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Diagnostic Evaluation of Immunoassay Kits for Early Pregnancy Detection in Cattle

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ABSTRACT

Diagnosis of pregnancy at an early stage is important for profitable dairy enterprise. The objective of present study was to evaluate diagnostic performance of immunoassay kits used for early pregnancy detection in cattle. Two ELISA based kits were used for detection of pregnancy-associated glycoproteins-1 (PAG-1) and pregnancy-specific protein B (PSP-B). Another RIA based kit was used for the estimation of serum progesterone (P_4). Ultrasound examination was done in all cows under controlled experimental conditions and used as a reference method. Based on availability of assay points, two pregnant groups of dairy cattle i.e. 30-35D (n=16) and 45-50D (n=10) were included for PAG-1/PSP-B estimation. However, all four Pregnant groups viz., 7-10D (n=11), 17-20D (n=8), 30-35D (n=16) and 45-50D (n=10) and three non-pregnant groups viz., Heifer (n=6), Cyclic Non-AI (n=13) and Post-partum (PP; n=13) were included for progesterone estimation. Ultrasound examination was done at 30-35D and 45-50D of pregnancy to screen the experimental animals and early pregnancy samples were confirmed retrospectively. Sensitivity of all three immunoassay kits for PAG-1, PSP-B and P_4 was reported 92.31%, 96.15% and 84.44%, respectively. Specificity, positive predictive value (PPV), negative predictive value (NPV) and Accuracy for P4 kit were observed 90.63%, 0.93, 0.81 and 87.01%, respectively. However, lack of values in non-pregnant animals in study for the estimation of PAG-1/PSP-B limits full reflection of diagnostic performance of respective kits. Henceforth, it is recommended to include large number of pregnant as well as non-pregnant animals to conclude comprehensively on the diagnostic performance of these assays.

HIGHLIGHTS

- ELISA kits based on PAG-1/PSP-B protein showed comparable sensitivity to screen the pregnant animals.
- RIA kit based on progesterone estimation showed better diagnostic values viz., sensitivity, specificity, PPV, NPV and accuracy for pregnancy detection in dairy cattle.

Keywords: ELISA/RIA, PAG-1/PSP-B, Pregnant, Non-pregnant, Progesterone, Ultrasound

Diagnosis of pregnancy at an early stage is important for efficient reproductive management in dairy cattle. The commonly available clinical methods for pregnancy diagnosis in cattle are per-rectal palpation of the genital organs and ultrasonography (Zemjanis, 1970; Thompson *et al*., 1994; Ginther, 1998; Fricke, 2002). Over the years, emphasis has been given to develop laboratory based methods of early pregnancy diagnosis in bovine, which evolved many assays based on the estimation of hormones and pregnancy specific protein/antigens in the blood. The possibility of using progesterone (P_4) for pregnancy diagnosis in farm animals was proposed in 1971 by

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Laing and Heap (Rioux and Rajotte, 2004) and further its quantification could help in pregnancy diagnosis as early as 21-24 days after breeding (Fricke *et al*., 2016). This has led to development of several commercial kits based on Radio Immunoassay (RIA) or Enzyme linked Immunosorbent Assay (ELISA) methods (Sah *et al*., 2017). The progesterone is most biologically active progestogen in cattle and primarily produced by the corpus luteum (CL) during the estrous cycle and by the CL as well as placenta during pregnancy. Further, blood based pregnancy-specific assay was developed by estimating pregnancy-specific protein B (PSP-B) or Pregnancy-associated glycoproteins (PAGs, Sasser *et al*., 1986; Humblot *et al*., 1988; Zoli *et al*., 1992; Vasquez *et al*., 1995) in dairy cattle around 21-30 days after artificial insemination (AI).

Pregnancy-associated glycoproteins belong to a large family of aspartic peptidases, of which pregnancy-specific protein B was the first member to be discovered (Butler *et al*., 1982). These glycoproteins are produced by specialized trophoblastic cells in the ruminant placenta (Eckblad *et al*., 1985; Resee *et al.,* 2016), which migrate from the trophectoderm to fuse with maternal uterine epithelial cells, and release their granular content containing PSP-B/PAG into the maternal circulation (Wooding, 1992). Blood concentrations of PAGs were initially measured by RIA (Humblot *et al*., 1988; Zoli *et al*., 1992; Szenci *et al*., 1998; Ayad *et al.,* 2007). More recently, ELISA based assays for the estimation of PSP-B (Gabor *et al*., 2007; Romano and Larson, 2010) and PAG (Friedrich and Holtz, 2010; Commun *et al.,* 2016) became commercially available under various trade names viz., BioPRYN, IDEXX, QuickVET etc., which provide a qualitative pregnancy classification based on measurement of PSP-B and PAG in the serum of pregnant ruminants. The information on the use of these immunoassays under field conditions is very meager. Therefore, the objective of the present study was to evaluate diagnostic ability of some commonly available ELISA kits viz., PSP-B (Biospes), PAG-1 (Biospes) and progesterone (Immunotecch) for pregnancy determination in dairy cattle using trans-rectal ultrasound as a reference method.

MATERIALS AND METHODS

The present study was a part of DBT funded project "Development of early pregnancy diagnostic assay through discovery of biomarkers in cattle and Buffalo." Approval from IAEC has been granted prior to study on experimental animals.

Experimental design

The study was conducted on the cattle (n=77) of different breeds (Sahiwal, Tharparkar and Crossbreds) which included pregnant (n=45) and non-pregnant (n=32) cattle. Pregnant animals were further divided into four groups i.e. 7-10D (n=11), 17-20D (n=8), 30-35D (n=16) and 45-50D (n=10). Non-pregnant animals included i.e. Heifer (n=6), Cyclic Non-AI (n=13) and Post-partum (PP; n=13).

Pregnancy was determined by tans-rectal ultrasound on two occasions i.e. about 30D and 45D post AI using 7.5 MHz linear probe through trans-rectal real time B mode USG (EXAGO ECM^{TN}, France), while it was retrospectively confirmed in 7-10D and 17-20D groups in pregnant cattle.

Blood sampling

Blood samples were collected aseptically from the both pregnant and non-pregnant cattle of C&B Farm, ICAR-IVRI, Izatnagar and other dairy farms including field samples. Approximately, 5-7 ml of blood from each selected cattle was collected by jugular venipuncture in vacutainers containing clot activator. The vacutainers containing blood sample were kept at 37ºC for coagulation of blood. The sera were separated by centrifugation at 800 × G for 15 minutes. The serum samples were labeled properly and stored at -20ºC till use.

Estimation of serum PAG-1 and PSP-B

Serum samples were assayed for PAG-1 (Biospes; Lot no.: E06d/2020M) and PSP-B (Biospes; Lot no.: E06e/2020M) using two different ELISA kits (Chongquing Biospes Co. Ltd, China) according to manufacturer's instructions. PAG-1 and PSP-B estimations were done only in two groups of pregnant animals i.e. 30-35D (n=16) and 45-50D (n=10), based on the availability of the assay points. Reading in terms of optical density (OD) for all the test samples and provided standards were taken at 450 nm in multimode plate reader (Biorad, Imark, USA). The sensitivities of the kits were 1.0 and 0.3 ng/ml for PAG-1 and PSP-B protein, respectively. Relative OD was

calculated for each well through provided formula in instruction manual and subsequently standard curve was generated for both the kits (Fig.1). Concentration of PAG-1 and PSP-B were determined using equation of standard curve and eventually pregnancy status was determined for each sample.

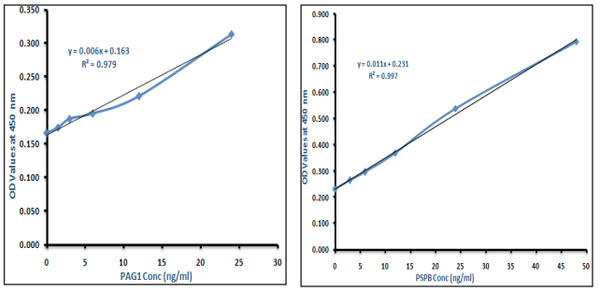

Fig. 1: Standard curve for ELISA kits of PAG-1 and PSP-B based on optical density of sera samples at 450 nm

Estimation of progesterone concentration

Progesterone concentrations in the sera were estimated by RIA using standard diagnostic kits (Immunotech, France). Progesterone estimation was done adopting the procedure described by the manufacturer. The radioactive count of all tubes measured in the gamma counter (Packard Cobra II Gamma Counter) calibrated for I^{125} for one minute and programmed to evaluate the concentration of progesterone in the serum samples from the standard curve drawn using values of known standards. The values were obtained in ng/ml. The analytical sensitivity of the kit was 0.05 ng/ml and the intra-assay and inter-assay coefficients of variation were d" 6.5% and d" 7.2%, respectively.

Ultrasound examination

Ultrasonographic (USG) examination was done to screen pregnant and non-pregnant animals. Embryonic vesicle, conceptus, embryonic membranes and embryonic fluid were observed to confirm the pregnancy, while empty uterine horns with rosette shape structure in cross-section or empty tube-like structure in longitudinal section were appreciated to confirm the non-pregnancy in experimental cattle (Fig. 2). Animals were considered doubtful, if failed to appreciate any of mentioned ultrasound features and the particular animal was not included in the study. Further, early pregnant groups (7-10D and 17-20D) were confirmed retrospectively through USG and animals found non-pregnant were dropped from the study groups. The accuracy of pregnancy diagnosis through ultrasound examination may be considered 100% in controlled experimental conditions (Szenci *et al*., 1995) and therefore used as a reference method in the present study with 100% sensitivity and specificity.

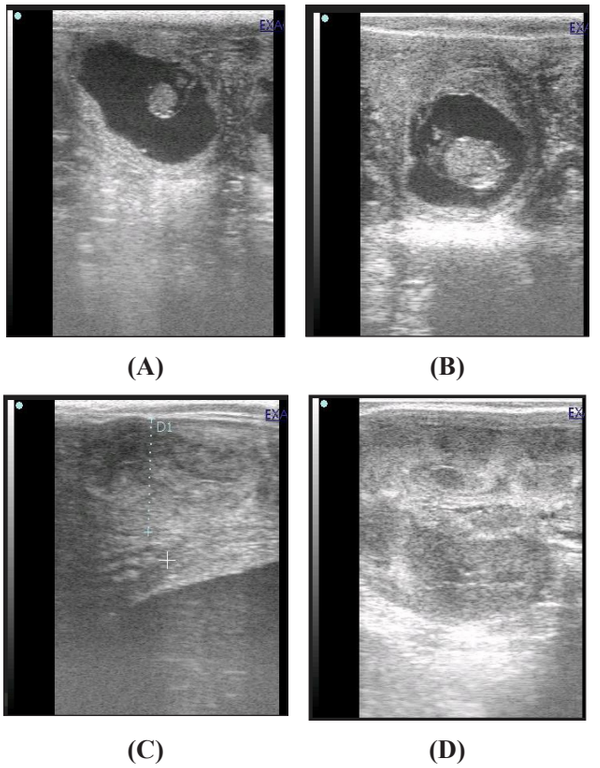

Fig. 2: Ultrasound examination of pregnant (A,B) and non-pregnant (C,D) animals. (A,B) Conceptus with embryonic vesicle and fluid is visible at 30 and 45 day post-breeding, respectively, (C) Cross-section of both empty uterine horns is visible in Non-AI cattle in circular rosette shape fashion, (D) longitudinal section of both empty uterine horns is visible in non-pregnant cow

Comparative assessment of diagnostic performance

Assessment of comparative diagnostic evaluation of three immunoassay kits was done for early pregnancy diagnosis using USG as reference method having ideal sensitivity and specificity of 100%. Cut-off value was decided for

three assays to categorize the result into pregnant and non-pregnant animals. ROC curve (AUC: 0.929) was generated for data of serum progesterone and 4.82 ng/ml concentration was decided as cut-off value through obtaining point farthest to x-axis and nearest to y-axis. ROC curve for other two biomarkers PAG-1 and PSP-B could not be generated as the values for non-pregnant groups were not available. Therefore, cut-off value to discriminate pregnant and non-pregnant was decided using available literature (Sasser *et al*., 1986; Piechotta *et al*., 2011). Cut-off value was 1.5 and 1.0 ng/ml for PAG-1 and PSP-B, respectively. Subsequently, true positive, false negative, true negative and false positive samples were screened based on cut off value for these immunoassay kits, eventually diagnostic parameters such as sensitivity (Sn), specificity (Sp), positive predictive value (PPV), negative predictive value (NPV), accuracy and Youden's Index (J) were calculated to interpret comparative diagnostic performance using USG as reference method.

STATISTICAL ANALYSIS

The data generated in the present study for assessment of pregnancy status was analyzed by one way ANOVA using GraphPad Prism version 8.0.2 and receiver operating characteristic (ROC) curve using SPPS software package for windows (SPSS-20). A probability value of $P<0.05$ indicated that the difference was statistically significant while $P>0.05$ indicated that difference was not significant.

RESULTS AND DISCUSSION

Serum PAG-1/PSP-B

The mean±SEM concentrations of PAG-1 and PSP-B proteins in serum samples of cattle in the two pregnant groups (30-35D and 45-50D) are presented in Table 1.

Standard curve for the PAG-1 kit was obtained in straight line ($R^2 = 0.979$) with $y = 0.006x + 0.163$ equation, where x-axis represents the concentration of PAG-1 and y-axis represents respective OD_{450}. The mean concentration of serum PAG-1 in two pregnant groups viz., 30-35D and 45-50D were 17.28±2.70 and 16.58±3.19 ng/ml, respectively. Lowest PAG-1 concentration 1.0 ng/ml was reported in group 45-50D, while 30-35D group showed highest concentration (38.67 ng/ml). Standard curve for the PSP-B kit was obtained in straight line ($R^2 = 0.997$) with $y = 0.011x + 0.231$ equation, where x-axis represents the concentration of PSP-B and y-axis represents respective OD_{450}. The mean concentration of serum PSP-B in two pregnant groups viz., 30-35D and 45-50D were 15.23±1.94 and 19.92±2.21 ng/ml, respectively. Both lowest (0.09 ng/ml) and highest (30.09 ng/ml) PSP-B concentration was reported in group 30-35D. Linear trend for mean concentration was non-significant ($P>0.05$) and mean values showed non-significant difference ($P>0.05$) across the groups.

Blood concentrations of PAGs/PSP-B were initially measured by radioimmunoassay (Humblot *et al*., 1988; Zoli *et al*., 1992; Szenci *et al*., 1998), and the results have been compared with those of ultrasonography (Szenci *et al*., 1995). However, ELISA based assays for PAG/PSP-B became commercially available later (Friedrich *et al*., 2010). Twenty-two PAG genes have been reported till date and it was observed that all the PAGs are not expressed simultaneously throughout the pregnancy rather some are expressed early while others at the different point of time with the progression of pregnancy (Ushizawa *et al*., 2004). The wide variation in PAG levels limits PAG testing as a reliable indicator of pregnancy until about 26 to 30

Table 1: Descriptive statistics for serum PAG-1 and PSP-B concentration in serum samples of cattle

Variables	PAG-1		PSP-B	
	30-35D	45-50D	30-35D	45-50D
Sample Size (n)	16	10	16	10
Min Conc. (ng/ml)	1.17	1.00	0.09	1.86
Max Conc. (ng/ml)	38.67	30.50	30.09	28.09
Mean±SEM	17.28±2.70	16.58±3.19	15.23±1.94	19.92±2.21
Median	16.25	18.50	15.86	21.00
SD	10.80	10.10	7.74	6.99

day post-breeding (Zoli *et al*., 1992; Humblot, 2001). The concentration of PAGs measured through RIA was recorded 1.0-6.0 ng/ml around 28 to 30 days of gestation (Zoli *et al*., 1992; Karen *et al*., 2014; Gajewski *et al*., 2008), which increased steadily up to day 36 (8.0-16.0 ng/ml). Our findings are in accordance with these reports. Green *et al*. (2005) used homologous ELISA with monoclonal PAG antibodies and reported average concentration of PAG about 8.75 and 12.3 ng/ml at 4th and 5th week post-breeding, respectively. However, Gajewski *et al*. (2008) measured PAG concentration in cattle at different stages of pregnancy using RIA and reported 3.47 ng/ml on 41 day, which is comparatively lower than the value recorded in the present study on 45-50 day (16.58 ng/ml) using ELISA kit for PAG-1. The differences in the values as compared to reported ones might be attributed to the period of blood collection, different breeds, method of estimation and type of kits. In addition, individual variation in placental production of different PAG molecules could also be a reason for these variations (Piechotta *et al*., 2011).

We have recorded very high concentration of PSP-B (15-17 ng/ml) around day 30-50 of gestation in contrast to 3 ng/ml by earlier report (Sasser *et al*., 1986). The first blood based PSP-B assay using RIA in ruminants was described by Sasser *et al*. (1986) and Humblot *et al*. (1988). Sasser *et al*. (1986) developed double antibody radioimmunoassay for the serological detection of PSP-B and observed 1 ng/ml around day 30 sera which increased up to 9 ng/ml around 90 days of gestation. Present study reported comparative higher value at around 30 to 50 days of gestation, however, our values are subjected to ELISA. Variable affinity of respective antibodies against targeted bio-molecules may be attributed to the recorded variations (Piechotta *et al*., 2011). In addition, effect of period of blood collection, breed differences, method of estimation and type of kits could not be ruled out.

Serum progesterone

The mean±SEM concentrations of serum progesterone in different pregnant as well as non-pregnant groups of cattle are presented in Table 2. The mean concentration of serum P_4 in different pregnant groups viz., 7-10D, 17-20D, 30-35D and 45-50D were 4.18±0.41, 7.75±0.79, 9.27±0.98 and 10.78±1.28ng/ml, respectively, while values for non-pregnant groups viz., Heifer, Non-AI and PP were 0.13±0.03, 1.76±0.46, 1.31±0.55 ng/ml, respectively. Lowest P_4 concentration was observed in heifer group (0.02 ng/ml) while 30-35D group showed highest concentration (16.37 ng/ml). However, all the non-pregnant groups sera have low level of progesterone. Linear trend for mean concentration was significant ($P<0.05$) and mean values showed significant difference ($P<0.05$) across the groups.

High levels of progesterone in serum or milk between days 18 to 24 post-insemination forms the basis of establishment of pregnancy in cattle (Shemesh *et al*., 1973; Sasser and Ruder, 1987). Mean plasma progesterone concentration in pregnant and non-pregnant cows were recorded 3.0-5.8 and 0.22-2.0 ng/ml, respectively around 19-22 days post-breeding or post-estrus (Shemesh *et al*., 1973) and we also observed similar trend in both pregnant as well as non-pregnant groups. Progesterone concentration was observed 2.0-6.2 ng/ml in non-pregnant cows and pregnant cows showed about 10.0 ng/ml concentration around day 24 post-conception (Humblot *et al*., 1988). We observed comparatively lower progesterone level in present study for both the groups. Serum progesterone level in early gestation around 4th week post-conception ranged between 4.7-5.5 ng/ml in dairy cow (Lobago *et al*., 2009). Further,

Table 2: Descriptive statistics for serum progesterone concentrations in serum samples of cattle

Variables	7-10D	17-20D	30-35D	45-50D	Heifer	Non-AI	PP
Sample Size (n)	11	8	16	10	6	13	13
Min Conc. (ng/ml)	2.24	5.57	1.12	1.19	0.02	0.10	0.09
Max Conc. (ng/ml)	6.21	11.71	16.37	14.76	0.26	4.67	6.97
Mean±SEM	4.18± 0.41	7.75± 0.79	9.27± 0.98	10.78± 1.28	0.13± 0.03	1.76± 0.46	1.31± 0.55
Median	4.43	7.34	9.60	12.08	0.11	1.53	0.35
SD	1.36	2.23	3.92	4.05	0.08	1.66	2.00

mean progesterone concentration was recorded 7.2±2.2, 6.6±2.8, 6.6±2.4, 7.8±3.0, and 6.0±3.4 ng/ml at 26-29, 30-33, 34-37, 38-41 and 42-58 days of gestation, respectively in dairy cows (Piechotta *et al*., 2011). However, we have recorded relatively higher level of serum progesterone during the same window. Variations among the findings might be due to the difference in the method of estimation, type of kits, days of blood collection and breeds.

Table 3: Comparative diagnostic performance of all the methods

Diagnostic parameters	PAG-1	PSP-B	P_4	USG
Sn (%)	92.31	96.15	84.44	100.00
Sp (%)	—	—	90.63	100.00
PPV	—	—	0.93	1.00
NPV	—	—	0.81	1.00
Accuracy (%)	—	—	87.01	100.00
Youden's Index (J)	—	—	75.07	100.00

In another study, serum progesterone level during the first three weeks post-partum period was reported about 0.28-0.75 ng/ml (Khatti *et al*., 2017) and our findings showed close agreement to this for PP group of animals. Most of the reports for estimation of progesterone to monitor the pregnancy are milk based (Zaied *et al*., 1979; Nebel *et al*., 1987; Kamboj and Prakash, 1993; Simersky *et al*., 2007; Samsonova *et al*., 2015). In addition, we did not find much reports showing level of progesterone around day 7 post-breeding in serum samples (Noakes *et al*., 2009). However, milk progesterone around day 9-10 post-conception reported to be around 11.1 ng/ml in dairy cows (Zaied *et al*., 1979), which is comparatively higher to the values observed in present study as level of progesterone in milk is known to be higher because of fat content (Noakes *et al*., 2009).

Diagnostic evaluation

In this study, all three immunoassay kits viz., PAG-1, PSP-B and P_4 showed high rate of sensitivity i.e. 92.31%, 96.15% and 84.44%, respectively. We could not calculate specificity of PAG-1 and PSP-B kits as the values for non-pregnant samples were not available. However, progesterone kit showed specificity of 90.63%. Positive predictive value, negative predictive values, accuracy and Youden's index for P_4 kit were 0.93, 0.81, 87.01 and 75.07, respectively. These diagnostic parameters could not be calculated for other two immunoassay kits.

The sensitivity of tests reported in this study for PAG-1 (92.31%), PSP-B (96.15%) and progesterone (84.44%) is comparable to previous reports (Romano and Larson, 2010; Piechotta *et al*., 2011; Sah *et al*., 2017; Northop *et al*., 2019; Masello *et al*., 2020) which also showed about 90 to 97% sensitivity around fourth week of pregnancy. Specificity of progesterone estimation was observed highest (90.63%) as compared to the earlier studies i.e. 57% by Sah *et al*. (2017) and 83% by Masello *et al*. (2020). Sah *et al*. (2017) reported 80% accuracy for serum progesterone estimation through ELISA kit, while present study reported about 87% using RIA kit in dairy cow. Muhammad *et al*. (2000) reported 71.4% and 100% accuracy for positive and negative samples, respectively for estimation of blood plasma progesterone in Holstein cows. Further, Masello *et al*. (2020) reported 85% and 96% PPV and NPV, respectively. However, we have reported 93% and 81% PPV and NPV, respectively. All these differences in recorded values might be attributed to the different method of detection, variation in sample size, days of blood collection, type of kits used and breed of animals.

CONCLUSION

All three immunoassays kits based on estimation of biomarkers of pregnancy showed comparable sensitivity to screen the pregnant cow. Diagnostic performance (PPV, NPV and Accuracy) of progesterone kit was relatively better for identification of pregnant and non-pregnant cows. However, lack of values for PSP-B/PAG-1 in non-pregnant animals limits full reflection of diagnostic performance of respective kits. Henceforth, it is recommended to include large number of pregnant as well as non-pregnant animals to conclude comprehensively on the diagnostic performance of these assays.

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