 15.42% cross-reactions with 11-α-OH-progesterone.[1] Waldman has developed a milk progesterone assay having sensitivity of 25 pg/well, with 45.06% cross-reactions with 5α-pregnane-3,20-dione, 40.68% cross-reactions with 11-α-OH-progesterone, 8.1% cross-reactions with 17-α-OH-progesterone, and 12.5% cross-reactions with pregnanolone.[8] Nakao et al. have developed a milk progesterone assay having sensitivity of 10 pg/tube.[9]

Different heterologous combinations have also been tested. The enzyme immunoassay composed of the P-11-HS-antibody with P-11-glucuronide enzyme conjugate showed a sensitivity of 25 pgm/well, with 8% cross-reaction with 5β-pregnane-3,20-dione.[10] Using P-11-HS antibody with P-3CMO-enzyme conjugate, Marcus and Hackett[11] developed a progesterone assay having a sensitivity of 1 pgm/tube, with 50% cross-reaction with 5α-pregnane-3,20-dione, 48% cross-reaction with 5β-pregnane-3,20-dione, 95% cross-reaction with 11-α-OH-progesterone, and 7% cross-reaction with 17-α OH-progesterone. Thus, the ELISA for milk progesterone with good sensitivity and high specificity has not been designed so far. In the present study, using an antigen heterologous combination of P-3-O-CMO-antibody with the 17-α-OH-P-3-O-CMO-HRP enzyme conjugate, we have developed a sensitive (0.07 ng/mL) as well as specific assay, having a cross-reaction of less than 0.1% with analogous C18, C19, C21, and C27 steroids with the exception of 17α-OH-progesterone (8.73%), pregnenolone (4.8%), and cortisone (1.56%).

The major problem in developing the ELISA for milk progesterone was the puzzling matrix interference posed by milk as compared to serum.[24] Matrix effect can be defined as the possible interference in the assay from sample constituents other than the hormone to be measured. Milk contains approximately 3.4% total fat, which varies with the stage of lactation. Progesterone, being a lipophilic molecule,[25] is highly soluble in the fat or lipid content of the milk, and that can severely affect its accurate estimation in milk. The milk fat exerts an influence on the shape of the calibration curve and brings variability in recovery, so it is recommended to prepare the standards in a constant milk fat concentration.[7] We have prepared standards in stripped milk that was further diluted five times with 3 M KCl solution to compensate for the nonspecific influence or intersample variation of milk constituents.[26] We have used different strategies such as low and high pH assay buffer, heating of milk samples, and use of a high ionic concentration of chaotropic salts to obtain the released progesterone from milk protein/fat. The use of a high ionic concentration of chaotropic salts gave good recovery as compared to the other method employed to obtain the released progesterone from milk protein/fat.

The developed assay is very sensitive, with a lower detection limit of 70 pg/mL. The lower limit further contributes in the reduction of interference from milk samples, as the milk sample volume required for assay is very low (10 μL or 50 μL, 1:5 diluted). We have measured the level of progesterone in full-cream and toned milk samples. The progesterone concentration is twice in full-cream milk as compared to single toned milk. As the progesterone, being a lipophilic molecule, is highly soluble in fat, removal of fat from milk brings down the concentration of progesterone in milk. The value of progesterone measured by the developed ELISA correlates well with established RIA kit measurement.

In conclusion, a sensitive, specific, and viable direct assay for milk progesterone has been developed, which requires low volume of milk sample, and the assay can be completed in 1.5 h. We have screened two varieties of milk samples for progesterone concentrations, and found that high progesterone concentrations exist in full-cream milk more than toned milk. This assay may be extended for the detection of pregnancy in dairy animals, using milk as the biological fluid.

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