Pregnancy Diagnosis in Sows by Using an On-Farm Blood Progesterone Test

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ABSTRACT : To improve animal production, a simple and accurate pregnancy diagnosis plays a very important role. Therefore, the purpose of this study was to develop an on-farm blood progesterone enzyme immunoassay (EIA) system for monitoring the early pregnancy in sows. Star tubes coated with mouse monoclonal anti-progesterone antibody were used for this proposed EIA system which was tested in field trials.

The results could be obtained within 30 minutes either by spectrophotometry or the naked eye. Heparinized fresh blood samples collected from the ear vein of sows 17-22 days after breeding (day 0) were tested qualitatively to diagnose sows as pregnant or non-pregnant with high (> 3 ng/ml) or low (≦ 3 ng/ml) progesterone in the blood. To provided a double check data, plasma progesterone levels were also measured quantitatively by the same EIA system with some modification. Total agreement of diagnosis by the on-farm EIA kit and by farrowing or abortion from 128 tested sows was found to be 92.2% accuracy (93.1% on pregnant diagnosis and 83.3% on non-pregnant diagnosis). It was concluded that the on-farm EIA blood progesterone test is a very useful method for monitoring the early pregnancy status of sows.
(Key Words: Sow, Pregnancy Diagnosis, Blood Progesterone, Enzyme Immunoassay)

INTRODUCTION

With the rising cost of keeping barren sows in the herd, the demand for accurate early pregnancy diagnosis is increasing. Currently available methods for pregnancy diagnosis in sows including those based on Doppler ultrasound reflection and the determination of plasma estrone sulfate concentration hardly meet the requirement due to the diagnosis beyonds an estrous cycle and time-consuming (McCaughey and Red, 1979; Cunningham et al., 1983; Sugiyama et al., 1985; Almond et al., 1987; Szenci et al., 1993). At the present time, determination of progesterone concentrations in the blood during days 17-22 postmating may be the most practicable technique for the early pregnancy diagnosis of sows (Lin et al., 1988). Following the development of enzyme immunoassay (EIA) makes this type of on-farm testing possible. Over the past few years, many commercial kits using EIA for cowside milk progesterone measurement have entered the market (Nebel, 1988; Nebel et al., 1989), however similar kits for sows have as yet not received equal popularity, only one experiment have been reported (Glossop et al., 1989).

Therefore, this study established a simple method of EIA for blood progesterone determination and its application in early pregnancy diagnosis in sows.

MATERIALS AND METHODS

Monoclonal antibody production

The process for production of progesterone monoclonal antibody was taken from Fantl et al. (1981) with certain modifications. BALB/c mice were immunized with a mixture of 4-pregnen-11-alpha-ol-3, 20 dione hemisuccinate: BSA and Freund adjuvant three times, with a two-week interval between each injection. Three days after the last immunization, the mice were sacrificed and splenectomized.

Splenocytes were isolated and then fused with NS-1 cells using PEG-1,500. The fused cells were cultured in 96-well microtiter plates with HAT (hypoxanthine, aminopterin, and thymidine) media for 14 days and then transferred to HT medium for maintenance. RIA was used to test the cultured media for progesterone antibody production, and limiting dilution method was employed to establish the monoclonal antibody hybridoma cell lines. Five cell lines, named G1, G4, G6, G7, and G9, were obtained and subsequently stored in liquid nitrogen. The G7 cell line was found to be the best for progesterone monoclonal antibody production. The G7 cells were then injected into the peritoneal cavity of BALB/c mice to induce ascites. The progesterone monoclonal antibody subsequently harvested from the ascitic fluid was used in

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the following procedures.

Preparation of enzyme conjugate

Horseradish peroxidase (HRP, type VI, Sigma P-8375) was coupled with the 11 alpha-hemisuccinate derivative of progesterone, using a modification of the mixed anhydride method described by Joyce et al. (1977). In a 10 ml test tube, 1 mg of 4-pregnen-11 alpha-ol-dione hemisuccinate (Steraloids Q-3252) was dissolved into 250 μl of 1, 4-dioxan. Then, ten μl of tri-n-butylamine and 5 μl isobutyl chlorocarbonate were mixed into the solution. After 40 minutes of constant stirring in an ice bath, 1 ml of HRP (2,500 units/ml D. W.) was added at a controlled temperature of 8-10℃ and pH 8.0. The reaction mixture was stirred for an additional 4 hours and then dialysed apart 3 times with 0.05 M phosphate buffer (pH 7.0). This dialysate was applied to a 1.6 × 100 cm Sephadex G-100 column and eluted with 100 ml of 0.05M phosphate buffer (pH 7.0). Each 2 ml fraction of the eluate was collected and measured for enzymatic and immunologic activities. The fraction with the highest activity was diluted 10 times and stored at −20℃.

Stock solutions

The following stock solution gave optimal results in the star tube assay system: (1) coating buffer: 0.1M sodium bicarbonate, pH 9.6; (2) washing solution: 0.01M phosphate buffer, pH 7.0, containing 0.15M NaCl and 0.05% Tween 20 for the quantitative laboratory tests and tap water for the qualitative field test; (3) blocking and assay buffer: 0.1M sodium phosphate buffer, pH 7.0, containing 0.15M NaCl and 0.1% gelatin (this phosphate buffer was also used for the dilution of the sample, standard, and conjugate); (4) substrate: 20 mg o-phenylene diamine dihydrochloride (OPD, Sigma P-1526) and 5 μl 30% H_2O_2 were added to 50 ml 0.1M phosphate buffer, pH 6.0, allowed to stand for 10 minutes before use, and were stored in the dark; (5) stopping reagent: 8N H_2SO_4. A volume of 50 ml of substrate solution was sufficient for the progesterone determination of a set 50 tubes. Each set included 5 standards and 20 samples in duplicate.

Preparation of antibody coated star tubes

Polystyrene star tubes (Nunc 470319, Denmark), 12 × 75 mm, with 6 intersecting riges forming a "star" at the bottom, were coated with 500 μl monoclonal antibody (G7) in a coating buffer at a dilution of 1:30,000. The tubes were then stored overnight at 4℃. Following cold storage, the antibody was decanted, and the tubes were washed twice with washing buffer. To cover any surface of the tube not coated by the highly dilute antibody solution, 1 ml of blocking buffer was added to each tube and incubated at room temperature for 2 hours. After this last incubation, the tubes were decanted, washed twice again with washing buffer, drained, and left inverted on paper towels to dry. The dried tubes are then ready for use and can be stored at 4℃ for at least 6 months.

Enzyme immunoassay procedure

For quantitative analysis, the plasma or serum samples were first diluted with 0.1 M phosphate buffer (30x). The dilution was made to facilitate aliquoting the samples for assay. All of the diluted samples were mixed with a vortex, and then aliquots of 300 μl were added to each antibody coated tube via duplicate pipetting. This was immediately followed by the addition of 500 μl of progesterone-HRP conjugate diluted in assay buffer (4,000x) to initiate a competition reaction for the coated antibody. The tubes were then incubated for 15 minutes at room temperature. Following incubation, separation of free bound progesterone was achieved by decanting the tubes and washing three times. The amount of conjugate was deterimined by adding 1 ml OPD substrate solution to each tube, then allowing this mixture to incubate at room temperature for 15 minutes, and then terminating the substrate reaction with 1 drop of 8N H_2SO_4 stopping solution.

The standard curve was made using the same procedure with 300 μl progesterone standard (1-1,000 pg/tube). Quantitative analysis of samples was completed by comparative spectrophotometric light absorbance analysis against this standard curve.

Light absorbance readings of the standards and samples were measued at 492 mm, with a Spectronic 1001 spectrophotometer (Bausch & Lomb). A standard dose-respones curve was constructed by plotting the percent of bound progesterone (E/Eo × 100) against the total amount of progesterone in the standard solutions using the log-logit transformation fitting program as described by Davis et al. (1980). The ratio, E/Eo, is defined as the average absorbance reading of a sample or standard/the average absorbance reading of zero standard. Inter- and intra-assay coefficiency of variation were calculated using the methods described by Rodbard (1974). Results are expressed as ng progesterone/ml.

For the qualitative analysis, heparinized blood samples of varing volumes (0.1-0.2 ml) were collected, in duplicate, directly into the antibody coated star tubes, then 500 μl of progesterone-HRP was immediately added to each tube. After a 15-20 miuntes incubation period at room temperature, the tubes were washed twice with tap

water. Following the wash, 1 ml of OPD solution was added to each tube and was allowed to incubate for another 15-20 minutes, depending on color formation.

Finally one drop of H_2SO_4 stopping solution was added to stop the enzyme-substrate reaction. The color change of the substrate-enzyme reaction in the star tubes was then evaluated by eye in comparison with a quality control (concentration = 3 ng progesterone/ ml buffer solution). If the color of the sample was found to be lighter than that of the quality control, a diagnosis of pregnancy was made. A non-pregnant diagnosis was made if the sample color was darker than that of control.

Radioimmunoassay procedure

The radioimmunoassay of plasma progesterone was carried out as previously described by Lin et al. (1988).

Pregnancy diagnosis

A total of reliable data from 128 sows in two commercial farms in Taiwan, were used in this study for qualitative and quantitative measurements. All of the sows were mated or artificially inseminated and a single sample of blood was taken from each sow during the period 17-22 days after service. Blood (1 to 2 ml) was drawn from ear vein with a heparinized syringe. The 2-3 drops blood sample from the syringe without the needle was immediately tested in the field to diagnose the pregnancy on-farm, compared with a quality control of 3 ng progesterone/ ml. When the color changes of samples were the same as or deeper than those of control, recognizing as non-pregnancy. The remainder of the samples were centrifuged and the plasma was then collected and stored at −20℃ until quantitatively assayed. The diagnosis of the pergnancy status was then confirmed by either the farrowing 114 ± 10 days after breeding or abortion. Estrus return was used only for reference in the hot summer season in this study.

RESULTS AND DISCUSSION

The mouse hybridoma system yielded 5 anti-progesterone cell lines. When compared with the other lines and with a rabbit source polyclonal antiserum (#4713), the G7 line was shown by RIA to produce the best antiserum. The RIA system data for cross reaction is shown in table 1. The affinity constant of G7 is $1.1 \times 10^{10}\ M^{-1}$, and the sensitivity is 3 pg. The functional titre of G7 is 30,000x.

The most common enzyme conjugated to progesterone with mixed anhydride for use in a progesterone EIA system is HRP VI (Erlanger et al., 1957; Joyce et al., 1977). There were 2 peaks found on chromatographic analysis of the progesterone-HRP conjugate fractionation: the first peak was found from fraction No. 35-39, and the second from No. 53-57 (figure 1). We collected the eluants of No. 54-56, because of the higher titre and enzyme activity found in the No. 53-57 range. Two peaks were evidenced, because HRP VI contains two isomers in solution. A 1:4,000 dilution of progesterone-HRP conjugate resulted in the best absorbance change for quantitative analysis of the progesterone standard. The various constituents of plasma, such as protein, lipids, calcium, etc., will absorb light in the spectrophotometer, thus, interfere with the quantitative spectrophotometric analysis of the progesterone assay (van de Wiel and Koops, 1986). This interference is termed the matrix effect. A parallelism test performed to find the volume of plasma at which this effect is negligible, thus, avoid its interference with the assay (Cekan, 1975; Chase, 1983).

Table 1. Cross-reactivity of anti-progesterone monoclonal antibody (G7) with various steroids and bovine serum albumin (BSA) determined by 50% displacement

Steroids	Cross-reactivity (%)
Progesterone	100
Pregnenolone	0.06
17α-OH progesterone	0.19
Corticosterone	< 0.01
Testosterone	< 0.01
Estrone	< 0.01
Estradiol-17β	< 0.01
Cholesterol	< 0.01
BSA	< 0.01

The immunogen is progesterone-11α-hemisuccinate-BSA and the cross-reactions are obtained by RIA system.

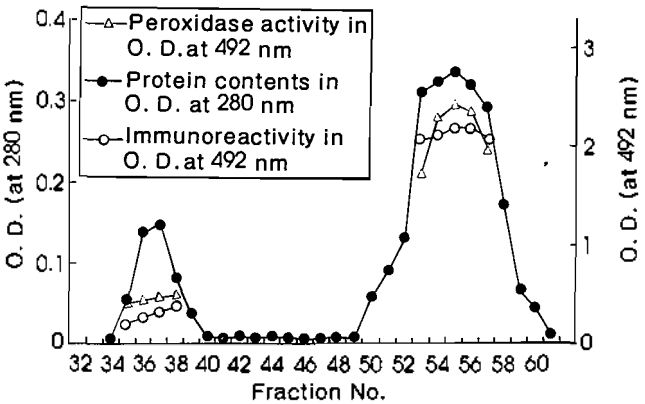

Figure 1. Sephadex G-100 chromatography of the progesterone-11α HS-horseradish peroxidase (type VI) conjugate.

To perform this test, 4 plasma standards were prepared to contain 0, 5, 10, 20, and 40 ng progesterone/ml boar plasma. These 4 plasma standards were assayed for progesterone in volumes of 10, 50, 100 and 200 μl and compared to assays of the buffer standard at the same volumes. Figure 2 shows that the plasma standard assays overlap those of the buffer at the 10 and 50 μl volumes. However, the 100 and 200 μl volumes of plasma standard showed an increase in light absorbance of 40-60% over. Figure 3 shows that there is a close correlation between progesterone standard curve made from buffer and boar plasma. So, we may use the buffer standard to accurately measure the progesterone concentration in plasma samples less than or equal to 50 μl. In volumes greater than 50 μl, the matrix effect will not be avoided, and absorbance readings will be inaccurate.

Five replicate analysis of progesterone concentration in control plasma sample (20 ng/ml), determined in five consecutive assays within one month, gave an intraassay coefficient of variation of 7% and an inter-assay coefficient of variation of 12%. Furthermore, correlation coefficients of the progesterone concentrations in the plasma samples were determined by EIA and by a validated RIA system. The excellent agreement between EIA and RIA was as the following regression line: EIA = 1.24 RIA- 1.97, r = 0.992, n = 20.

We conducted the qualitative analysis of our study just as would be done by a farmer or veterinarian in the field. In this way, we could test the practicability of the kit on the farm, as well as its reliability. Therefore, the blood sample volumes ranged from 50-200 μl were examined. These samples were taken from a pool of sows in luteal and estrous stages. From figure 4, we see the large difference of color changes between luteal and estrous stages among the various volume, suggesting that blood volumes from 50-200 μl work well for the

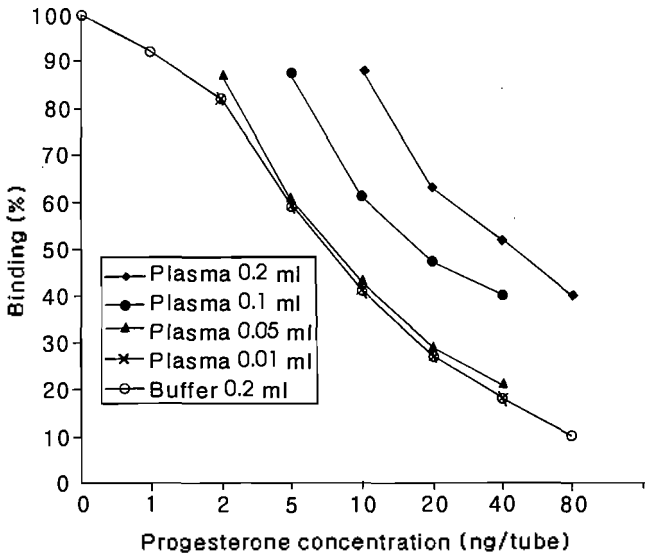

Figure 2. The parallelism test of progesterone concentrations in different plasma volumes.

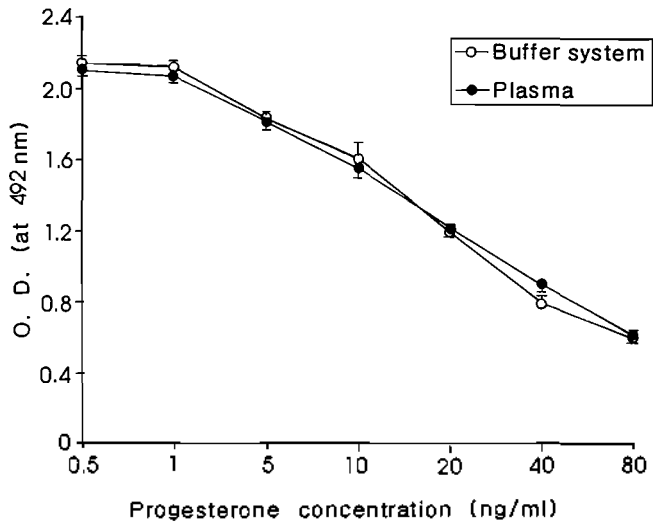

Figure 3. The standard curves of progesterone in buffer and plasma systems determined by direct enzyme immunoassay. Each point is the mean of five observations, standard errors are indicated by vertical lines.

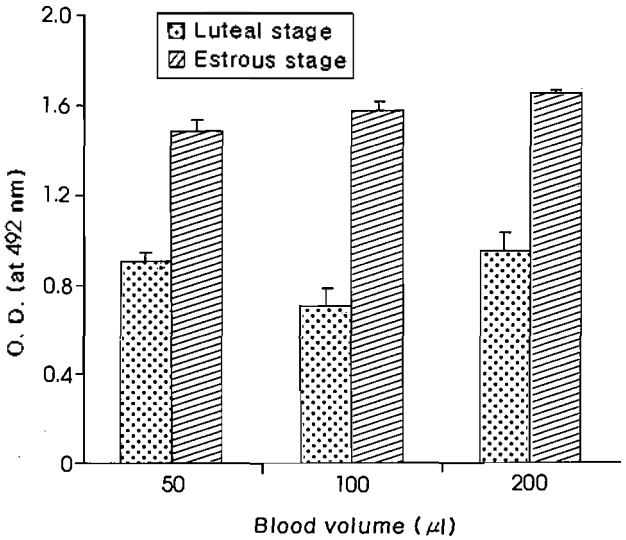

Figure 4. Color responses between the blood samples with different volumes, obtained from luteal and estrous stages, as measured by enzyme immounoassay. Each column is the mean of five observations, standard errors are indicated by vertical lines. The progesterone concentrations in these blood samples obtained from luteal and estrous stages were 15.2 ± 4.3 and 1.5 ± 0.3 ng/ml, respectively.

qualitative test. It should be pointed out here that although the greater volumes adversely affect the outcome of the quantitative analysis, as was previously noted, this is not the case for the qualitative analysis. The reason for this discrepancy is unknown.

The results of pregnancy diagnosis made by the on-farm blood progesterone test are summarized in table 2. The total accuracy of pregnancy diagnosis by this method was 92.2% (118/128) i. e. the rate for correct pregnant diagnosis was 93.1% (108/116) and that for non-pregnant diagnosis was 83.3% (10/12), as confirmed by farrowing and abortion. A highly agreement of pregnancy diagnosis (92.2% vs 94.5%) was found between the on-farm test and the quantitative test of plasma progesterone in laboratory based on 3 ng progesterone/ml. Those of miss-diagnosis ones may be due to the early embryonic death, irregular cycle length, reproductive disease, the assay problems etc. Measurement of whole blood progesterone by enzyme immunoassay has advantages in that only small volumes need to be collected and no centrifugation is required. In a report to investigate the value of whole blood progesterone measurement by using Ovucheck 'Sowside' kits on 17 to 20 days after service. The results showed that the accuracy of identification of pregnent and non-pregnant sows was 94.6% and 35.7%, respectively (Glossop et al., 1989). This earlier trial has identified problems with blood sampling that not encountered in our study. This discrepancy may be due to the higher quality control (7 ng/ml) in his study. In conclusion, we have developed a blood progesterone direct assay kit that is highly reliable and simple to use for sow's early pregnancy diagnosis. When the rate of detection of empty sows is low, especially in hot summer season, this assay may be very useful in practice.

Table 2. Early pregnancy diagnosis of sows by the on-farm blood progesterone test and the plasma progesterone quantitative test

no. of sows bred	pregnant		non-pregnant		total accuracy (%)
	farrowed on-farm test		non-farrowed on-farm test		
128	108	116	10	12	92.2 (118/128)
	farrowed quantitative test		non-farrowed quantitative test		
128	105	110	16	18	94.5 (121/128)

Pregnant recognition determined by the progesterone values over 3 ng/ml and % accuracy based on parturition or abortion.

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