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Correlation between Homologous and Heterologous Enzymeimmunoassays for Progesterone Determinations in Milk from Cows

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Two enzymeimmunoassays, homologous and heterologous, have been used for measuring progesterone in unextracted bovine milk using HRP as the enzyme label. Antibody raised by immunization of the rabbit against 11α-hemisuccinate-BSA was used for the homologous system (EIA-11α) and 7α-carboxyethylthioether-BSA (EIA-7α) for the heterologous. The progesterone derivatives used for the enzyme-hormone conjugates were 11α-hemisuccinate and 6β-OH-hemisuccinate respectively. Milk progesterone in 60 samples measured by EIA-11α and EIA-7α were highly correlated (r = 0.93). Both systems were further compared with a conventional direct progesterone radioimmunoassay (RIA) in regular use for the same samples showing a good correlation. The sensitivity estimated was much higher in the EIA-7α (0.5 pg/well) than in the EIA-11α (32 pg/well).

Key words: EIA-homologous, EIA-heterologous, Progesterone.

Since the time when artificial insemination in domestic animals was introduced, the determination of milk progesterone concentrations has been used as a valuable method of early pregnancy diagnosis (3, 17). The concentration of progesterone in milk reflects that in plasma and gives valuable information about the physiological status of the cow (11, 14). The method most extensively used so far for the measurement of hormones is RIA, however, has several drawbacks which are inherent to the use of radioactive isotopes such as the short life of the labelled hormone radioactivity and the expensive equipment needed. These problems have prompted the search during the last decade of other labels such as enzymes instead of radioactive isotopes (4, 15, 22).

Enzymeimmunoassay (EIA) is now

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being widely applied for progesterone determinations in milk (2, 18) using either homologous or heterologous systems. In the present study the determinations of progesterone levels in unextracted whole milk samples from cows were done using both systems: homologous-EIA (11-α-hemisuccinate for antiserum and tracer) and heterologous-EIA (7-α-carboxyethylthioether-BSA for antiserum and 6-β-hydroxyhemisuccinate for the tracer). The results were compared between both methods mentioned above as well as between EIA and the conventional RIA procedure using the same milk samples.

Materials and Methods

Reagents. — Horseradish peroxidase grade 1, was obtained from Boehringer (Mannheim, W. Germany). Tetramethylbenzidine (TMB) was from Fluka AG (Buchs. Switzerland). Progesterone 6-β-hydroxyhemisuccinate (P-6β) from Steraloids (Wilton, N. Y.); progesterone-11-α-hemisuccinate (P-11α), progesterone-11-α-hemisuccinate-BSA, progesterone and thimerosal were from Sigma. Bovine serum albumin (BSA) fraction V, crystallized and lyophilized, was obtained from Fluka AG.

Tracers. Enzyme-labelled progesterone (P-HRP) was prepared by conjugating progesterone-6-β-hydroxyhemisuccinate for the heterologous system and progesterone-11α-hydroxyhemisuccinate for the homologous system, to horseradish peroxidase (HRP) using the mixed anhydride reaction method of ERLANGER *et al.* (8) as modified by DAWSON *et al.* (7).

Separation of the conjugate from any remaining low molecular weight material was achieved by gel chromatography (Sephadex G 25 fine; column: 1.6 cm wide and 50 cm length). The HRP-progesterone conjugate so obtained was immediately tested for its titer, freeze dried and stored in dark ampoules at −20 °C until use.

Antibodies.— The antiserum used in the homologous system was raised in rabbits against progesterone 11-α-hydroxyhemisuccinate conjugated to bovine serum albumin (BSA) by multiple intradermal injections. The immunoglobulin fraction (IgG) was purified by ammonium sulfate precipitation followed by dyalisis against 0.04 M phosphate-buffered saline (PBS) pH 7.2 and after titration freeze-dried to store.

In the heterologous system the antiserum raised against progesterone 7-α-carboxyethylthioether-BSA was used.

For titration of antibodies, polystyrene microtiter plates (Costar, Europe, Badhoevedrop. The Netherlands) were coated with serial dilutions of antiprogesterone IgG stock solution, in order to determine the optimal dilution to be used in the EIA. The optimal antibody dilution was 1:10,000 for the homologous system and 1:20,000 for the heterologous system.

Substrate solution. — Tetramethylbenzidine (TMB) was used as chromogen, because of its non-carcinogenic and non-mutagenic properties (4). The substrate solution consisted of 1 % H_2O_2 and 0.6 % tetramethylbenzidine-dimethylsulfoxide mixed in 0.1 M acetate-citrate buffer. The solution should be about 25 °C and was used within 30 min.

Standard progesterone. — A working solution of progesterone in ethanol was prepared (10 mg/ml) and stored at 4 °C. When required dilutions were prepared in phosphate buffer. Tubes containing standards were dried under nitrogen. Standard curves for both EIA systems were obtained by plotting the absorbance against the amount of added progesterone.

Milk samples.— Samples were collected from 60 cows on the 21st day after arti-

ficial insemination and maintained with 0.1 % potassium dichromate at 4 °C until assayed.

Results

The term homologous system indicates that the progesterone derivative coupled to the enzyme is identical to that used for the preparation of the immunogen for raising the antiprogesterone serum (11α-hemisuccinate), while in the heterologous system the hormone derivatives are different (progesterone 7α-BSA for the immunogen and progesterone 6β-HRP for the tracer).

Homologous system.— Figure 1 shows the standard curve obtained by EIA for progesterone determination with the homologous system (EIA-11α) from 8 consecutive assays.

Precision was tested by replicate measurement of two milk samples within one assay, and of other two milk samples (controls) in five consecutive assays. Mean values ± S.D. (ng/ml) and their corresponding inter-assay coefficient of variation (CV) were 2.84 ± 0.54 (n = 5; CV = 19.14 %) and 9.4 ± 1.16 (n = 5, CV = 12.39 %), respectively. Mean values and intra-assay CV were 3.28 ± 0.56 (n = 14; CV = 17.34 %) and 14.45 ± 1.46 (n = 14, CV = 10.12 %), respectively.

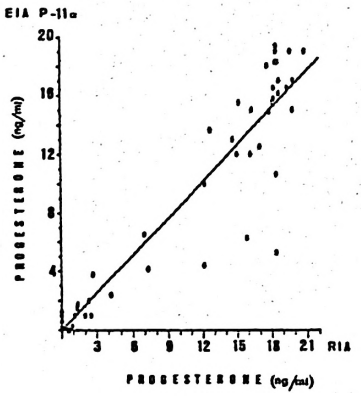

Fig. 2. *Correlation between progesterone values in milk samples as determined by RIA and EIA-11α.*

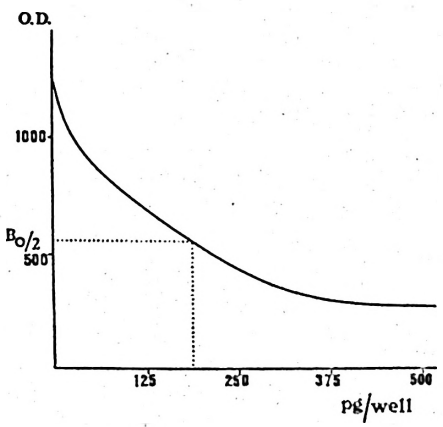

Fig. 1. *Standard curve from 8 consecutive assays plotted as the mean optical density at each standard concentration with homologous EIA system (P-11α).*

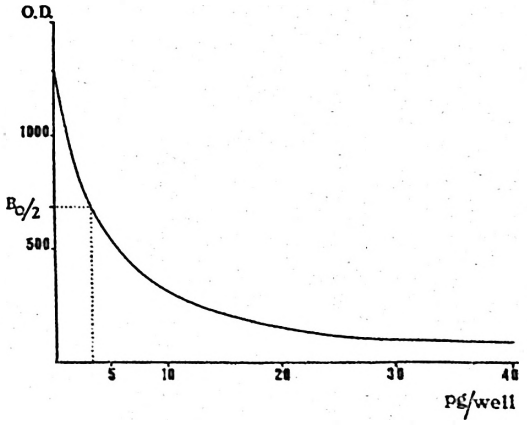

Fig. 3. *Standard curve for enzymeimmunoassay of progesterone using rabbit anti-progesterone-7α-carboxyethylthioether-BSA and progesterone-6β-hydroxyhemisuccinate-HRP (heterologous system).*

Comparison of progesterone concentrations determined by EIA-homologous system and RIA in the same 60 milk samples is shown in figure 2. The correlation between the values obtained by these two methods was $r = 0.94$ ($p < 0.001$) and its equation of the regression line was EIA-11α = 0.83 RIA + 1.25 × 10^{-2}.

The sensitivity of the homologous system, calculated as the smallest amount of progesterone which differed significantly from zero (B_0-2 S.D.) (1) was 32 pg/well; and the sensitivity defined as the amount of progesterone which causes a 50 % reduction of the initial binding ($B_0/2$) in the standard curve was 80 pg/well.

Heterologous system. — The standard curve of the heterologous-EIA system obtained from 7 consecutive assays is shown in figure 3. The sensitivity of this system was $B_0/2$ = 4 pg/well and B_0-2 S. D. = 0.5 pg/well. The interassay CV of two milk samples were 13.8 % and 8.42 % (n = 5) with a mean value (± S. D.) of 3.9 ± 0.54 and 10.3 ± 0.86 ng/ml, respectively. Mean values (± S. D.) and

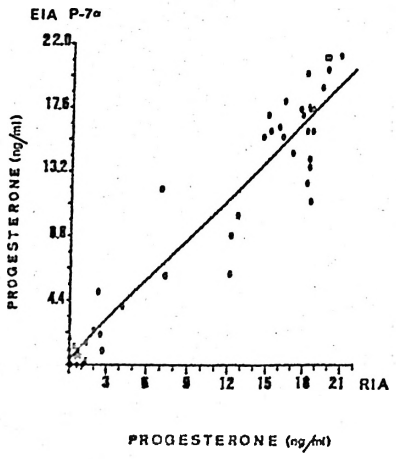

Fig. 4. *Relationship between the concentrations of progesterone as measured by EIA-7α and RIA in 60 milk samples.*
Equation of the regression line was EIA-7α = 0.90 RIA + 0.35.

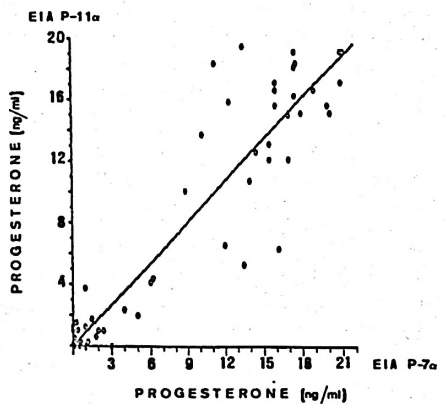

Fig. 5. *Correlation of milk progesterone levels as obtained by EIA-homologous and EIA heterologous systems.*

their corresponding intra-assay CV were 19.8 ± 1.56 (n = 10, CV = 7.8 %) and 10.88 ± 1.94 (n = 10, CV = 17.90 %).

The correlation coefficient obtained between the EIA-heterologous system and RIA is shown in figure 4 ($r = 0.96$; $p < 0.001$). The correlation of progesterone determined by heterologous and homologous systems is shown in figure 5 ($r = 0.93$; $p < 0.001$).

Discussion

Analysis of progesterone in milk plays an important role in reproduction management and veterinary control of cows. In particular, progesterone determination is applicable to oestrus detection and early pregnancy diagnosis. Following insemination the earliest possible confirmation of pregnancy is essential in order to make profitable rebreeding.

Radioimmunoassay has been widely used for milk progesterone assays. However, this method has some disadvanges, radiation being the most serious hazard. In the past decade enzymeimmunoassay has been widely applied for determination

of progesterone in milk (2, 6) as an alternative method to early pregnancy diagnosis. The EIA has been developed by several authors using either heterologous or homologous systems obtaining different conclusions. JOYCE *et al.* (12) found EIA homologous systems to be as sensitive as heterologous for progesterone and oestradiol. BOSCH *et al.* (5) found the highest sensitivities for oestrogen when using an oestradiol 16/17-monosuccinyl-BSA antiserum in an homologous system. On the other hand, greater sensitivity with a heterologous system, compared with a homologous one, has been demonstrated for progesterone (10, 13), for oestrogen (19, 20) and for testosterone and oestradiol (9, 21). However, SUGIYAWA *et al.* (16) found the same sensitivity with EIA homologous and heterologous systems for oestrone sulphate.

Recently, VAN DE WIEL and KOOPS (18) have developed a sensitive microtiterplate EIA for progesterone measurement in bovine milk or blood plasma without extraction or centrifugation, using a heterologous system.

In this paper we compare the results obtained in milk samples of 60 cows for pregnancy diagnosis using the same heterologous system developed by the above mentioned authors and a homologous EIA system, both without extraction. Though the sensitivity obtained with the homologous system (32 pg/well) was much lower than that obtained with the heterologous one (0.5 pg/well) however the good correlation coefficient between both systems, as well as the coincidence of the pregnancy diagnosis results (96 % for the non pregnant animals and 90 % for the pregnant ones) suggests that the homologous system is also valid for early pregnancy diagnosis in bovine milk.

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Resumen

Se determinan los niveles de progesterona en leche de vaca mediante dos sistemas de enzimoinmunoensayo, homólogo y heterólogo, utilizando HRP como enzima marcadora. Los anticuerpos utilizados en el sistema homólogo (EIA-11α) se obtienen mediante inmunización de conejos frente a progesterona 11α-hemisuccinato-BSA y para el sistema heterólogo (EIA-7α): 7α-carboxietiltioeter-BSA. Los niveles de progesterona en 60 muestras de leche determinados (r = 0,93). Los resultados obtenidos con ambos sistemas se comparan con los obtenidos por RIA convencional en las mismas muestras de leche obteniendo una buena correlación. La sensibilidad estimada es mayor en el EIA-7α (0,5 pg/pocillo) que en el EIA-11α (32 pg/pocillo).

Palabras clave: EIA-homólogo, EIA-heterólogo, Progesterona.

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