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Original article

Agreement of different methods for assessment of progesterone concentrations in beef cattle serum

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Abstract

Progesterone (P4) is responsible for the main reproduction processes. Concentration of P4 varies widely among different determination methods, and interpretation of these values may be difficult. The objective of the current study was to assess the agreement of three different enzyme immunoassays (ELISA) in relation to radioimmunoassay (RIA) of P4 concentration assessment of beef cow serum samples.

Samples were collected randomly considering high (pregnant cows) and low (non-pregnant cows) P4 concentrations. Depending on the P4 assessment method, four groups were created as follows: Group 1 – direct samples assessed by ELISA, Group 2 – extracted samples assessed by ELISA, Group 3 – samples assessed by automated ELISA, and Group 4 – samples assessed by RIA.

The mean progesterone concentration was 4.50 ng/mL, 1.24 ng/mL, 4.07 ng/mL and 4.39 ng/mL from Group 1 to Group 4, respectively. The mean difference (MD) between Group 1, Group 2 and Group 3 individually compared with Group 4 was −0.10 ± 1.24 ng/mL, 3.15 ± 3.58 ng/mL and 0.33 ± 1.42 ng/mL, and the 95% confidence interval (CI) for the differences (*s*) was from −0.99 to 0.78 ng/mL, from 0.59 to 5.71 ng/mL, and from −0.69 to 1.34 ng/mL, respectively. The confidence interval for the lower and upper limit of the agreement ranged from −4.12 to −1.05 ng/mL and from 0.84 to 3.91 ng/mL between Group 1 and Group 4, from −8.45 to 0.42 ng/mL and from 5.88 to 14.75 ng/mL between Group 2 and Group 4, from −4.29 to −0.76 ng/mL, and from 1.41 to 4.94 ng/mL between Group 3 and Group 4.

Our findings show that the best agreement with RIA was observed for Group 1 and Group 3, while the agreement in the extraction method was least accurate.

Key words: agreement, confidence interval, assessment methods, progesterone, sample extraction, ELISA, RIA

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Introduction

Progesterone is an important factor for proper occurrence of the estrous cycle and embryo development (Mann and Lamming 1999, Lonergan et al. 2016). Progesterone gives valuable information about ovarian activity. On day 0 of estrous, low progesterone levels may be detected due to an inactive corpus luteum, and during the luteal phase, or in the case of pregnancy, production of progesterone increases (Isobe and Nakao 2003, Forde et al. 2011). Progesterone circulates in the blood mainly bound to corticosteroid binding globulin (CBG), sex hormone binding globulin (SHBG) and albumin. Over 80% of circulating P4 is bound to plasma proteins and only 2–10% of the total concentration circulates as free hormone (Lagana et al. 1986, Carrière and Lee 1994, Mekonnin et al. 2017).

A number of scientists have reported a sensitive and specific RIA and ELISA for progesterone determination (Sugden 1978, Farahmand et al. 1998, Boggs et al. 2016). Historically, the RIA has been called the gold standard for quantifying hormones in bovine serum (Skenandore et al. 2017). Due to the potential radiation hazards from RIA, radioactive waste disposal and inactivation, alternative methods have also been be used (Farahmand et al. 1998, Colazo et al. 2008, Skenandore et al. 2017).

One of these is ELISA – the most frequently used method for determining the progesterone level in direct samples. There is an emerging risk for assay interference from other sample constituents, but extraction of the desired hormone from samples may eliminate this problem. Therefore, the loss of steroid hormone during extraction is critical. The results of another parallel study indicated that there was a probability of antibody-ligand binding interference in serum samples (Skenandore et al. 2017). Commercial ELISA relies on the use of progesterone-enzyme conjugates and these lack an efficient and convenient purification method (Mitchell et al. 2004). Conventional assays involve extraction of progesterone from serum by organic solvent; however, the direct assay of progesterone in unextracted serum has also been described (Haynes et al. 1980, Ratcliffe 1982, Lagana et al. 1986).

There are many methods for measurement of suitable analytes; therefore, it is not easy to decide which one is the most accurate. Quantitative values differ between different methods and vary widely. Detection methods must be accurate, rapid, specific and economical, and should not require specialized equipment or dangerous chemicals (Lagana et al. 1986, Cardoso et al. 2014). In order to make a comparison of different methods, Bland and Altman (2010) have suggested assessing the degree of agreement. Comparison is carried out by calculating the difference, the 95% confidence interval and the limits of agreement of the confidence interval.

Calculation of difference provides information for assessing possible relationships between the measurement error and the true value, and the plot is used for visual analysis of the agreement between two different methods assaying the same parameter. The width of a 95% confidence interval is related to distribution of differences, and the size and the variability of the samples. Normal distribution or actual mean will be located within the interval (mean±2SD). Variables outside the out-layer represent 5% of the intervals that are not expected to cover the true mean limit of agreement (Wiles 2013). Limits of agreement of the confidence interval demonstrate the range of variation: the wider the range, the less the accuracy, and *vice versa*.

The aim of this study was to compare and assess the agreement of P4 concentration with three ELISA setup methods in relation to RIA in beef cow serum samples.

Materials and Methods

All the procedures were carried out in accordance with the guidelines of the State Food and Veterinary Service. The study included clinically healthy limousin beef cows (n=10) in according with general clinical investigation. The reproductive condition was confirmed by rectal-ultrasound (iScan, Draminski) examination. The beef cows were reared in a barn with loose housing system throughout the year and fed (three times daily) a ration formulated to meet or exceed physiological nutritional requirements: ~113 MJ/ME, ~1200 g/CP, ~830 g/DP. Blood samples were taken from the coccygeal vein into vacutainer tubes from each cow once at the same time from 10:00 to 11:00, in January and delivered to the laboratory in four hours. Samples were divided into those with high P4 concentration (pregnant cows) and low (non-pregnant cows). Each serum sample was divided into 4 replicates and a total of 40 samples were stored at −20°C until further assessment. ELISA was carried out in the Animal Reproduction Laboratory and Small Animals Clinic, and RIA was performed in a certified Laboratory of General Endocrinology at the Institute of Endocrinology, Lithuanian University of Health Sciences. Depending on the progesterone determination method, four groups were created:

Group 1
Direct serum samples assessed by ELISA

A commercially available diagnostic kit based on competitive binding and quantitative determination of progesterone in animal blood serum was used.

Table 1. Comparison of progesterone concentration determined by different methods.

	Group 1	Group 2	Group 3	Group 4
Mean, ng/ml	4.50±5.18	1.24±1.31	4.07±5.08	4.39±4.67
Pregnant cows, ng/ml	8.75±4.17	2.29±1.12	8.05±4.46	7.89±4.37
Non-pregnant cows, ng/ml	0.24±0.10	0.20±0.07	0.09±0.05	0.89±0.23

A total of 25 µL of each standard, controls and samples were poured into appropriate wells and incubated for 5 minutes; 200 µL of enzyme conjugate was then poured into each well and thoroughly mixed for 10 seconds. The microplate was incubated for 60 min, following which the wells were washed three times with a diluted wash solution (400 µL per well). A total of 200 µL substrate solution was added to each well. The plates were then incubated for 15 min and 100 µL of a stop solution was then added. The absorbance was determined at 450 ± 10 nm with a microplate reader, and the results were calculated automatically using a 4 parameter logistics curve (Bio-TEK Synergy HT, USA). The standard concentration ranged from 0 to 40 ng/mL. The variability within the assay was 5.4–6.99 % and between the assay 4.34–9.96 %. The sensitivity of the current method was 0.045 ng/mL.

Group 2
Extracted serum samples assessed by ELISA

Sample extraction was carried out by pouring 60 µL of serum and 1000 µL of ethyl acetate into glass tubes which were immediately closed with stoppers. The tubes were vortexed for 10 minutes and following 2 minutes of incubation 500 µL of the upper solvent phase was poured into a second glass tube, and nitrogen evaporations were then performed. The samples were reconstituted with 60 µL of steroid-free serum and vortexed.

The extracted samples were analyzed according to the procedure described for Group 1. The final steroid concentration was calculated by multiplying the obtained values by a factor of 2.

Group 3
Serum samples assessed by automated ELISA

A commercially available diagnostic kit based on competitive binding and quantitative determination of progesterone in serum samples was used. The method is fully automatic and entirely performed in the test cups (TOSOH AIA Analyzer, Japan). A standard concentration ranged from 0.1 to 40 ng/mL. The intra- and inter-assay variations were 2.1–2.4% and 2.8–3.1%, respectively. The sensitivity of the method was 0.06 ng/mL.

Group 4
Serum samples assessed by RIA

A commercially available diagnostic kit based on competitive binding and quantitative detection of progesterone in direct blood serum samples was used. The microplate wells have a fixed amount of labelled ^{125}I steroid which competes with the steroid to be measured in the direct sample.

Following 2-hour incubation at 37°C in a water bath, an aspiration step terminates the competition reaction. The tubes are then washed with 3 mL of a wash solution and aspirated again. The P4 concentrations of the samples were evaluated by means of a γ-ray counter (BERTHOLD, Germany). The standard concentration ranged from 0 to 36 ng/mL. The intra--assay and inter-assay variations were 4.0–5.2 % and 6.5–8.6 %, respectively. The sensitivity of the method was 0.05 ng/mL.

The statistical analysis of data was performed using the SSPS 18 (SPSS Inc., Chicago, IL, USA) software package and variables were analyzed using the *t* test, and significance was considered $p<0.05$. The results were expressed as the mean difference (MD) and the differences (*s*), the 95 % confidence interval (CI), and the interval of normal distribution (mean±2SD).

Results

The mean value of P4 concentration varies between groups and was: for Group 1, 4.50±5.18 ng/mL (range from 0.19 to 15.52 ng/mL); for Group 2, 1.24±1.31 ng/mL (range from 0.10 to 4.26 ng/mL); for Group 3, 4.07±5.08 ng/mL (range from 0.02 to 14.78 ng/mL); and for Group 4, 4.39±4.67 ng/mL (range from 0.57 to 16.09 ng/ml). The progesterone concentration in pregnant cows was 8.75 ng/mL, 2.29 ng/mL, 8.05 ng/mL and 7.89 ng/mL; in non-pregnant cows it was 0.24 ng/mL, 0.20 ng/mL, 0.09 ng/mL and 0.89 ng/mL, respectively (Table 1).

Significant correlations were established between Group 1 and Group 4, as well as between Group 2 and Group 4 (r=0.977), and also between Group 3 and Group 4 (r=0.965). There was also a correlation between Group 1 and Group 2 (r=0.962), Group 1 and Group 3 (r=0.995), and Group 2 and Group 3 (r=0.944, $p<0.001$).

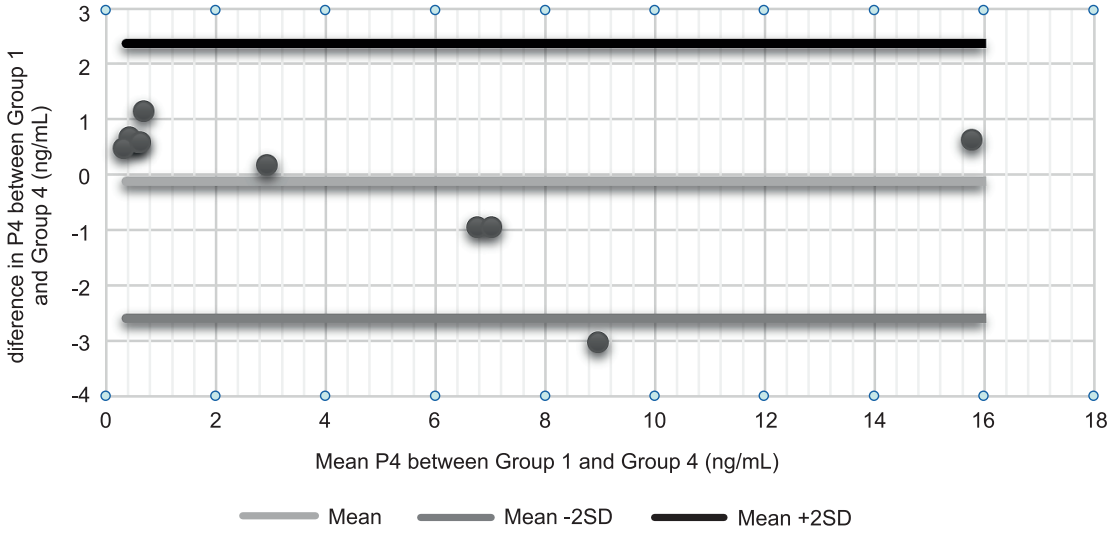

Fig. 1A. Differences of progesterone concentration against means between Group 1 and Group 4.

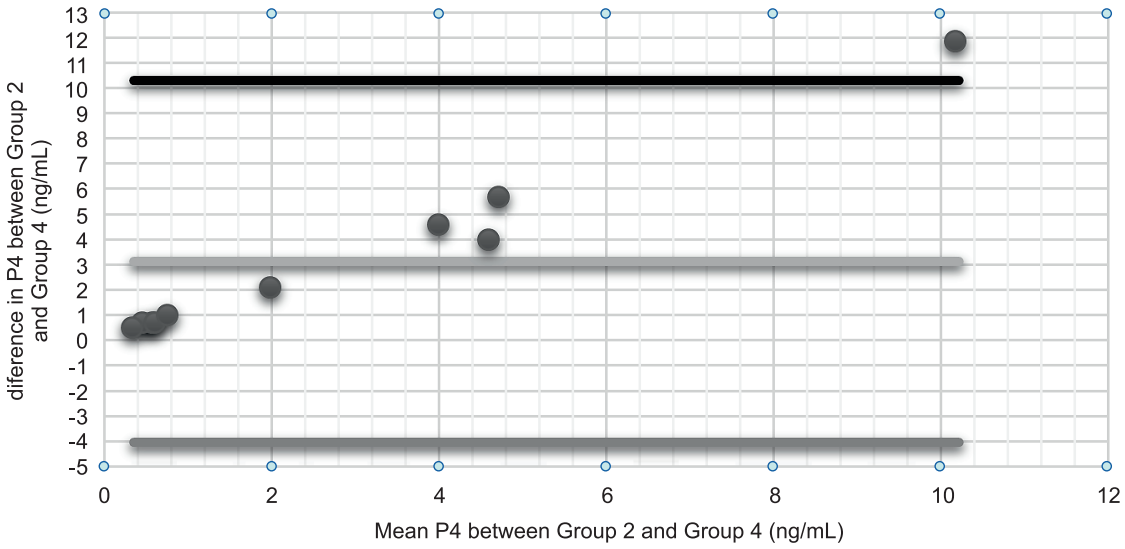

Fig. 1B. Differences of progesterone concentration against means between Group 2 and Group 4.

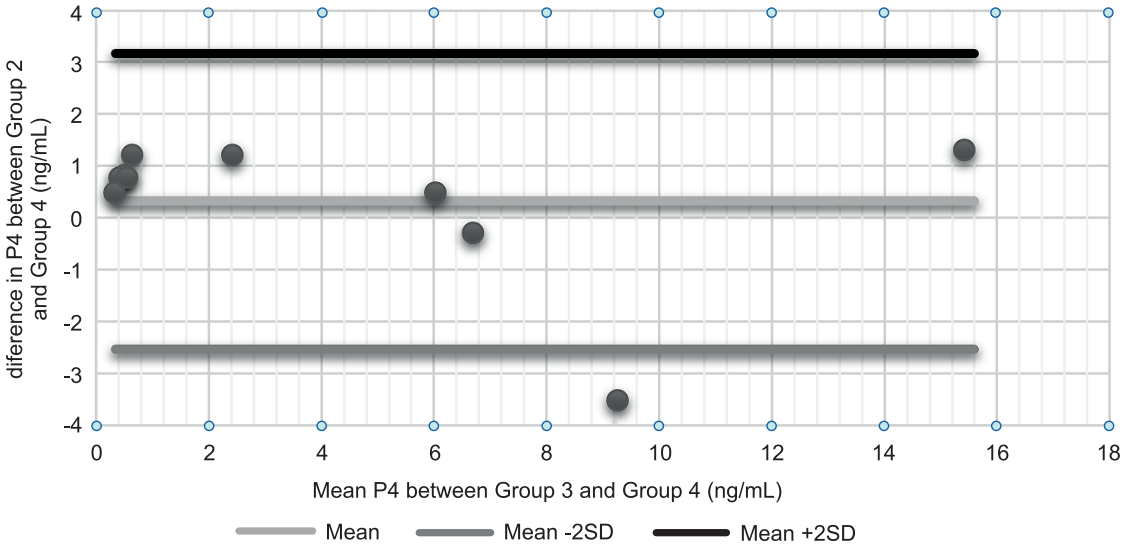

Fig. 1C. Differences of progesterone concentration against means between Group 3 and Group 4.

Table 2. Limits of agreement and, lower and upper range of 95 % confidence interval between different methods and RIA.

	Group 1 x Group 4	Group 2 x Group 4	Group 3 x Group 4
MD–2*s*	–2.58 ng/mL	–4.01 ng/mL	–2.52 ng/mL
MD+2*s*	2.38 ng/mL	10.31 ng/mL	3.17 ng/mL
Range of 95 % CI for the lower limits of agreement*	–4.12 to –1.05 ng/mL	–8.45 to 0.42 ng/mL	–4.29 to –0.76 ng/mL
Range of 95 % CI for the upper limits of agreement*	0.84 to 3.91 ng/mL	5.88 to 14.75 ng/mL	1.41 to 4.94 ng/mL

* CI – confidence interval

The differences and the mean between the groups are shown in Fig. 1A, 1B and 1C. The mean progesterone concentration was estimated by computing the confidence interval for the mean. The interval of mean±2SD is expected to capture 95 % of the samples with a possible 5% of variables out of the interval (Bland and Altman 2010, Wiles 2013).

It is difficult to link the relation between the methods based on the data in the plots, but it is possible to calculate the bias by calculating the mean difference (MD) and the standard deviation of the differences (*s*). For the P4 concentration of Group 1 with Group 4, the MD was –0.10 ng/mL and *s* was 1.24 ng/mL. The differences (MD±2*s*) will be as limits of the agreement: MD–2*s* = –0.10 – (2 x 1.24) = –2.58 ng/mL and MD+2*s* = –0.10 + (2 x 1.24) = 2.38 ng/mL. Progesterone in Group 1 may be –2.58 ng/mL below or 2.38 ng/mL above the Group 4 progesterone values.

The mean difference and the difference between Group 2 and Group 4 were 3.15 ng/mL and 3.58 ng/mL, and between Group 3 and Group 4 were 0.33 ng/mL and 1.42 ng/mL, respectively. The limits of agreement for Group 2 may be –4.01 ng/mL below or 10.31 ng/mL above, and for Group 3 it may be –2.52 ng/mL below or 3.17 ng/mL above the Group 4 progesterone values.

The standard error (SE) and the confidence interval (CI) may be useful for evaluating the precise measurements. For the P4 concentration between Group 1 and Group 4 *s*=1.24 and SE for MD=0.39. The 95 % CI with 10 degrees of freedom (df) *t*=2.26 (Student *t* distribution) and CI for bias is –0.10 – (2.26 x 0.39) and –0.10 + (2.26 x 0.39) giving a range from –0.99 to 0.78±0.68 ng/mL, respectively. The 95 % CI for the lower limit of agreement between Group 1 and Group 4 is –2.58 – (2.26 x 0.68) and –2.58 + (2.26 x 0.68) giving a range from –4.12 to –1.05 ng/mL. The 95% CI for the upper limit of the agreement between Group 1 and Group 4 is calculated as 2.38 – (2.26 x 0.68) and 2.38 + (2.26 x 0.68) giving a range from 0.84 to 3.91 ng/mL.

For P4 concentration between Group 2 and Group 4 *s*=3.58 and SE for MD=1.13. The bias for the CI is calculated as 3.15 – (2.26 x 1.13) and 3.15 + (2.26 x 1.13) giving a range from 0.59 to 5.71±1.96 ng/mL. The 95 % CI for the lower limit of the agreement between Group 2 and Group 4 is –4.01 – (2.26 x 1.96) and –4.01 + (2.26 x 1.96) giving a range from –8.45 to 0.42 ng/mL. The 95 % CI for the upper limit of the agreement between Group 2 and Group 4 is calculated as 10.31 – (2.26 x 1.96) and 10.31 + + (2.26 x 1.96) giving a range from 5.88 to 14.75 ng/mL.

For P4 concentration between Group 3 and Group 4 *s*=1.42 and SE for MD=0.45. The bias for the CI is 0.33 – (2.26 x 0.45) and 0.33 + (2.26 x 0.45) giving a range from –0.69 to 1.34±0.78 ng/mL. The 95 % CI for the lower limit of the agreement between Group 3 and Group 4 is –2.52 – (2.26 x 0.78) and –2.52 + + (2.26 x 0.78) giving a range from –4.29 to –0.76 ng/mL. The 95 % CI for the upper limit of the agreement between Group 3 and Group 4 is calculated as 3.17 – (2.26 x 0.78) and 3.17 + (2.26 x 0.78) giving a range from 1.41 to 4.94 ng/mL. The data discussed is presented in Table 2.

Discussion

Beef cow blood serum samples were assayed in order to assess the agreement of three different ELISA assays in relation to RIA. Three methods (Group 1, Group 3 and Group 4) did not differ in their ability to determine the P4 concentration when overall mean and pregnant samples were analyzed, but differ more in non-pregnant samples. The comparison of the methods is based on calculating the difference, the 95% confidence interval and the limits of agreement of the confidence interval. Here we will discuss the key criteria for assessing agreement and selection of the method.

We observed a positive correlation between groups; however, according to Bland and Altman (2010) and Marcus and Hackett (1986), the correlation coefficient measures the strength of the relationship and indicates a possible link and a positive linear direction of the selected variable, but not agreement between measurements. Therefore, in this study we do not use a correlation coefficient to assess agreement between two measurements.

The literature has shown that direct sample methods have resulted in lower concentrations than extracted samples (Skenandore et al. 2017). Our findings indicate

that the mean progesterone concentration tends to be three times lower in the method with sample extraction compared with other methods. Colazo et al. (2008) study data shows that RIA has detected 8.1±1.0 ng/ml P4 concentration in pregnant cow plasma, ELISA with sample extraction showed 10.9±2.0 ng/ml, and ELISA with direct sample analysis was 8.9±0.4 ng/ml; P4 concentration in non-pregnant cows was 0.2±0.06 ng/ml, 0.4±0.1 ng/ml and 1.1±0.2 ng/ml, respectively. We have found that the P4 concentration in extracted samples of pregnant cows is several times lower (2.29±1.12 ng/ml) than in other methods (where it ranged from 8.75±4.17 to 7.89±4.37 ng/ml); the highest values in non-pregnant cows were observed by RIA (0.89±0.23 ng/ml) the lowest were assessed with automated ELISA (0.09±0.05 ng/ml), and in methods with direct and extracted samples the values were 0.24±0.10 ng/ml and 0.20±0.07 ng/ml, respectively. Skenandore et al. (2017) claimed that the loss of steroid hormone during extraction is critical. We observed that the lowest P4 concentration of non-pregnant cows was determined with automated ELISA. Perhaps, depending on the range of analyte to be analyzed, different determination methods may be selected.

We further observed a negative relationship of mean difference between Group 1 and Group 4, and a positive relationship between Group 2 and Group 4, and between Group 3 and Group 4 it was –0.10 ng/mL, 3.15 ng/mL and 0.33 ng/mL, respectively. This shows the mean difference for the groups – the lower the difference (null hypothesis) the better is the agreement and accuracy.

The width range of the 95% confidence interval was established in the extracted method (Group 2), which indicates the distribution of the variables. This should also be minimized by comparing the values of two different methods. The limits of agreement of the confidence interval was two times higher for the lower limits and three-four times higher for the upper limits of agreement comparing Group 2 with other groups. This wide range of limits may be misleading and is unlikely to provide accuracy when interpreting determined values of progesterone. However, in Group 1 and Group 3, an acceptable degree of agreement was assessed by evaluating the same key criteria.

We can conclude that the use of a direct sample method is more reliable then the extraction method for progesterone determination in serum samples. It is also necessary to consider not only the statistical parameters when choosing which method to use.

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