

Early pregnancy diagnosis using a commercial ELISA test based on pregnancy-associated glycoproteins in Holstein-Friesian heifers and lactating cows

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Abstract: In this study, we aimed to investigate the efficacy of a commercial ELISA test kit for detecting pregnancy-associated glycoproteins (PAGs) in peripheral blood for early pregnancy diagnosis and compare plasma PAG levels during early pregnancy in both Holstein-Friesian heifers and lactating cows. A total of 231 plasma samples were collected from heifers and lactating cows on days 25, 28, and 32 after insemination. Pregnancies were confirmed 30 days after the collection of plasma samples. Plasma PAG levels were measured using a commercial ELISA test kit for diagnosing bovine pregnancy. The effects of examination date and animal status (heifers vs. lactating cows) on PAG levels in heifers and lactating cows were analyzed using two-way ANOVA. Plasma PAG levels were significantly higher in heifers than in lactating cows; levels also increased significantly with the date of examination, i.e. days 25, 28, and 32 for heifers ($P < 0.001$), but not for lactating cows ($P > 0.05$). Although the sensitivity, specificity, and accuracy of pregnancy diagnosis using the ELISA test were acceptable in both groups, the performance of the test was superior in pregnant heifers compared to lactating cows.

Key words: Pregnancy-associated glycoproteins, early pregnancy diagnosis, lactating cows, heifers, ELISA

1. Introduction

In dairy herds, economic profitability is directly related to the reproductive performance of cows and milk yield. Low reproductive performance results in decreased peak milk yields owing to extended calving intervals and the delayed onset of lactation periods (1). Intensive selection for milk yield and improvement in husbandry conditions have helped to achieve a dramatic increase in milk yield. However, fertility has not increased at the same rate (2,3). In Turkey, extending calving intervals for 1 day corresponds to 11 L of milk per dairy cow (4). Similarly, in England it has been reported that each day without pregnancy, beginning from the 60th day after calving, costs 3 pounds sterling (5).

One strategy for improving reproductive performance aims to shorten the calving–conception interval by rapidly diagnosing pregnant cows and inseminating nonpregnant cows (6,7).

As an alternative to transrectal ultrasonography and rectal palpation for diagnosing pregnancy, laboratory

methods based on detecting specific antigens, such as pregnancy-associated glycoproteins (PAGs), are increasingly used for large cattle herds (6). Certain PAGs produced by trophoblastic giant cells on the outer sheet of the placenta pass into the maternal peripheral blood after implantation (8). Antibodies developed against PAGs can be detected by techniques such as radioimmunoassay (RIA) or enzyme-linked immunosorbent assay (ELISA) as early as 21 days after insemination, although the most reliable results are obtained after day 27 (9,10).

The ELISA test can be used under farm conditions and avoids the health risks for users associated with the radioactive substances used in RIA-based tests (11). To date there are several reports (10–12) that confirm the utility of PAG levels for pregnancy diagnosis in cows. In this present study, we aimed to investigate the suitability of a commercial ELISA test kit to detect PAGs in the peripheral blood for early pregnancy diagnosis, as well as to compare plasma PAG levels during early pregnancy in both Holstein-Friesian heifers and lactating cows.

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2. Materials and methods

2.1. Study animals and sample collection

A total of 231 Holstein-Friesian cows, including heifers (18–26 months old, $n = 119$) and lactating cows (3–6 years old, 137 ± 7 days in milk, 36 kg/day milk, $n = 112$), were used in this study. All cows were in good health and reared on two different dairy farms in Adana and Aydın provinces of Turkey. Ethics committee approval was received for all applications made in this research (reference number: #2012-51). Animals were synchronized by two intramuscular injections of PGF_{2 α} (Lutelen, Topkim, İstanbul, Turkey) at 11-day intervals. Estrus was detected using three 30-min observation periods per day. Animals in standing heat were inseminated artificially. Blood samples (10 mL) were collected from coccygeal venipuncture into vacutainer tubes containing K₃-EDTA as an anticoagulant (Vacutainer, Becton, Dickinson and Co., Franklin Lakes, NJ, USA) on day 25 ($n = 61$), 28 ($n = 84$), or 32 ($n = 86$) after insemination. The day of insemination was designated day zero.

The blood samples were centrifuged within 2 h for 20 min at 4000 rpm under ambient temperatures (20 °C) on the farms where the animals were reared. After centrifugation, plasma samples were frozen in Eppendorf tubes (1.5 mL) and transported on dry ice to the Dicle University Faculty of Veterinary Medicine laboratory and stored at -20 °C until analysis.

2.2. Ultrasonographic examination

Transrectal ultrasonography was carried out for each cow or heifer on days 25, 28, and 32 after insemination using a real-time B-mode ultrasound scanner (Scanner 480 Vet, Esaote/Pie Medical, Maastricht, the Netherlands) equipped with a 5/7.5-MHz linear endorectal transducer. Pregnancy was determined as positive based on the detection of anechoic allantoic fluid or a viable embryo. The ovaries were also examined for the presence of the corpus luteum. The cows and heifers were reexamined by transrectal ultrasonography on days 55–62 (30 days after collecting plasma samples) in order to confirm the pregnancy based on the detection of an embryonic heartbeat. Heifers and lactating cows determined by these successive examinations to be pregnant or nonpregnant were included in the study.

2.3. PAG-ELISA analysis

The pregnancy diagnoses based on PAG-ELISA analyses of plasma samples were performed in the Dicle University Faculty of Veterinary Medicine Laboratory using a commercial test kit (IDEXX Bovine Pregnancy Test Kit, Westbrook, ME, USA). The IDEXX Bovine Pregnancy Test Kit diagnoses pregnancy based on the PAG concentration

in bovine plasma or serum. The working principle of the PAG-ELISA kit was described by Byrem et al. (13).

Plasma PAG ELISA tests were conducted according to the manufacturer's instructions by trained technicians who were blinded to the pregnancy status of the heifers and lactating cows. Briefly, 100- μ L plasma samples were added to 96-well ELISA plates commercially coated with monoclonal antibodies directed against bovine PAGs and incubated for 60 min at 37 °C. The wells were thoroughly washed using an automatic 8-channel plate washer (Flexiwash, Asys Hitech, Eugendorf, Austria) to remove all components other than those bound to antibodies. The wells were incubated with 100 μ L of detector solution for 30 min at room temperature (RT). Following three serial washes, 100 μ L of TMB substrate was added to the wells and incubated for 15 min at RT. Reactions were stopped with stop solution. Positive and negative controls were included in the ELISA procedure.

After stopping the reaction, the optical density of each well was read at a wavelength of 450 nm (Biochrom Anthos Zenyth 200rt microplate reader, Cambridge, UK). Results were calculated and expressed sample - negative ($S - N$) (with both values corrected by subtraction of the reference wavelength OD of the negative control). For plasma samples, if the result was ≥ 0.3 samples are classed as positive (pregnant), and those below 0.3 were classed as negative (nonpregnant).

2.4. Statistical analysis

Data for assays were arranged as follows: (a) correct positive diagnosis (PAG-ELISA test and ultrasound examinations positive), (b) incorrect positive diagnosis (PAG-ELISA test positive and ultrasound examinations negative), (c) incorrect negative diagnosis (PAG-ELISA test negative and ultrasound examinations positive), and (d) correct negative diagnosis (PAG-ELISA test negative and ultrasound examinations negative). From these data, the sensitivity ($100 \times a / a + c$), specificity ($100 \times d / d + b$), positive predictive value ($100 \times a / a + b$), negative predictive value ($100 \times d / c + d$), and accuracy ($100 \times (a + d) / (a + b + c + d)$) of the PAG-ELISA test were calculated (14).

A two-way analysis of variance (ANOVA) was used to assess the effects of the date of examination and animal status (heifers vs. lactating cows) on plasma PAG levels (based on optical density values). Differences among examination dates within the pregnant and nonpregnant groups were tested using one-way analyses of variance. Multiple comparisons between examination days were performed with Tukey's HSD test. SPSS 16.0 was used for statistical analyses. For all statistical analyses, $P < 0.05$ was considered significant.

3. Results

Among the 231 samples screened using the PAG-ELISA test, there were 95 correct positives, 125 correct negatives, 7 incorrect positives, and 4 incorrect negatives. Therefore, the sensitivity, specificity, positive predictive value, negative predictive value, and accuracy of the PAG-ELISA test were 95.9% (98%–93.7%), 94.7% (98.5%–90.6%), 93.1% (98%–88.2%), 96.9% (98.5%–95%), and 95.2% (98.3%–91.9%) among both heifers and lactating cows (Table 1). Among nonpregnant animals, examination date and animal status (heifers vs. lactating cows) had no effect on plasma PAG levels ($P > 0.05$). In pregnant animals, there were significant effects of examination date and animal status (heifers vs. lactating cows) on plasma PAG levels ($P < 0.001$). The interaction between these two factors was also significant ($P < 0.001$). Among pregnant animals, plasma PAG levels obtained from heifers were higher than those from lactating cows ($P < 0.001$). Multiple comparisons between examination days within each animal status group (heifers vs. lactating cows) showed that plasma PAG levels increased from the 25th to 32nd days of pregnancy in heifers ($P < 0.001$). In contrast, no change in the plasma PAG levels of lactating cows was observed ($P > 0.05$) (Table 2; Figure).

4. Discussion

Early pregnancy diagnosis in lactating cows is an essential tool for shortening calving intervals. Diagnosing

pregnancy before days 30–35 after insemination using rectal palpation or transrectal ultrasonography is still done on dairy farms (15–17). Alternatively, laboratory tests have been developed to diagnose early pregnancy by detecting PAGs released from binucleated cells of embryonic trophoblasts (18–20).

The sensitivity and specificity of the PAG-ELISA test have been reported as 93.9%–100% and 66.7%–95.5%, respectively (10,12,21). In the present study, we found the sensitivity and specificity of the PAG-ELISA test to be 95.9% and 94.7% in heifers and lactating cows, respectively. These results were consistent with those reported in the literature. However, test performances varied owing to differences among commercial kits, user experience, and individual variability in serum PAG concentration. These differences limit the reliability of PAG-ELISA for diagnosing pregnancy between the 26th and 30th days of pregnancy (19,22).

In a survey of 1673 dairy cows, Silva et al. (10) detected 29 incorrect negative results and estimated the negative predictive value of the test to be 97.1%. In accordance with those results, we observed four incorrect negative cases between the 25th and 32nd days after insemination, which suggested a negative predictive value of 96.9%. Incorrect negative findings might result from low and varying levels of PAGs among different individuals between the 25th and 32nd days (19). After conception, PAGs can be detected in maternal blood as early as 22 to 24 days after insemination.

Table 1. Sensitivity, specificity, predictive values, and accuracy of plasma pregnancy associated glycoprotein (PAG) ELISA tests in heifers and lactating cows.

	Days of pregnancy	n	a	b	c	d	Se(%)	Sp(%)	PPV(%)	NPV(%)	ACC(%)
Heifers	25	18	6	0	0	12	100	100	100	100	100
	28	33	13	0	0	20	100	100	100	100	100
	32	68	31	1	1	35	96.8	97.2	96.8	97.2	97
	Total	119	50	1	1	67	98	98.5	98	98.5	98.3
Lactating cows	25	43	17	3	2	21	89.4	87.5	85	91.3	88.3
	28	51	21	0	1	29	95.4	100	100	96.6	98
	32	18	7	3	0	8	100	72.7	70	100	83.3
	Total	112	45	6	3	58	93.7	90.6	88.2	95	91.9
Heifers + lactating cows	25	61	23	3	2	33	92	91.6	88.4	94.2	91.8
	28	84	34	0	1	49	97.1	100	100	98	98.8
	32	86	38	4	1	43	97.4	91.4	90.4	97.7	94.1
	Total	231	95	7	4	125	95.9	94.7	93.1	96.9	95.2

a, correct positive diagnosis (pregnant); b, incorrect positive diagnosis (nonpregnant); c, incorrect negative diagnosis (pregnant); d, correct negative diagnosis (nonpregnant); Se, sensitivity; Sp, specificity; PPV, positive predictive value; NPV, negative predictive value; ACC, accuracy.

Table 2. The effects of examination date and animal status (heifers vs. lactating cows) on plasma PAG levels.

Animal status	Examination days	Pregnant animals		Nonpregnant animals	
		n	Mean ± SE	n	Mean ± SE
Heifers	25	6	1.212 ± 0.254 ^a	12	0.022 ± 0.001 ^a
	28	13	2.085 ± 0.246 ^b	20	0.031 ± 0.003 ^a
	32	32	2.767 ± 0.068 ^c	36	0.040 ± 0.006 ^a
	Total	51	2.416 ± 0.109 ¹	68	0.034 ± 0.003 ¹
			P < 0.001		n.s.
Lactating cows	25	20	1.602 ± 0.158 ^a	23	0.037 ± 0.012 ^a
	28	21	1.396 ± 0.130 ^a	30	0.041 ± 0.011 ^a
	32	7	1.691 ± 0.268 ^a	11	0.046 ± 0.022 ^a
	Total	48	1.528 ± 0.095 ²	64	0.040 ± 0.008 ¹
			n.s.		n.s.

^{a, b, c} Different letters show significant differences between rows (examination dates) in the same column (within each animal status).

^{1, 2} Different numbers show that differences between heifers and lactating cows within each pregnancy status were significant (P < 0.001). n.s.: nonsignificant.

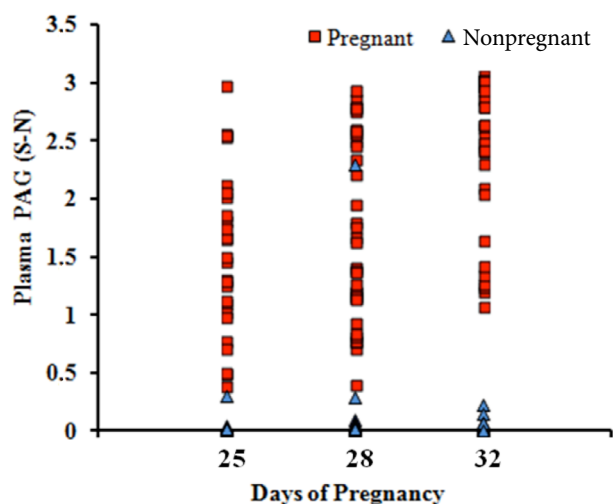


Figure. Plasma PAG levels measured by PAG-ELISA on days 25, 28, and 32 after insemination in heifers and lactating cows.

Their concentration increases during pregnancy and reaches the highest level before calving (18–20). Therefore, the sensitivity of the test is expected to increase as pregnancy progresses, while the rate of incorrect negative results is expected to decrease. Accordingly, Szenci et al. (23) reported that the sensitivity of the PAG-RIA test before the 29th day after insemination is lower than that observed on subsequent days, and it is nearly 100% on the 37th day. Increases in the sensitivity of the test have been

associated with increasing PAG levels in maternal blood. The mean plasma PAG levels observed in the present study also increased from the 25th to 32nd days. Therefore, the sensitivity of the test increased with plasma PAG levels, while the frequency of incorrect negative results decreased.

It has been reported that incorrect positive results are more likely to be obtained during the early postpartum period (10). In the present study, we observed seven incorrect positive results among 231 samples between the 25th and 32nd days, of which three were on the 25th day. Incorrect positive results might be obtained shortly after embryonic death, before PAG levels in maternal blood fall below the threshold for detection using the ELISA test. The half-life of PAGs in maternal blood after induced embryonic death has been reported to be 2.7 to 7 days (24,25). Therefore, incorrect positive results from the PAG-ELISA test may sometimes be due to embryonic death or fetal loss. However, the incorrect positive results in the present study were not associated with embryonic death, since no pregnancy loss was observed.

One other potential cause of incorrect positive results from the PAG-ELISA test may be residual PAGs from previous pregnancies. Kaya et al. (26) indicated that the clearance of PAGs from plasma is completed within 45 days after calving in dairy cows. Thus, measuring PAG levels to determine pregnancy loss does not seem to be feasible in practice, since PAGs can still be detected in circulation even if the cow is no longer pregnant. Zoli et

al. (19) reported that PAG levels in dairy and beef cattle increase from the 22nd day of pregnancy until calving and continually decrease to undetectable levels 100 ± 20 days after calving. Silva et al. (10) reported that incorrect positive results were observed in certain cows up to 100 days after calving following their first pregnancy. In this study lactating cows with an average of 137 days in milk were used. Conversely, Zoli et al. (19) detected PAG-like immunoreactivity in seven individuals among 30 noninseminated cows, and in three individuals among 20 bulls. Green et al. (20) detected the presence of PAGs in blood from five individuals among 42 cows 15 days after artificial insemination. However, PAGs detected at this stage are unlikely to be due to pregnancy, since a placental connection has not yet developed. Therefore, incorrect positive results may be due to cross-reactions with foreign proteins. We suggest that the incorrect positive results observed in the present study were related to cross-reactions with proteins other than PAG, resulting in the observed reduced sensitivity.

In the present study, the sensitivity, specificity, positive predictive value, negative predictive value, and accuracy of the PAG-ELISA test were higher for heifers than for lactating cows. While plasma PAG levels increased significantly over time in heifers, i.e. from day 25 to 28, they did not change in lactating cows. Furthermore, plasma PAG levels in heifers were significantly higher than those in lactating cows. Because PAGs can be detected in milk (27) and there was a negative correlation between plasma PAG levels and milk yield (28), lower plasma PAG levels in lactating cows may be explained by the removal of PAGs from the plasma owing to their excretion in milk.

Although the sensitivity, specificity, and accuracy of pregnancy detection using the ELISA test were acceptable in both groups, the performance of the test was superior in pregnant heifers compared to lactating cows. We conclude that the PAG-ELISA test is sufficiently specific and sensitive for diagnosing pregnancy on dairy farms and can be used as a different method for pregnancy diagnosis.

References

1. LeBlanc S. Assessing the association of the level of milk production with reproductive performance in dairy cattle. *J Reprod Dev* 2010; 56: 1-7.
2. Lucy MC. Fertility in high-producing dairy cows: reasons for decline and corrective strategies for sustainable improvement. *Soc Reprod Fertil* 2007; 64: 237-254.
3. Walsh SW, Williams EJ, Evans ACO. A review of the causes of poor fertility in high milk producing dairy cows. *Anim Reprod Sci* 2011; 123: 127-138.
4. Sarıözkan S, Aral Y, Murat H, Aydın E, Sarıözkan S. Süt sığırcılığı işletmelerinde fertilité bozukluklarından kaynaklanan finansal kayıpların hesaplanması. *Ankara Üniv Vet Fak Derg* 2012; 59: 55-60 (in Turkish).
5. Kossaibati MA, Esslemont RJ. The costs of production diseases in dairy herds in England. *Vet J* 1997; 154: 41-51.
6. Ott TL, Dechow C, O'Connor ML. Advances in reproductive management: pregnancy diagnosis in ruminants. *Anim Reprod* 2014; 11: 207-216.
7. Whitlock BK, Maxwell HS. Pregnancy-associated glycoproteins and pregnancy wastage in cattle. *Theriogenology* 2008; 70: 550-559.
8. Peter AT. Bovine placenta: a review on morphology, components, and defects from terminology and clinical perspectives. *Theriogenology* 2013; 80: 693-705.
9. Beckers JE, Drion PV, Garbayo JM, Perenyi ZS, Zarrouk A, Sulon J, Remy B, Szenci O. Pregnancy associated glycoproteins in ruminants. Inactive members of the aspartic proteinase family. *Acta Vet Hung* 1999; 47: 461-469.
10. Silva E, Sterry RA, Kolb D, Mathialagan N, McGrath MF, Ballam JM, Fricke P. Accuracy of a pregnancy-associated glycoprotein ELISA to determine pregnancy status of lactating dairy cows twenty-seven days after timed artificial insemination. *J Dairy Sci* 2007; 90: 4612-4622.
11. Karen A, Sousa NM, Beckers JF, Bajcsy ÁC, Tibold J, Mádl I, Szenci O. Comparison of a commercial bovine pregnancy-associated glycoprotein ELISA test and a pregnancy-associated glycoprotein radiomimmunoassay test for early pregnancy diagnosis in dairy cattle. *Anim Reprod Sci* 2015; 159: 31-37.
12. Romano JE, Larson JE. Accuracy of pregnancy specific protein-B test for early pregnancy diagnosis in dairy cattle. *Theriogenology* 2010; 74: 932-939.
13. Byrem TM, Velek K, Pearse HL. The Detection of Pregnancy Associated Glycoproteins (PAG) in Routine Milk Recording Samples as an Indicator of Pregnancy in Dairy Cattle. Rome, Italy: ICAR; 2012.
14. Kastelic JP. Critical evaluation of scientific articles and other sources of information: an introduction to evidence-based veterinary medicine. *Theriogenology* 2006; 66: 534-542.
15. Romano JE, Thompson JA, Forrest DW, Westhusin ME, Tomaszewski MA, Kraemer DC. Early pregnancy diagnosis by transrectal ultrasonography in dairy cattle. *Theriogenology* 2006; 66: 1034-1041.
16. Youngquist RS. Pregnancy diagnosis. In: Youngquist RS, Threlfall WR, editors. *Current Therapy in Large Animal Theriogenology*. 2nd ed. St. Louis, MO, USA: Saunders; 2007. pp. 294-303.

17. Lucy M, Green J, Poock S. Pregnancy determination in cattle: a review of available alternatives. *Applied Reproductive Strategies in Beef Cattle* 2011; 1: 367-375.
18. Sasser RG, Ruder CA, Ivani KA, Butler JA, Hamilton WC. Detection of pregnancy by radioimmunoassay of a novel pregnancy-specific protein in serum of cows and a profile of serum concentrations during gestation. *Biol Reprod* 1986; 35: 936-942.
19. Zoli AP, Guilbault LA, Delahaut P, Ortiz WB, Beckers JF. Radioimmunoassay of a pregnancy associated glycoprotein in serum: its application for pregnancy diagnosis. *Biol Reprod* 1992; 46: 83-92.
20. Green JA, Parks T, Avalle MP, Telugu BP, McLain AL, Peterson AJ, McMillan W, Mathialagan N, Hook RR, Xie S et al. The establishment of an ELISA for the detection of pregnancy-associated glycoproteins (PAGs) in the serum of pregnant cows and heifers. *Theriogenology* 2005; 63: 1481-1503.
21. Green JC, Volkmann DH, Poock SE, McGrath MF, Ehrhardt M, Moseley AE, Lucy MC. Technical note: A rapid enzyme-linked immunosorbent assay blood test for pregnancy in dairy and beef cattle. *J Dairy Sci* 2009; 92: 3819-3824.
22. Humblot P. Use of pregnancy specific proteins and progesterone assays to monitor pregnancy and determine the timing, frequencies and sources of embryonic mortality in ruminants. *Theriogenology* 2001; 56: 1417-1433.
23. Szenci O, Beckers JF, Humblot P, Sulon J, Sasser G, Taverne MAM, Varga J, Baltusen R, Scheckk G. Comparison of ultrasonography, bovine pregnancy-specific protein B, and bovine pregnancy-associated glycoprotein 1 tests for pregnancy detection in dairy cows. *Theriogenology* 1998; 50: 77-88.
24. Semambo DKN, Eckersall PD, Sasser RG, Ayliffe TR. Pregnancy-specific protein B and progesterone in monitoring viability of the embryo in early pregnancy in the cow after experimental infection with *Actinomyces pyogenes*. *Theriogenology* 1992; 37: 741-748.
25. Szenci O, Beckers JF, Sulon J, Bevers MM, Börzsönyi L, Fodor L, Kovács F, Taverne MAM. Effect of induction of late embryonic mortality on plasma profiles of pregnancy associated glycoprotein 1 in heifers. *Vet J* 2003; 165: 307-313.
26. Kaya MS, Kose M, Coban S, Mutlu H, Ucar EH, Guzeloglu A, Atli MO. Profile of Pregnancy Associated Glycoproteins in bovine plasma during postpartum period; is it usable for detecting pregnancy loss? *Reprod Dom Anim* 2015; 50 (Suppl. 3): 41.
27. Friedrich M, Holtz W. Establishment of an ELISA for measuring bovine pregnancy-associated glycoprotein in serum or milk and its application for early pregnancy detection. *Reprod Domest Anim* 2010; 45: 142-146.
28. Ricci A, Carvalho PD, Amundson MC, Fourdraine RH, Vincenti L, Fricke PM. Factors associated with pregnancy-associated glycoprotein (PAG) levels in plasma and milk of Holstein cows during early pregnancy and their effect on the accuracy of pregnancy diagnosis. *J Dairy Sci* 2015; 98: 2502-2514.