Evaluation of pregnancy-associated glycoprotein (PAG) test in early pregnancy diagnosis in dairy cows

Research Article

ABSTRACT

In this study, it was aimed to evaluate and compare the effectiveness of pregnancy-associated glycoprotein (PAG) levels in the milk samples of dairy cows in their early pregnancy diagnosis. A total of 229 Holstein cows, aged between 3 and 7 years old, inseminated in the first natural estrus after completing the voluntary waiting period and not returning to their estrus at 18-24 days after insemination, were used as animal material. Ultrasound examination was performed on the 29th day after insemination to diagnose early pregnancy, and milk samples were collected on the same day to determine PAG levels. The ultrasound examination was used as a reference test. The sensitivity, specificity, predictive values, and accuracy rates of the milk PAG test were found to be 92%, 100%, 100%, 91.7%, 95.8%, respectively, and the agreement between the ultrasound and milk test results was excellent according to the Kappa value (κ=0.919). The overall validity of the milk test was found to be 95.75%. The false negative rate of the milk PAG test was found as 8%. The false negative results were thought to be caused by embryonic deaths. In conclusion, the milk PAG test is a reliable test for early pregnancy diagnosis in dairy cows.

Keywords: Dairy cows, diagnosis, early pregnancy, milk PAG

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INTRODUCTION

Correct pregnancy diagnosis is very important for field conditions. It is important to determine pregnancy as early as possible and by the most reliable method. This importance lies in identifying non-pregnant cows as soon as possible and reinseminating them to ensure conception in order to shorten the calving interval. This way, infertile animals in the herd can be detected and treated in a shorter time, or economic losses can be reduced by removing them from the herd. Sound reproductive management depends on establishing and maintaining pregnancy as soon as possible after the end of the voluntary waiting period after birth or after the breeding season in seasonal systems (Pohler et al., 2016).

Pregnancy detection can be performed by using both direct and indirect methods. Direct methods include transrectal palpation and transrectal ultrasound. Indirect methods include chemical pregnancy markers, such as milk or plasma progestogen concentrations or pregnancy-associated protein and pregnancy-associated glycoprotein (PAG) measurements (Bekele et al., 2016; Doğan and Köse, 2022; Fosgate et al., 2017; Gajewski et al., 2008; Purohit, 2010).

Bovine PAGs are produced by the binuclear trophoblastic cells of the placenta immediately after implantation. PAGs can be detected in maternal circulation from the 22nd day of pregnancy to 2-3 months after delivery (Çiplak, 2024; Schlafer et al., 2000).

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Evaluation of pregnancy-associated glycoprotein (PAG) test is important in the control of caruncles and uterine gland morphogenesis, differentiation, and functions, which are necessary for fetal nutrition and the maintenance of intrauterine viability (Igwebuike, 2006). The sensitivity and specificity of the milk PAG testing method used as a pregnancy test are very high. As a stress-free method in pregnancy management in ruminant animals, it is particularly useful in the diagnosis of pregnancy and the determination of embryonic deaths (Gajewski et al., 2008).

This study aimed to evaluate diagnostic power of the milk PAG test used in the early diagnosis of pregnancy in of cows after insemination.

MATERIALS AND METHODS

Animal material

The study was carried out in a dairy farm with 3,000 dairy cows in Aydın, where intensive feeding and herd management systems are used.

The animal material of the study included healthy and clinically normal 229 cows without reproductive disorders and aged from 3 to 7 years that were artificially inseminated by the farm's technical staff after 60 days of calving and whose estrus did not return 18-24 days after insemination. The average daily milk production per cow was about 55 kg during the study period. Efforts were made to reduce variation in the general condition of cows so that milk PAG changes could be attributed to factors other than the clinical condition of the cows at the time of the study.

The cows were housed in a closed barn with a red-light system and a barn air conditioning system. In the farm, cows were milked 3 times a day and fed a Total Mixed Ration (TMR) prepared with feed ingredients calculated according to their needs (Ethics committee approval 2016/1-2).

Method

Estrus detection and insemination

Artificial insemination was performed by the technicians in the first natural estrus of the cows, and after the voluntary waiting period, a herd management program (activity meter, decrease in milk yield) was applied, and the results were evaluated in combination with their observations.

Collection of milk samples

On the 29th day after insemination, following the expression of a few shots of foremilk from any of the healthy udder lobes from the cows whose estrus did not return, milk samples were taken into a tube in an amount of approximately 20 ml.

Performing ultrasound examinations

Ultrasound examination was accepted as a reference test in the study. In the examination, device (Shenzhen Well. D Wed-3000v, China) has a B mode, 7.5 MHz frequency, and with a transrectal linear probe was used. Ultrasound examinations and milk sampling were performed on the same day (29th day). In cases where the pregnancy diagnosis was uncertain, a second examination was performed within the first week after the first examination (up to the 35th day after insemination) to confirm the diagnosis.In the ultrasonic examination, the quality of the ovary note CL (>22 mm, luteal tissue density, compactness) found in rectal examination was determined. Likewise, the other ovary was also evaluated. This way, when ultrasound-guided pregnancy diagnosis emerged while scanning the uterus, the corn where CL was found was revealed. The animal was considered pregnant when at least 3 of the following findings were observed while scanning the corn uterus: amniotic fluid, fetal membranes, umbilical cord, fetal heartbeat, fetus itself, and compartmental structures in the uterus. In animals without quality CL in one of the ovaries (not in sufficient size), one of the cornu uteri was scanned in detail in terms of possible pregnancy in longitudinal

and transverse sections starting from the bifurcation area. The animals in which these symptoms were not observed were considered non-pregnant.

PAG Determination in milk samples

A commercial ELISA kit for measuring PAG levels (IDEXX Milk Pregnancy Test-Diagen, Türkiye) was used to test the collected milk samples. The test was performed by experienced technical personnel, in line with the instructions of the manufacturer.

Interpretation of the results

According to the value obtained from the reader: <0.100 was considered empty or non-pregnant (negative result), 0.100-0.250 was considered suspicious, and >0.250 was considered pregnant (positive result). The test result was considered as a false positive result for the test when a non-pregnant cow was diagnosed as pregnant on ultrasound, and as a false negative result for the test when a pregnant cow was diagnosed as not pregnant on ultrasound. Among the cows diagnosed as suspicious as a result of the test, the positive suspicious result was accepted for pregnant animals according to the ultrasound examination, and the negative suspicious result was accepted for non-pregnant animals (Akkose, 2023).

Statistical analysis

In the early pregnancy diagnosis process, the results of ultrasound examinations, whose validity was previously determined and was quite high, were used as reference values. In the validity and reliability analyses of the milk PAG test, sensitivity and specificity values were determined by identifying positives, negatives, and their accuracy rates. The agreement between the ultrasound and milk PAG test results was evaluated using the Kappa test.

The Kappa coefficient takes values between 0 and 1. Accordingly, values of 0.93-1 are considered excellent, values of 0.81-0.92 are considered very good, values of 0.61-0.80 are considered good, values of 0.41-0.60 are considered moderate, values of 0.21-0.40 are considered somewhat poor, and values of 0.01-0.20 are considered poor (Altman, 1991).

The general strength or validity of the milk PAG test was found using the following formules:

Sensitivity: True positive /(true positive + false negative) x 100

Specificity: True negative /(false positive + true negative) x 100

Positive predictive value: True positive /(true positive + false positive) x 100

Negative predictive value: True negative /(false negative + true negative) x 100

Accuracy ratio: (True positive+true negative)/(true positive + true negative + false positive + false negative) x 100

RESULTS

Pregnancy findings obtained by two methods from 229 cows whose pregnancy statuses were examined on the 29th day after insemination by ultrasound and the milk PAG test are given in Table 1.

Table 1: Pregnancy findings detected by ultrasound and milk PAG test on the 29th day after insemination.

Parameters	Ultrasound	Milk PAG
Pregnant	104	103
Not pregnant	125	100
False positive	0	9
False negative	0	0
Positive suspect	0	1
Negative suspect	0	16
Total	229	229

In the ultrasound examination, which was accepted as the reference method in the study, it was determined that 104 cows out of the 229 cows included in the study were pregnant, and 125 cows were not pregnant.

In the milk PAG tests of the same 229 cows, it was found that 103 cows were pregnant, 100 cows were not pregnant, and 17 cows were suspicious.

Nine out of the 125 cows diagnosed as not pregnant by ultrasound were diagnosed as pregnant with the milk PAG test, and 16 were diagnosed as suspicious. Similarly, while none of the 104 cows diagnosed as pregnant by ultrasound were found to be not pregnant, 1 cow was diagnosed as suspicious with the milk PAG test. While 9 cows were diagnosed as false positive by the milk PAG test, no false negative diagnosis was made.

The overall strength or validity of the milk PAG test (103 + 100/103 + 100 + 0 + 9=) was found to be 95.75%.

The sensitivity, specificity, indication of pregnancy and non-pregnancy, accuracy, and compatibility rates of the milk PAG tests were found as shown in Table 2.

Table 2: Sensitivity, specificity, predictive values, accuracy, and agreement rates of the milk PAG test.

	Sensitivity %	Specificity %	Positive Predictive Value %	Negative Predictive Value %	Accuracy %	Kappa
Milk PAG	92	100	100	91.7	95.8	0.919*

* Kappa value> 0.8: excellent agreement.

The accuracy rate of the milk PAG test was found to be 95.8%, and the agreement between the two tests was excellent (kappa value>0.8). The false positive rate of the milk PAG test was found as 8.03%.

DISCUSSION

In this study, which aimed to evaluate the effectiveness of PAG testing that has the potential to be used in early pregnancy diagnosis and has attracted the attention of researchers in recent years, ultrasound examination, whose validity was previously determined and was quite high, was accepted as the reference test method.

Dairy farms should determine whether their animals are pregnant as early as possible, using a method that is low-cost, highly accurate, and easily applied in field conditions (Balhara et al., 2013). Determining pregnancy early and with high accuracy plays a key role in establishing the appropriate delivery interval.

Early pregnancy can be observed by performing daily ultrasound examinations. Additionally, it is reported that chemical analyses can be used in cows. In chemical methods used in pregnancy diagnosis, the presence or quantity of reproductive hormones is measured at a specific time after insemination. Another approach is to investigate the existence of a living embryo based on offspring-specific structures in the mother's blood. Progesterone analysis was the first to come to mind and the most researched method about pregnancy diagnosis with chemical methods. In recent years, PAG, which was found to be synthesized only from the pregnant uterus, has attracted attention. Moreover, PAG testing has advantages over progesterone-based pregnancy diagnosis. One of these advantages is that PAG can always be checked after an appropriate number of days following insemination, while the timing of progesterone testing is limited to 19-23 days after insemination. Secondly, the reliability of positive results in progesterone testing was reported as 80% by the best estimates by Sasser and Ruder (1987), while the reliability of positive results in PAG testing was determined to be 95-100% (Sasser et al., 1986; Zoli et al., 1992).

Routine pregnancy examination by ultrasound is recommended in cows on the 28th day after insemination (Dinç, 2008). Based on extensive studies today, ultrasound has been widely accepted as a valid method for the diagnosis of pregnancy. In addition to the

detection of a viable embryo in the uterus, ultrasound can quickly detect pregnancy loss, embryonic deaths, and embryonic degeneration (Moharrami et al., 2013). It also provides additional information about the non-pregnant animal. Furthermore, it allows the determination, direction, and rapid implementation of synchronization or resynchronization protocols according to the existence of functional structures in the ovaries (Dinç, 2008).

From an economic point of view, the sensitivity of the early non-pregnancy test (correct diagnosis of the pregnant animal) was found to be more important than its specificity (correct determination of the non-pregnant animal). It was reported that the sensitivity of a test should be higher than 96% if the test is performed 31 days after insemination and higher than 94% if it is performed after 24 days to gain potential economic benefit from the early diagnosis of the non-pregnant animals with chemical tests (Fricke and Giordano., 2011; Giordano at al., 2013).

Le Blanc (2013) found a sensitivity value of 99.2% and a specificity value of 95.5% in a study using 683 animals in 8 dairy farms. Lawson et al. (2014) reported that they found these values as 100% and 97.9% in 112 cows tested on the 33rd and 52nd days after insemination.

In this study, similar to previous studies, the sensitivity of the milk PAG test in determining pregnancy was determined as 92% (true positive rate). Similarly, the milk PAG test was found to have 100% specificity in revealing non-pregnant cows (true negative rate). In this study, the accuracy rate of the milk PAG test was found to be 95.8%. It was concluded that the milk PAG testing method performed above the economic gain limits (Lavon et al., 2022).

While none of the 104 cows diagnosed to be pregnant with ultrasound were diagnosed to be not pregnant by milk PAG testing, 1 cow was diagnosed with suspected pregnancy. While 9 cows (8.3/7.2%) were diagnosed as false positive in the milk PAG tests, no false positive diagnosis was made. Embryonic deaths indicative of false negative results in a test are important in terms of economic losses (Fosgate et al., 2017). Akkose (2018; 2023), determined that 95% of cattle with PAG values below 1.4 ng/ml on the 31st day of gestation experienced embryonic death until the 60th day of gestation. It was stated as a result that this test can be used as a good marker between the 31st and 59th days to identify embryonic deaths (Ask-Gullstrand et al.,2023; Yang et al.,2024).

In the milk PAG tests in this study, 1 cow was diagnosed with suspected pregnancy (positive suspicion), and 16 were diagnosed with suspected pregnancy (negative suspicion). When the value read in the evaluation made according to the milk PAG levels was in the range of 300-1,000 pg/ml, the result was considered suspicious. According to this assumption, the reading values of 17 cows (7.42%) were found to be suspicious. One of these 17 animals was evaluated as a positive (pregnant) suspect, and the remaining 16 were evaluated as negative (non-pregnant).

CONCLUSION

As a result, in this study, it was determined that the milk PAG test performed on the 29th day after insemination is a reliable and practical method for early pregnancy diagnosis.

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Author contributions: HA carried out the created the records and examinations, HA and DAD conceived the idea and prepared the original draft of the article. HA did the writing and DAD helped to finalize the manuscript. All authors made corrections and approved the final version of the manuscript.

Availability of data and materials: The data for this study are available on reasonable request from the corresponding author.

REFERENCES

Akköse, M. (2023). Comparative evaluation of two commercial pregnancy-associated glycoproteins tests for early detection of pregnancy in dairy cattle. *Theriogenology*, *200*, 11-17. https://doi.org/10.1016/j.theriogenology.2023.01.022

Akkose, M. (2018). Effect of synchronization protocols on pregnancy associated glycoproteins (Doctoral dissertation). *Harran University Institute of Health Sciences, Department of Rotation and Artificial Insemination YL Thesis.*

Ask-Gullstrand, P., Strandberg, E., Båge, R., & Berglund, B. (2023). Genetic parameters of pregnancy loss in dairy cows estimated from pregnancy-associated glycoproteins in milk. *Journal of Dairy Science*, *106*(9), 6316-6324. https://doi.org/10.3168/jds.2022-23007

Altman, D.G. (1991). Practical statistics for medical research. *Chapman and Hall, London*. https://doi.org/10.1002/sim.4780101015

Balhara, AK., Gupta, M., Singh, S. (2013). Early pregnancy diagnosis in bovines:current status and future directions. *The Scientific World Journal*, *958540*, 1-10. https://doi.org/10.1155/2013/958540

Bekele, N., Addis, M., Abdela, N. (2016). Pregnancy diagnosis in cattle for fertility management: A review. *Global Veterinaria*, *16*(4), 355-364. https://doi.org/10.5829/idosi.gv.2016.16.04.103136

Çiplak, A. Y. (2024). Pregnancy diagnosis methods in cows. *Journal of Animal Science and Economics*, *3*(1), 23-29. https://doi.org/10.5281/zenodo.10731544

Dinç, DA. (2008). Ultrason fiziği ve ineklerde reprodüktif ultrasonografi. *Ankara: Pozitif Matbaacılık.*

Doğan, A. A., and Köse, A. M. (2022). Evaluation of bovine visual ELISA test for detection of pregnancy-associated glycoproteins in early pregnancy diagnosis in goats. *Small Ruminant Research*, *212*, 106722. https://doi.org/10.1016/j.smallrumres.2022.106722

Fosgate, GT., Motimele, B., Ganswindt, A. (2017). A Bayesian latent class model to estimate the accuracy of pregnancy diagnosis by transrectal ultrasonography and laboratory detection of pregnancy-associated glycoproteins in dairy cows. *Preventive Veterinary Medicine*, *145*, 100-109. https://doi.org/10.1016/j.prevetmed.2017.07.004

Fricke, P. M. and Giordano, J. O. (2011). Use of chemical tests for pregnancy diagnosis in a reproductive management program. *Proc. Dairy Cattle Reprod. Conf, Kansas City, MO. Dairy Cattle Reproduction Council, Hartland*, WI. 48–56.

Gajewski, Z., Melo de Sousa, N., Beckers, J. F. (2008). Concentration of bovine pregnancy associated glycoprotein in plasma and milk: its application for pregnancy diagnosis in cows. *Journal of Physiology and Pharmacology*, *59*(Suppl 9), 55-64.

Giordano, J. O., Fricke, PM., Cabrera, V. E. (2013). Economics of resynchronization strategies including chemical tests to identify non pregnant cows. *Journal of Dairy Science*, *96*, 949–961. https://doi.org/10.3168/jds.2012-5704

Igwebuike, U. M. (2006). Trophoblast cells of ruminant placentas – A mini review. *Animal Reproduction Science*, *93*, 185–198. https://doi.org/10.1016/j.anireprosci.2005.06.003

Lavon, Y., Friedman, S., Shwimmer, A., Falk, R. (2022). Performing early pregnancy tests in milk and their effect on cow welfare and reproductive performance compared to rectal pregnancy tests 40 to 45 days post insemination. *Dairy*, *3*, 465–473. https://doi.org/10.3390/dairy3030034

Lawson, B. C., Shahzad, A. H., Dolecheck, K. A. (2014). A pregnancy detection assay using milk samples: Evaluation and considerations. *Journal Dairy Science*, *97*, 6316–6325. http://dx.doi.org/10.3168/jds.2014-8233

Le Blanc, S. J. (2013). Short communication: Field evaluation of a pregnancy confirmation test using milk samples in dairy cows. *Journal Dairy Science*, *96*, 2345–2348. https://doi.org/10.3168/jds.2012-6414

Moharrami, Y., Khodabandehlo, V., Ayubi, M. R. (2013). Accuracy rate of early pregnancy diagnosis in Holstein heifers by transrectal ultrasonography using 7.5MHz linear array transducer. *European Journal of Experimental Biology*, *3*(3), 678-686.

Pohler, K. G., Franco, G. A., Reese, S. T. (2016). Past, present and future of pregnancy detection methods. *Applied Reproductive Strategies in Beef Cattle – Des Moines, Iowa* – September 7-8, 251.

Purohit, G. (2010). Methods of pregnancy diagnosis in domestic animals: The Current Status. *Reproduction*, *1*, *WMC001305*. https://doi.org/10.9754/journal.wmc.2010.001305

Sasser, R. G., Ruder, C. A., Ivani, K. A. (1986). Detection of pregnancy by radioimmunoassay of a novel pregnancy-specific protein in serum of cows and a profile of serum concentrations during gestation. *Biology Reproduction*, *35*(4), 936–942. https://doi.org/10.1095/biolreprod35.4.936

Sasser, R. G. and Ruder, C. A. (1987). Detection of early pregnancy in domestic ruminants. Journal Reproduction Fertilty, Supplement, 34, 261–271.

Schlafer, D. H., Fisher, P. J., Davies, C. J. (2000). The bovine placenta before and after birth: Placental development and function in health and disease. *Animal Reproduction Science*, *60-61*, 145–160. https://doi.org/10.1016/s0378-4320(00)00132-9

Yang, M. K., Yeh, R. H., Lee, C. J., Yeh, Y. H., Chen, Y. H., Banhazi, T., Tu, P. A. (2024). Pregnancy maintenance and fetal loss assessment in Holstein cows through analyzing pregnancy-associated glycoproteins in milk. *Theriogenology*, *217*, 11-17. https://doi.org/10.1016/j.Theriogenology.2024.01.010

Zoli, A. P., Guilbault, L. A., Delahaut, P. (1992). Radioimmunoassay of a bovine pregnancy-associated glycoprotein in serum: its application for pregnancy diagnosis. *Biology Reproduction*, *46*(1), 83-92. https://doi.org/10.1095/biolreprod46.1.83