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COMPARISION OF IGF-1 (INSULIN LIKE GROWTH FACTOR-1) LEVELS IN BOVINE SERUM SAMPLED THREE TIMES DURING THE DAY USING VALIDATED EQUINE IGF-1 *ELISA*

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Cows in negative energy balance exhibit reduced fertility, mediated by metabolic signals that influence the reproductive system. Measurement of IGF-1 contributes to the diagnosis of negative energy balance. The aim of this study was to investigate possible variations in IGF-1 levels in samples taken at different times of the day. Equine IGF-1 ELISA was used for measuring IGF-1 in bovine samples. Statistical analysis was applied to the results. Using scatter diagrams fitted with Deming regression lines, we established that paired measurements on samples obtained at different times were close to the line of equality. 90% of the absolute differences were less than the reproducibility coefficient, confirming that the level of agreement between the IGF-1 levels at different times of the day was satisfactory. IGF-1 levels in bovine samples were independent of the time of day at which samples were taken.

Key words: IGF-1, dairy cows, ELISA, method agreement

INTRODUCTION

Nutritional status is a major factor influencing the ability of an animal to reproduce (Raush *et al*., 2002). In adult females, dietary intake acts at various levels within the hypothalamus-pituitary-ovarian axis to influence ovarian activity and is a key factor regulating embryo survival during pregnancy (Armstrong *et al*., 2003). Dairy cows enter a period of negative energy balance in early lactation, which may last up to 20 weeks in high yielding cows. Predisposing factors to this state include calving difficulties, inappropriate diet, reduced intake of dry matter and high rate of body condition score loss (Wathes *et al*., 2003). The shortfall in energy intake is met by extensive mobilization of body tissues. Cows in negative energy balance release non-esterified fatty acids (NEFAs) from fat, and ketone bodies (acetone, acetoacetate and β-hydroxybutyrate) are produced by the liver as the fatty acids are metabolized. Therefore, circulating concentrations of both NEFA and β-hydroxybutyrate (BHB) increase after calving to an extent that is related to the degree of fat mobilization (Wathes *et al*., 2003). Increased milk fat occurs in ketotic cows, presumably because of the increased availability of BHB and fatty acids for milk fat synthesis (Podpečan *et al*., 2007). The change in body

condition score (BCS) after calving provides a subjective estimate of the energy shortfall in an individual cow (Edmunson *et al*., 1989). Insulin, the insulin-like growth factor (IGF) system and leptin are nutritionally influenced mediators of energy balance and reproduction function. High BCS loss corresponds to low IGF-1 and increased NEFA and BHB concentrations in the early postpartum period, during the first lactation and in older cows. In recent studies on the influence of nutrition on the reproductive function in cattle, metabolic hormones as nutritional signals have been proposed to exert a direct effect at the ovarian level. This was based on the well established principle that profiles of metabolic hormones such as GH, insulin, and IGF, are closely associated with nutrition induced alterations in body energy and protein balance. A negative relationship was observed between BCS loss in the early postpartum period and the interval to first ovulation and to conception (Beam and Butler, 1999; Wathes *et al*., 2001; Podpečan *et al.*, 2008). Circulating concentrations of IGF-1 in the peripartum period are good indicators of the capacity of energy-restricted cows to resume cyclicity after parturition (Roberts *et al*., 1997). Cows with poor energy status have low circulating concentrations of IGF-1. The association of low circulating IGF-1 concentrations with poor conception rates supports proposals that the IGF-1 system plays an important role in the reproductive tract (Wathes *et al*., 1998). Subsequent studies, both *in vitro* and *in vivo*, have highlighted the importance of IGF-1 and /or insulin acting in synergy with follicle stimulating hormone (FSH) and luteinizing hormone (LH) (Gong, 2002). IGF-1 and leptin are proposed as endocrine signals involved in the negative energy balance and/or in the level of body reserves that may explain the reproductive performance (Meikle *et al*., 2004).

Thus, it is important diagnostically to ascertain accurate values of the level of IGF-1. Its level in bovine samples is measured mostly by radio immuno assay (RIA) following acid-ethanol extraction (Ciccioli *et al*., 2003; Kane *et al*., 2004; Hidalgo *et al*., 2004).

ELISA is easy to perform and does not involve the use of radioactive tracers and a human *ELISA* kit has already been used to determine porcine IGF-1 according to previous validation (Corson *et al*., 2002). We have therefore evaluated the use of an equine IGF-1 *ELISA* for measuring IGF-1 in bovine samples. The influence of different times of sampling was also tested. Moreover, if the same test could be used for simultaneous determination of IGF-1 in bovine and equine samples, it could be of benefit to clinical veterinary laboratories.

MATERIAL AND METHODS

Animals, blood sampling and study design

Blood samples were taken from 18 dairy cows of Slovenian domestic brown breed, with average milk production of 7000 kg during standard lactation. Cows were kept in a free-stall barn system and fed total mixed rations. The basic ration was composed of hay, grass and maize silage. Depending on the production and milk yield, the ration was supplemented with protein concentrate (19 % digestible

raw protein), as well as roughly crushed maize grains and vitamin-mineral mixture. All cows had access to food and water *ad libidum* during the whole year.

Blood samples were taken three times a day, in the morning (8 a.m.), at noon (12 a.m.) and in the evening (18 p.m.) to determine possible variations in the level of IGF-1. Blood samples were collected from the tail vein, clotted and centrifuged at 2000 x g for 15 min. Serum was separated and stored at –20°C until IGF-1 was quantified. All serum samples were analysed at the same time after 4 months of storage at -20°C.

IGF-1 concentration was measured using IGF-1 equine *ELISA* (DRG Diagnostics) after test validation, described in detail below.

Analytical methods - IGF-1 ELISA

IGF binding protein, IGFBP-3, interferes in the determination of IGF-1, and therefore has to be separated. Serum extraction and the *ELISA* procedure were performed according to the User's Manual for IGF-1 equine *ELISA* (DRG Diagnostics). Standards (recombinant human IGF-1) were not extracted as it's defined in the user's manual. Serum extraction was performed before *ELISA*. Standards and extracted serum samples were then analysed using *ELISA*. The *ELISA* uses antibodies with high affinity and specificity for two epitopes on IGF-1. A monoclonal anti-IGF-1 antibody bound to the polystyrene well is used to capture the IGF-1 from the sample in the presence of a different monoclonal anti-IGF-1 antibody conjugated to alkaline phosphatase. Following incubation and one step formation of the solid phase-IGF-1-conjugated monoclonal antibody sandwich, the well is washed to remove excess of unbound conjugated antibody. The chromogenic substrate is then added, which turns from colourless to yellow and is measured using a spectrophotometer at a 405 nm wavelength. Sample concentrations were read from a calibration curve, constructed with standard solutions of recombinant human IGF-1, according to the User Manual.

Precision of the test

Repeatability of the test procedure was determined by intra-assay precision. The intra-assay coefficient of variance (CV) was calculated by measuring 10 replicates of each of three samples. Reproducibility of the test procedure was determined by inter-assay precision. The inter-assay CV was calculated by measuring three samples in duplicates in three different assays. Satisfactory repeatability of the assay was defined when intra-assay CV was lower than 10% and satisfactory reproducibility as inter-assay CV lower than 20%.

Accuracy of the test

Recovery

To test the recovery of the test procedure, known concentrations of IGF-1 (recombinant human IGF-1) were added to 3 bovine serum samples. The recovery % was calculated from expected and assayed concentrations. Recommended acceptable limits of recovery in the range from 80% to 120 % were used in the evaluation of the assay.

Dilution test

The immunological identity of the IGF-1 present in bovine serum samples and the IGF-1 used for the standards to calibrate the standard curve was tested by a dilution assay. Three samples were assayed undiluted and at a dilution of 1:2 and 1:4. Assayed to expected ratios in the range of 80% to 120% were defined as acceptable.

Statistical analysis

The levels of IGF-1 in sera were determined at three sampling times – morning, noon and afternoon and the results compared. We used the statistical programme Analyse-it, General + Clinical Laboratory statistics, version 1.71.

Bias determination

Differences between pairs of measurements of samples obtained at different sampling were calculated for pairs of samples; morning – noon, morning – evening, noon – evening. In absolute bias plots, the biases were plotted against their average value for each sample. In order to assess how well the paired measurements agreed with each other, the limits of agreement were determined. The upper and lower limits of agreement were calculated as $\bar{d} \pm 2s_{diff}$; where $\bar{d}$ is the mean of difference for all the samples (average bias) and s_{diff} is the standard deviation of the differences. $2s_{diff}$ indicates the maximum difference likely to occur between two measurements. This is the value below which the bias between paired results may be expected to be (Petrie and Watson, 1999).

Deming regression

To compare the measurements of IGF-1 in samples obtained at different sampling times, scatter graphs to which we fitted a Deming regression line were constructed. Deming regression was used to solve the problem of describing the relationship between two measurements, both measured with a possible error. The intercept, standard error (SE) of the intercept and confidence intervals of the slope and the intercept were calculated (Jones and Payne, 1997).

RESULTS

Performance characteristics

Repeatability of the assay was estimated by measuring 10 replicates each of three samples. The intra-assay coefficient of variance (CV) varied from 7.9% to 8.2% (Table 1), confirming satisfactory repeatability of the test procedure. Inter-assay CV varied from 11.1% to 21.3% (Table 2), indicating suboptimal reproducibility of the assay.

Table 1. Repeatability (intra-assay precision) of equine IGF-1 ELISA to measure bovine IGF-1

	Mean (ng/mL)	Intra-assay CV (%)
Sample 1 (low)	108	8.2
Sample 2 (medium)	188.9	7.9
Sample 3 (high)	305.0	7.9

Three serum samples were assayed 10 times during the same assay.
CV = coefficient of variation (CV = SD/mean)

Table 2. Reproducibility (inter-assay precision) of equine IGF-1 ELISA to measure bovine IGF-1

	Mean (ng/mL)	Inter-assay CV (%)
Sample 1 (low)	122.8	17.0
Sample 2 (medium)	222.4	21.3
Sample 3 (high)	335.8	11.1

Three serum samples were assayed 3 times during different assays. All samples were measured in duplicates. CV = coefficient of variation (CV = SD/mean)

The accuracy of the assay was evaluated by recovery and dilution tests. When known concentrations of IGF-1 are added to sera of known IGF-1 concentrations, a satisfactory correlation between expected (endogenous + added hormone) and assayed IGF-1 was obtained. Recovery was calculated to be between 71.4 and 95.92 % (Table 3). Observed to expected ratios of 95.5% to 104.2% exhibit excellent parallelism for dilution at 1:2, whereas the ratios of 125.0 ±19.5 % at dilution 1:4 are out of the acceptable limits (Table 4).

Table 3. Recovery of equine IGF-1 ELISA for measuring bovine IGF-1

Sample No.	Concentration added (ng/mL)	Sample		
		Expected concentration (ng/mL)	Assayed concentration (ng/mL)	% recovery
1	0	NA	85	NA
	66.5	151.5	120	79.2
	135	220	170	77.3
	285	370	315	85.1
2	0	NA	110	NA
	66.5	176.5	160	90.6
	135	245	235	95.9
	285	395	370	93.7
3	0	NA	65	NA
	66.5	131.5	100	76.0
	135	200	165	82.5
	285	350	250	71.4

NA: not applicable; Recovery is calculated as ratio of assayed to expected concentration given in %.

Table 4. Dilution test of equine IGF-1 *ELISA* for measuring bovine IGF-1

Sample No.	Dilution	Sample		
		Expected concentration (ng/mL)	Assayed concentration (ng/mL)	% recovery
1	/	NA	393.1	NA
	1:2	196.6	205.0	104.2
	1:4	98.3	145.0	147.5
2	/	NA	377.3	NA
	1:2	188.7	190.0	100.7
	1:4	94.3	100.1	106.0
3	/	NA	419.1	NA
	1:2	209.5	200.0	95.5
	1:4	104.8	130.0	124.09

NA: not applicable; Recovery is calculated as ratio of assayed to expected concentration given in %.

Agreement between IGF-1 concentrations at different sampling time

Using Deming regression, we described the relationship between IGF-1 measurements at different time sampling by determining the straight line that most closely approximates the data points on a scatter diagram including two time sampling (morning – noon, noon – evening, morning – evening) (Fig. 1-3).

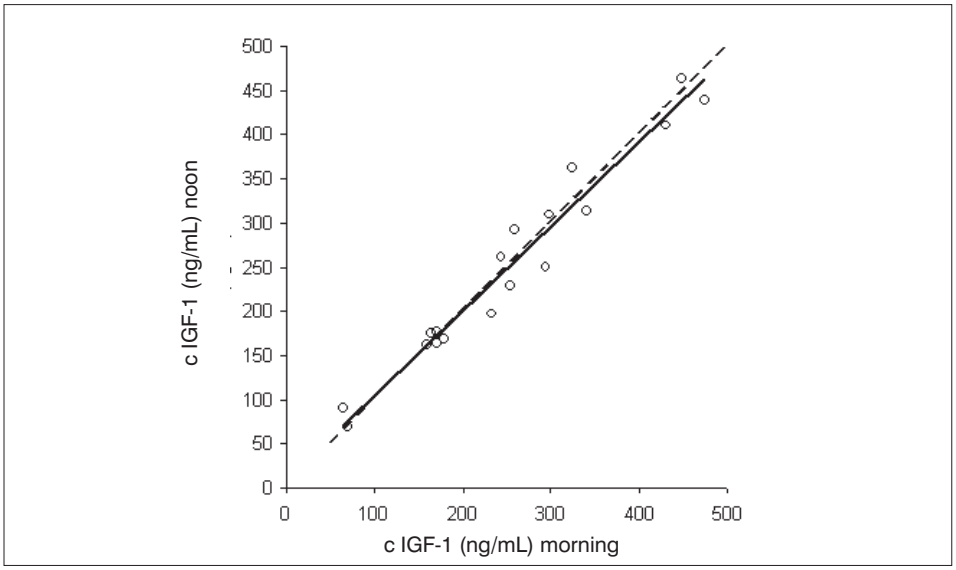

Figure 1. Deming regression noon – morning.
Deming regression line: c (noon) = 0.9634 c (morning) + 6.2897
Dash line represents the identity line (slope=1)

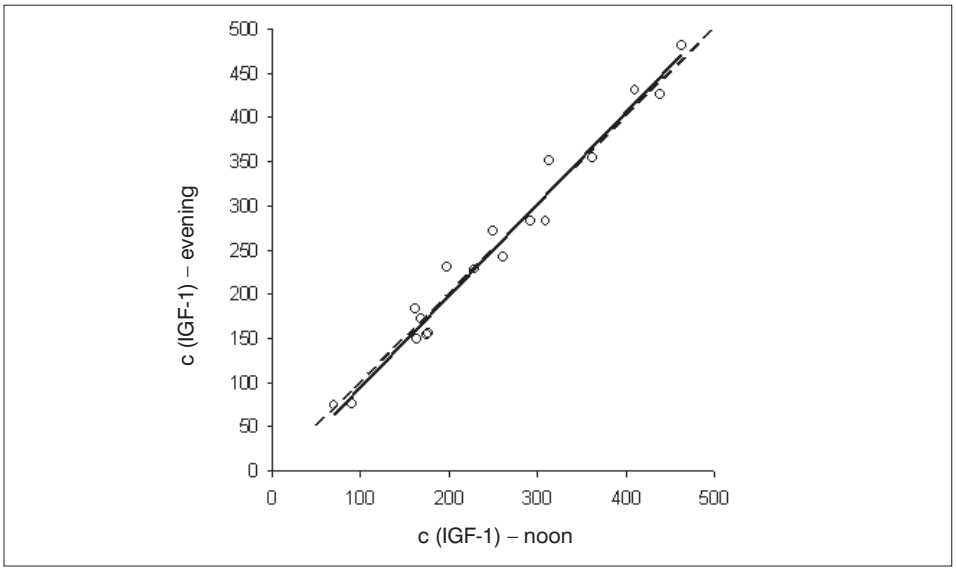

Figure 2. Deming regression noon – evening.
Deming regression line: c (evening) = 1.0427 c (noon) + 9.3818
Dash line represents the identity line (slope=1)

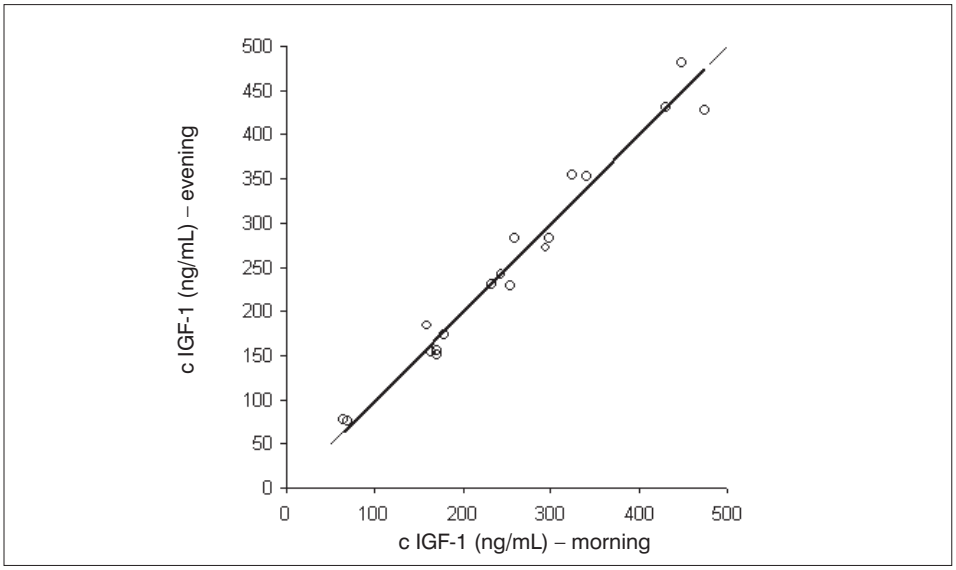

Figure 3. Deming regression morning – evening.
Deming regression line: c (evening) = 1.0049 c (morning) – 2.8914
Deming regression line. Dash line represents the identity line (slope=1)

The estimated intercept for the regression line does not differ much from zero (from 6.2897 ng/mL to -9.3818 ng/mL) for all three comparisons. The estimated regression equation indicates that the points are close to the line of equality, i.e. slope = 1 and SE of the slope (0.0462 to 0.0505) indicates that there is almost no pivoting of the line about a central point through the means of x and y (Table 5).

Table 5. Results of Deming method of comparison for IGF-1 values measured at different times

	Coefficient	SE	95% Confidence interval
Comparison between sampling times: morning – noon Deming regression line: c (noon) = 0.9634 c (morning) + 6.2897			
Intercept	6.2897	14.1011	-23.6033 to 36.1827
Slope	0.9634	0.0505	0.8564 to 1.0705
Comparison between sampling times: morning – evening Deming regression line: c (evening) = 1.0427 c (noon) + 9.3818			
Intercept	- 9.3818	12.3330	- 35.5265 to 16.7629
Slope	1.0427	0.0449	0.9476 to 1.1379
Comparison between sampling times: noon – evening Deming regression line: c (evening) = 1.0049 c (morning) – 2.8914			
Intercept	- 2.8914	12.9041	- 30.2468 to 24.4640
Slope	1.0049	0.0462	0.9069 to 1.1028

The mean percentage bias was close to zero for all three comparisons. The percentage biases for individual pairs of measurements lies in the interval –15 to 15%, which is in the range of satisfactory inter-assay CV. From the absolute bias plot (Fig. 4-6) it is also evident that the scatter is random, indicating that the sizes of differences between IGF-1 concentrations obtained at different sampling times are not related to the magnitude of the concentration. Thus, no proportional bias has been detected. No detectable average absolute bias was observed between measurements at different sampling times. IGF-1 concentrations measured in the morning, at noon and in the evening agree; more than 90% (94.5% and 100%) of the differences lie within the limits of agreement (Fig. 4-6).

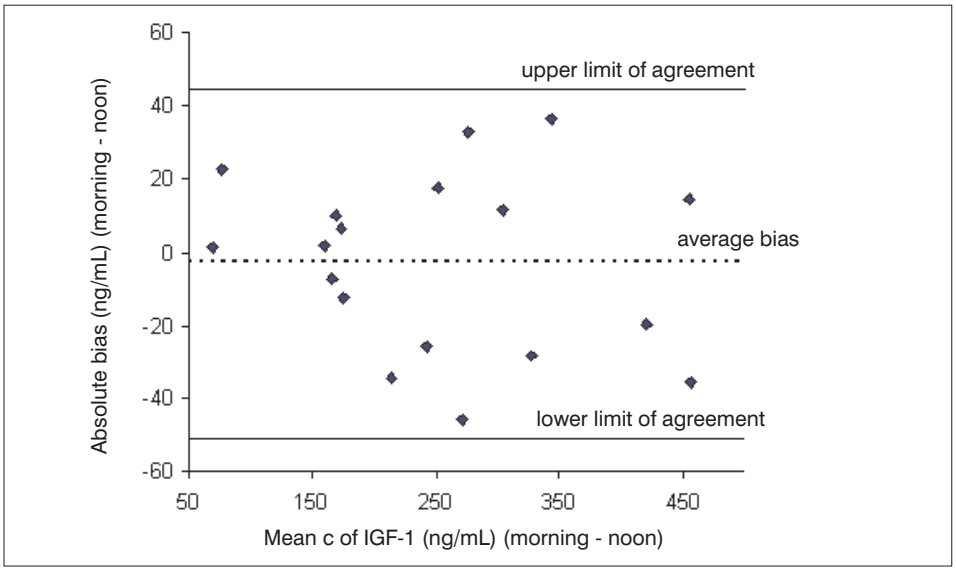

Figure 4. Absolute bias plot of IGF-1 measured at noon versus IGF-1 measured at morning showing average bias and limits of agreement

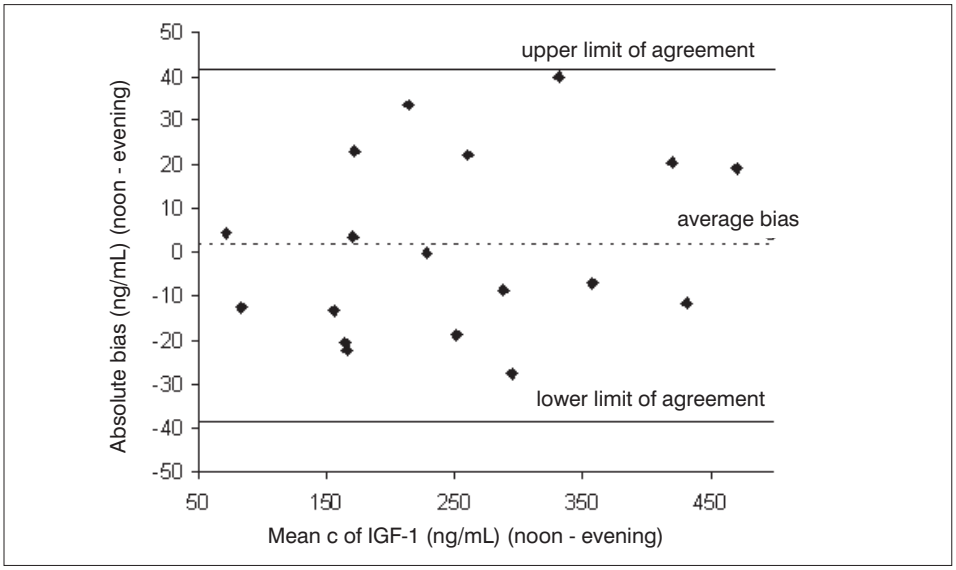

Figure 5. Absolute bias plot of IGF-1 measured at noon versus IGF-1 measured at evening showing average bias and limits of agreement

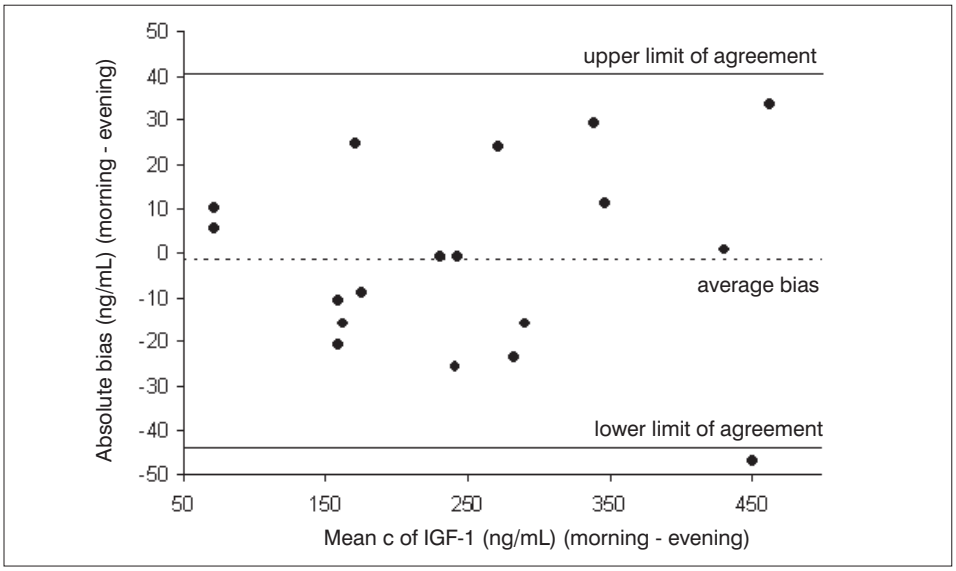

Figure 6. Absolute bias plot of IGF-1 measured at morning versus IGF-1 measured at evening showing average bias and limits of agreement

DISCUSSION

Cows in negative energy balance have reduced fertility, mediated by metabolic signals influencing the reproductive system. High BCS loss corresponds to increased NEFA and BHB concentration and decline in the level of IGF-1 (Pushpakumara *et al*., 2003). Therefore the measurement of IGF-1 contributes to the diagnosis of negative energy balance. In our study we analysed for possible changes in IGF-1 level during the day.

The ELISA for IGF-1 measurement uses antibodies with high affinity and specificity for two different epitopes on IGF-1. The nucleotide sequence of equine IGF-1 is 90% homologous to that of cows and 88% homologous to that of humans and sheep, but the amino acid sequence of bovine IGF-1 is identical to that of humans, horses, dogs and pigs (Nixon *et al*., 1999). Therefore, in the equine ELISA (DRG Diagnostics) human IGF-1 is used as a standard for the calibration curve. However, performance characteristics have to be analysed before measuring bovine IGF-1 with an ELISA that was constructed for measuring equine IGF-1. In our study the dilution test confirmed the immunological identity between bovine IGF-1 and human IGF-1 used to calibrate the standard curve of equine ELISA. The performance characteristics in the user's manual for IGF-1 equine ELISA confirm that there is immunological identity between human and equine IGF-1. A similar immunological identity was also confirmed when a human ELISA kit was used for determining porcine IGF-1 according to previous validation (Corson *et al*., 2002). The commercially available immunoradiometric assay for

determination of human IGF-1 was also used to measure plasma IGF-1 in dogs (Jensen and Hóier, 1995). On the basis of the recovery test we confirmed a satisfactory correlation between expected and assayed concentrations when known concentrations of IGF-1 were added to serum samples. Recovery was calculated to be between 71.4 and 95.92 %. Suboptimal recovery of 71.4% was found for the highest spiked concentration added to the sample of the lowest original IGF-1 concentration. The results of performance characteristics of ELISA are in agreement with the fact that bovine IGF-1 could not be distinguished from human IGF-1 on the basis of identical amino acid sequence, immunological and receptor cross-reactivity (Honegger and Humbel, 1986). Dilution test indicates that there is immunological identity between the IGF-1 present in serum and the IGF-1 used to calibrate the standard curve; observed to expected ratios of 95.5% to 104.2% exhibit excellent parallelism for dilution test at 1:2. Although the ratios of 125.0 ±19.5 % at dilution 1:4 are out of acceptable limits, it seems to be of only minor importance. Lower level of dilution parallelism at dilution of 1:4 in comparison of 1:2 is also observed by other authors who validated ELISA tests (Steiner *et al*., 2000).

It is necessary to establish that a method is repeatable before comparing two measurements for reproducibility (Petrie and Watson, 1999). In our study, CVs varied between 7.9 and 8.2 %, confirming the acceptable repeatability of the method (Porstman and Kiessig, 1992). CV was not related to the concentration value of IGF-1. According to inter-assay CV for medium sample, which exceeds the 20%, the assay shows suboptimal reproducibility.

In our study we tested the possible variation of the IGF-1 level with the time of sampling. Pairs of IGF-1 measurements were compared on samples obtained in the morning (at 8 a.am), at noon 12 a.m.) and in the evening (20 p.m). We performed the method in agreement with Deming regression because it is appropriate for describing the relationship between two variables, both measured with error (Jones and Payne, 1997; Mrkun *et al.*, 2007).

We were interested in assessing the similarity between IGF-1 values obtained at different times, so we compared pairs of measurements. Scatter plots and absolute and relative bias plots give the best overview of data comparisons (Twormey, 2004). Using scatter diagrams with fitted regression lines, we established that the paired measurements, obtained from samples of different sampling time, were close to the line of equality. We calculated the differences between IGF-1 values obtained at different times on the same day for each cow. The mean of these differences ($\bar{d}$) is an estimate of the average bias of one method (different sampling time) relative to that of the other. If this value is zero, then the two measurements agree on average. However, this does not imply that they agree for each individual measurement. In order to assess how well the measurements agree on an individual basis, we determined the limits of agreement (Honegger and Humbel, 1986). Of the absolute differences 90% are within the limits of agreement, confirming that the level of agreement between the methods was satisfactory. Moreover, the percentage biases for individual pairs of measurements are in the interval –15 to 15 %, which is in the range of satisfactory

inter-assay CV. Therefore measurements obtained at different sampling time are equally appropriate for IGF-1 measurement.

The use of equine-ELISA for IGF-1 determination in bovine samples greatly enhanced the simplicity of bovine IGF-1 measurement. The use of such test should be of benefit to practitioners in determining the negative energy balance in cows.

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Mrkun J *et al*.: Comparision of IGF-1 (insulin like growth factor-1) levels in bovine serum sampled three times during the day using validated equine IGF-1 *ELISA*

POREĐENJE KONCENTRACIJA IGF-1 (INSULINU SLIČAN FAKTOR RASTA 1) U UZORCIMA GOVEĐEG SERUMA PRIKUPLJENIM TRI PUTA TOKOM DANA UZ UPOTREBU KONJSKOG ELISA SETA

MRKUN J, KOSEC M i ZRIMŠEK PETRA

SADRŽAJ

Određivanje koncentracije IGF-1 može da doprinese postavljanju dijagnoze negativnog energetskog bilansa kod krava. Cilj ovih istraživanja je bio da se utvrde moguće varijacije koncentracije IGF-1 u uzorcima prikupljenim u različito doba dana. Za merenje koncentracije IGF-1 u serumu goveda korišćen je konjski ELISA set. Korišćenjem Deming regresione linije utvrđeno je da su parna merenja u uzorcima seruma bliska liniji jednakosti. Koncentracija IGF-1 nije bila zavisna od vremena uzorkovanja.