RESEARCH ARTICLE

Development of a Semi Quantitative Progesterone Enzyme Immunoassay for Determination of Ovulation Time in Bitches

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ABSTRACT

A solid phase residual binding progesterone enzyme immunoassay (EIA) for determination of ovulation time in bitches was developed using polyclonal antibodies against progesterone-BSA and progesterone-3-CMO-HRPO conjugate. The assay was optimized with 1:100 dilution of progesterone polyclonal antibody, 1:1000 dilution of progesterone -3-CMO HRPO conjugate and 1:10 dilution of serum samples. Validation of the developed assay for its sensitivity, specificity and accuracy by comparing it with vaginoscopy showed an overall agreement of 97 per cent between EIA and vaginoscopy. The diagnostic sensitivity of the progesterone EIA in predicting the preovulatory, ovulatory and post ovulatory periods were 96.42, 91.66 and 100 per cent respectively. The diagnostic specificity of the assay was 100 per cent. Comparison of the developed EIA with Chemiluminescence assay (CIA) gave a correlation coefficient (r) of 0.92.

INTRODUCTION

The determination of ovulation time is of major importance in the management of normal reproduction in the bitch as it indicates the period most appropriate for mating or insemination. The bitch is unique in that there is a preovulatory rise in plasma progesterone concentration brought about by the luteinisation of the granulose cells of the follicle (Concannon *et al*., 1977). Serial monitoring of plasma progesterone concentrations allow anticipation of ovulation by about 1 to 2 days and if continued allows confirmation of ovulation and detection of fertilization period (England and Concannon, 2002). However, quantitative progesterone estimations either by Radio Immuno Assay or Enzyme Immunoassay require the use of equipment, which are usually available in laboratories. In contrast, the semi quantitative type of progesterone assay kits allow progesterone concentrations to be assessed either qualitatively or semi quantitatively. The major disadvantage is the cost and the fact that these kits have to be necessarily imported. Thus, the indigenous development of a rapid, reliable and cost effective test for prediction of ovulation in bitches would have a great

impact on canine reproduction. Keeping this in mind the present study was taken up (i). To develop a semi quantitative progesterone enzyme immunoassay for determination of ovulation time in bitches and (ii) To evaluate the efficacy of the developed semi quantitative progesterone assay in predicting ovulation time in bitches.

MATERIALS AND METHODS

Preparation of Progesterone Standards, Polyclonal Anti Progesterone Antibody and Progesterone 3-CMO-HRPO Conjugate

Fifty milligrams of progesterone reference standard (P 9776, Sigma Aldrich, Bangalore) was dissolved in 1000 µl of chloroform and was kept frozen as a concentrated stock solution. This solution was used each time for fresh preparation of various progesterone standards viz. 0.5,1 to 10, 12, 15 and 30 ng/ml by serial dilution in Phosphate buffered saline and in turn used for standardization of the semi quantitative progesterone assay.

One vial of polyclonal anti progesterone antibody raised in rabbits (Cat. No. P 5289-100 TST, Sigma

Aldrich, Bangalore) was diluted with 5 ml of Tris HCL Buffer (pH: 8) and made to 200 µl aliquots and kept frozen at -20ºC and was used as the stock antibody for further dilution and coating.

Progesterone 3-CMO-HRPO conjugate (Cat. No. P 91-92-11 H, East Coast Bio, Inc.) was stored at 2-4ºC and was diluted with PBS just before the addition of conjugate to the wells. It was found that 1:1000 dilution of conjugate was optimum for the developed assay.

Standardization of Assay

The wells of the microtitre ELISA plate were coated with 100 µl of different dilutions (1:50, 1:100 and 1:200) of anti-progesterone polyclonal antibody in carbonate-bicarbonate buffer, pH 9.6. The plate was kept at 4ºC overnight. To remove the antiserum not bound to polystyrene, the wells of the plate were emptied by inversion, washed 3 times with 300 µl of washing buffer. The unreacted sites were blocked with 100 µl of 5 per cent skimmed milk powder in PBST and kept incubated at 37º C for 1 hr. The plate was then washed 3 times with washing buffer and100 µl of progesterone standards (0.5,1 to 10, 12, 15 and 30 ng/ml) were added and kept incubated at 37ºC for 1 hr. The washing step was repeated again and 100 µl of different dilutions of Progesterone-3 CMO HRPO conjugate (1:100, 1: 500, 1: 1000, 1: 2000, 1:5000 and 1:10000) were added to the wells and left for 1 hr incubation at 37ºC. Separation of the free from bound progesterone was achieved by emptying the plate and washing three times. The amount of conjugate binding was determined by the addition of 100 µl of ABTS substrate solution to each well and kept at 37ºC for 15 min. The substrate reaction was terminated by adding 100 µl of 0.1 M Sodium duodecyl sulphate stopping solution and the OD values were read at 405 nm.

A standard curve was drawn by plotting the mean absorbance on the Y-axis and standard progesterone concentrations on the X-axis of a semi log graph paper. Progesterone concentrations of the samples were extrapolated from the standard curve using linear regression equation $Y= ax + b$, where 'a' is slope of the curve and 'b' the intercept of the curve.

Validation of the Developed Semi Quantitative Progesterone Enzyme Immuno Assay

Once the assay design was completed and test parameters were identified the validation of the assay was done based on the data obtained during the development phase. The assay was validated for its sensitivity, specificity, accuracy, detection limit and intra assay co-efficient of variation as described previously (Jacobson, 1998). To measure the sensitivity and specificity of the developed assay, vaginoscopy was taken as the "gold standard" and the results of the developed EIA were compared with it. Twenty serum samples from diestrus or pregnant dogs and 20 serum samples from male dogs (known negatives) were quantified for progesterone by the developed immunoassay and the data were used for estimating the diagnostic specificity (DSp) of the EIA. Eleven serum samples with progesterone values determined by Chemiluminescence Immuno Assay (CIA) were retested using the developed EIA and the percent deviation was calculated to determine the accuracy of the test.

Determination of Ovulation Time using Vaginoscopy

Twelve bitches brought to the small animal obstetrics and gynaecology ward of the Madras Veterinary College Teaching Hospital were subjected to vaginoscopic studies at intervals of two days starting from day 5 of proestrual bleeding till day 1 of diestrus. Vaginoscopy was performed using a rigid endoscope (Karl Storz, GmBH, Tiuttlingen, Germany). The changes in the mucosal fold contours, colour of mucosa and fluid present, during proestrus, estrus LH surge, ovulation and fertile breeding time were assessed vaginoscopically and vaginoscopically and vaginal mucosal scores were assigned to each of these changes as per the vaginal mucosal score (VMS) system described previously (Lindsay, 1983).

Determination of Ovulation Time using the Developed Progesterone Enzyme Immuno Assay

Blood samples obtained from the 12 bitches at intervals of two days starting from day 5 of proestrual bleeding till day 1 of diestrus were centrifuged at 3000 rpm for 10 minutes for separation of serum which was kept stored at -80ºC for further assay. A total of 67 serum samples obtained from these 12 dogs were quantified for progesterone using the developed assay. The efficacy of the developed assay in determining the time of ovulation was correlated with the time of ovulation as determined by vaginoscopy.

RESULTS

A solid phase residual binding progesterone enzyme immunoassay was developed using polyclonal antibodies against progesterone –BSA and progesterone 3-CMO-HRPO conjugate. In this assay free progesterone in standards/samples was allowed to bind to the antibodies and then the progesterone HRPO conjugate was allowed to bind to the residual sites. This provided more opportunity for the free progesterone to bind to antibodies. 1:100 dilution of progesterone antibody, 1:1000 dilution of -3-CMO-HRPO conjugate and 1:10 dilution of serum samples were found to be optimal for detecting progesterone concentrations of 0-12 ng/ml in canine serum.

Sixty-seven serum samples collected from the twelve dogs were quantified for progesterone using the developed assay. Based on the progesterone levels, samples were categorised into the preovulatory (<3ng/ml), ovulatory (4-10ng/ml) and post ovulatory periods (>10ng/ml) periods. Progesterone values for 65 of 67 samples (97 per cent) were in agreement with Vaginoscopy which was taken as the "Gold standard" in predicting the preovulatory, ovulatory and post ovulatory periods. The diagnostic sensitivity of the progesterone EIA in predicting the preovulatory, ovulatory and post ovulatory periods was 96.42, 91.66 and 100 per cent respectively.

Twenty serum samples from diestrus or pregnant dogs and 20 serum samples from male dogs (known negatives) were quantified for progesterone by the developed assay. The diagnostic specificity (defined as the percentage of animals that did not have a given condition and were diagnosed by the assay as negative for the condition) was 100 per cent for all the known negative samples. The developed EIA had an overall diagnostic

sensitivity and specificity of 97.01 and 100 per cent respectively.

The detection range of the developed assay was 0.12ng/ml to 12.5ng/ml. The precision of the intra assay was estimated as the coefficient of variation (COV) between two readings for a sample. When the COV of the sample was more than 20 per cent, the samples were repeated. In the developed assay the maximum COV observed was only 9.18.

Eleven serum samples whose progesterone levels were quantified using Chemiluminescence Assay (CIA) were retested with the developed EIA to calculate its accuracy. Comparison of the developed EIA and CIA gave a correlation coefficient of 0.92 (n=11, P<0.01).

DISCUSSION

A semi quantitative progesterone enzyme immuno assay developed using polyclonal antibodies against progesterone, by adopting the residual binding assay technique described previously (Mirnal, 2000) was optimized with 1:100 dilution of progesterone polyclonal antibody, 1:1000 dilution of progesterone -3CMO-HRPO conjugate and 1:10 dilution of serum samples. The earlier reports on the increased sensitivity of progesterone EIA with the use of a heterologous system employing 3-CMO-HRPO as conjugate (Gross *et al*., 1980; Munro and Stabenfeldt, 1984; Hatzidakis *et al*., 1993) prompted us to use the same conjugate in our study.

The assay was validated for its sensitivity, specificity, accuracy, detection limits and coefficient of variation. For validation of the assay, vaginoscopy was taken as the "gold" standard. Progesterone values for 65 out of 67 serum samples were in agreement with vaginoscopy in predicting the preovulatory, ovulatory and post ovulatory periods, giving an overall agreement of 97 per cent and a diagnostic sensitivity of 96.42, 91.66 and 100 per cent respectively. The diagnostic specificity of the progesterone EIA in predicting known negatives was 100 per cent. The assay had an overall diagnostic sensitivity and specificity of 97.01 and 100 per cent respectively. The ability of a commercially available semi quantitative EIA kit (Ovucheck ® premate 10) originally designed for determining serum or plasma progesterone in dogs and cats, to determine progesterone in horse plasma, by comparing it with Radio immuno assay (RIA) showed the overall agreement between EIA and progesterone RIA to be 92.15 per cent. The sensitivity and specificity for determination of concentration of plasma progesterone < 3 ng/ml was 100 and 95.6 per cent respectively, for concentrations >10 ng/ml, the sensitivity and specificity were 92.3 and 93 per cent respectively (Pinto *et al*., 2006).

In the present study, low and high detection limit of the developed assay was 0.12 ng/ml and 12.5 ng/ml respectively. A previously EIA developed for measuring progesterone in serum reported the measurable range of progesterone in 0.1 ml of bovine serum to be 0.25 ng/ml and 10 ng/ml (Nakao,1980). Another study on the quality of the Target enzyme linked immunosorbent assay kit for determining progesterone concentration in dog plasma reported that the kit had a satisfactory specificity and sensitivity of 83.1 and 83.0 per cent respectively.

However, its predictive value of positive results (> 5ng/ml) which was indicative of ovulation was relatively weak indicating that the test was insufficient for confirming ovulation precisely (Fieni *et al*., 1993).

Although vaginoscopy was taken as the "gold" standard in the present study, it is usually considered to be subjective and hence, it was decided to check the reliability of the assay in diagnosing ovulation time by comparing it with another assay. For this purpose, Chemiluminescence assay was preferred as it was comparable to or even better than RIA in terms of sensitivity, accuracy and precision (Forseberg *et al.,* 1993). Eleven serum samples whose progesterone levels were quantified using Chemiluminescence assay were tested using the developed EIA. The correlation coefficient between these two was found to be 0.92 which was more than the table value of 9 degrees of freedom. The significant positive correlation value justifies the suitability of this test in diagnosing the ovulatory period.

The validation results indicate that the rapid and convenient EIA for progesterone has performance characteristics which are at least good as that of vaginoscopy and CIA. The standard curve and data indicate that the sensitivity, specificity and accuracy of the assay were satisfactory and the assay is capable of quantifying progesterone without interference in the presence of closely related hormones and plasma proteins.

Conclusion

In conclusion, effective progesterone EIA for determination of ovulation time in bitches has been developed which has proved to be both analytically and clinically valid thus, opening the possibility of the reliable onsite diagnostic technique.

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