316 (2026) 106584

Contents lists available at ScienceDirect

The Veterinary Journal

journal homepage: www.elsevier.com/locate/tvjl

The field evaluation of the diagnostic performance of a commercial pregnancy-associated glycoprotein-based lateral flow assay for early pregnancy detection in ewes

Gökhan Uyanık [a,*], Murat Yüksel [a], Murat Abay [b], Ahmet Gözer [a], Filiz Kara [a], Ebru Arslanhan [a], Ishak Gökcek [c], Ufuk Kaya [d]

[a] Hatay Mustafa Kemal University, Faculty of Veterinary Medicine, Department of Obstetrics and Gynaecology, Hatay, Türkiye
[b] Erciyes University, Faculty of Veterinary Medicine, Department of Obstetrics and Gynaecology, Kayseri, Türkiye
[c] Hatay Mustafa Kemal University, Faculty of Veterinary Medicine, Department of Physiology, Hatay, Türkiye
[d] Hatay Mustafa Kemal University, Faculty of Veterinary Medicine, Department of Biostatistics, Hatay, Türkiye

ARTICLE INFO

Keywords:
Ewes
Pregnancy diagnosis
Pregnancy-associated glycoprotein
Lateral flow assay
Enzyme-linked immunosorbent assay

ABSTRACT

The present study aimed to determine the diagnostic accuracy of the commercial Alertys OnFarm Pregnancy Test (AOPT) based on pregnancy-associated glycoprotein (PAG) for early pregnancy diagnosis in ewes. A total of 100 multiparous Awassi ewes were synchronised for estrus and mated during the non-breeding season. Whole blood samples were collected from the jugular vein of all ewes on days 21, 28, and 35 after mating. The AOPT was first performed on the collected whole blood samples. Plasma PAG concentrations were then determined by Enzyme-Linked Immunosorbent Assay (ELISA). Pregnancy diagnosis was made on the same days by transrectal ultrasonography (USG), which was accepted as the reference standard. The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy of AOPT compared to USG on days 21, 28, and 35 after mating were calculated. In addition, the diagnostic performance of plasma PAG concentrations for predicting pregnancy status was evaluated by determining cut-off values and conducting receiver operating characteristic (ROC) curve analysis. When the results were examined, the sensitivity of AOPT was calculated as 77.55 %, specificity as 92.15 %, PPV as 90.47 %, NPV as 81.03 % and accuracy as 85.00 % at 21 days after mating and showed strong agreement with transrectal USG (Kappa= 0.699; $P < 0.001$). In contrast, the sensitivity (95.55 %) and accuracy (93.00 %) of AOPT on day 28 improved significantly, showing almost perfect agreement with USG (Kappa= 0.859; $P < 0.001$). On day 35, AOPT achieved the highest diagnostic performance compared to other pregnancy days with 100 % sensitivity, 96.49 % specificity, and 98.00 % accuracy (Kappa= 0.959; $P < 0.001$). Plasma PAG levels (OD) were significantly higher in pregnant ewes than in non-pregnant ewes at all time points ($P < 0.001$). The optimal cut-off values for pregnancy detection were determined as > 0.28 on day 21 (AUC= 0.941), > 0.48 on day 28 (AUC= 0.986), and > 0.43 on day 35 (AUC= 1.000). In conclusion, AOPT can be considered a practical, rapid, and reliable method for early pregnancy detection in ewes, particularly from day 28 post-mating. However, possible false-negative results that may occur very early in pregnancy and false-positive results due to embryonic loss later in pregnancy necessitate careful interpretation and, if necessary, repeat testing or confirmation with USG.

Introduction

The early and accurate detection of pregnancy in ewes is critical to improve reproductive management and flock productivity (Akbulut and Çelik, 2021; Pickworth et al., 2020; Redden and Passavant, 2013). Rapid identification of non-pregnant ewes at an early stage allows these animals to be resynchronised and mated or removed from the flock (De Carolis et al., 2020). Thus, enterprise profitability can be increased by shortening the lambing interval and optimising feed costs (Ali et al., 2024; Goldansaz et al., 2022).

* Correspondence to: Hatay Mustafa Kemal University, Faculty of Veterinary Medicine, Antakya, Hatay 31001, Türkiye.
E-mail addresses: gokhan.uyanik@mku.edu.tr (G. Uyanık), murat.yuksel@mku.edu.tr (M. Yüksel), mabay@erciyes.edu.tr (M. Abay), ahmetgozer@mku.edu.tr (A. Gözer), filizkaratr@gmail.com (F. Kara), arslanhan@gmail.com (E. Arslanhan), igokcek@mku.edu.tr (I. Gökcek), u.kaya@mku.edu.tr (U. Kaya).

https://doi.org/10.1016/j.tvjl.2026.106584
Accepted 4 February 2026
Available online 5 February 2026
1090-0233/© 2026 Elsevier Ltd. All rights are reserved, including those for text and data mining, AI training, and similar technologies.

Although approximately 30 different methods have been described for the diagnosis of pregnancy in ewes, ultrasonography is the most widely preferred method in the field (Ali et al., 2024; Jones and Reed, 2017). In particular, transrectal ultrasonography (USG) is considered to be the gold standard for early pregnancy diagnosis in ewes as it allows direct visualisation of the embryo and heartbeat (Karen et al., 2014; Romano and Christians, 2008; Yadav et al., 2019). However, the requirements for pregnancy examination with transrectal USG, such as fasting the ewes for at least eight hours before, emptying their rectum, and fixing them in the dorsoventral position (Allabban and Erdem, 2020), lead to significant negativities in terms of animal welfare. In addition, although ultrasonography can be widely used in intensive and well-equipped production systems, the requirement for high-cost equipment, the need for experienced personnel, and the limited portability of the devices limit its practical applicability particularly in extensive production systems and rural field conditions (Erdem et al., 2020).

Recently developed chemical pregnancy tests, as an alternative to conventional methods such as USG, stand out with their advantages, including simplicity of use and not requiring costly equipment or special training (Kline et al., 2024). These tests are based on the qualitative or quantitative detection of pregnancy-specific biomarkers (progesterone, interferon tau, pregnancy-specific protein B, pregnancy-associated glycoproteins etc.) in the maternal circulation (Ott et al., 2018). Among these, pregnancy-associated glycoproteins (PAGs) are glycoproteins belonging to the family of inactive aspartic proteinases that are synthesised by trophoblast giant cells in the ruminant placenta and can be detected in the maternal circulation from 18 to 20 days of pregnancy (Adeyeye et al., 2021; Sousa et al., 2006). The development of Enzyme-Linked Immunosorbent Assays (ELISA) to detect PAGs has enabled this biomarker to be used effectively in pregnancy diagnosis (De Carolis et al., 2020). It has been reported in many reports that pregnancies can be detected with high accuracy rates from 26 to 30 days of pregnancy in cattle by looking at serum PAG levels (Akköse, 2023; Karen et al., 2015; Kline et al., 2024; Northrop et al., 2019; Szenci, 2021). The high structural similarity (70–80 %) of PAG molecules among ruminant species (Roberts et al., 2019; Xie et al., 1991) has brought to the spotlight that these tests developed for cattle can also be used effectively in ewes. Indeed, many studies have shown that PAG-ELISA tests developed for cattle can successfully diagnose pregnancies in ewes (Akköse, 2020; Alkan et al., 2020; da Costa et al., 2017; De Carolis et al., 2020; Moussafir et al., 2018). However, PAG-ELISA pregnancy tests are still not practical enough for routine use in ewes under extensive and rural on-farm conditions due to the need for laboratory infrastructure and sample processing. The main reason for this is that the tests can only be performed with plasma or serum samples, and the preparation of these samples requires equipment such as centrifuges, which are not usually available under farm conditions. In addition, the ELISA method requires specialised laboratory equipment, such as a microtiter plate reader for optical density measurement, so these tests are mostly performed in veterinary clinics or centralised laboratories (Mayo et al., 2016). All these factors significantly limit the applicability of PAG-ELISA tests directly under field conditions.

Recently, a PAG-based lateral flow (immunochromatographic) pregnancy test kit has been developed that can provide results in approximately 20 min directly with a few drops of whole blood sample (Rial et al., 2024). This test enables pregnancy diagnosis in the field without the need for laboratory equipment and stands out with its ease of application. This commercial lateral flow test for pregnancy detection in cattle (Alertys On-Farm Pregnancy Test [AOPT]) has attracted attention with successful field trials in the cattle industry. Akköse (2023) reported that AOPT showed an accuracy rate of 95.6 % on the 28th day of pregnancy in Holstein cows. Similarly, Şafak et al. (2025) emphasised that this test offers high sensitivity and specificity in the diagnosis of early pregnancy in cattle.

Considering the high structural similarity of PAG molecules in ruminant species and the results of existing reports, there is a strong argument that the AOPT developed for cattle can detect pregnancy with similar accuracy in ewes. However, to the best of our knowledge, the efficacy of this test in ewes has not been systematically evaluated to date, and validation studies are required for application in ewes. This study aimed to evaluate the efficacy and diagnostic accuracy of AOPT in the diagnosis of pregnancy on whole blood at 21, 28, and 35 days after mating in ewes, using transrectal ultrasonography as the reference standard.

Materials and methods

Ethical statement

This study was carried out with project-based authorization from the Ministry of Agriculture and Forestry of the Republic of Türkiye (Permit No: E-76063909–325.04.02-18663027), and all experimental procedures were conducted in strict accordance with the Regulation on the Welfare and Protection of Animals Used for Experimental and Other Scientific Purposes, thereby ensuring animal welfare and public health throughout the study period. Furthermore, the study protocol was reviewed and approved by the Hatay Mustafa Kemal University Animal Experiments Local Ethics Committee (Approval No: 2025/03–14). The study was designed and conducted in accordance with the STARD reporting standards for diagnostic accuracy studies (Bossuyt et al., 2015).

Animals and estrus synchronisation

The study was carried out in Reyhanlı, a district of Hatay Province situated in the Southeastern Anatolia Region, during the non-breeding season (April-June 2025). The area is located at 36°15'55.6308"N latitude and 36°32'46.4424"E longitude, with an altitude of 148 m above sea level.

The animal material used in the study consisted of 100 Awassi ewes that were clinically healthy, 2–5 years old, 40–50 kg, postpartum 90 days completed, multiparous, with a documented history of normal pregnancy and parturition in the previous breeding season, and no evidence of reproductive disorders or infertility. The ewes were grazed on natural pastures. No external roughage or concentrate feed was given but they were given access to water ad libitum. For estrus synchronisation, all ewes ($n = 100$) received intravaginal sponges (Esponjavet®, Hipra, Spain) containing 60 mg medroxyprogesterone acetate (MAP) for 7 days. On the day the sponges were removed, 500 IU eCG (Oviser®, Hipra, Spain) and 75 µg *d*-cloprostenol (Senkrodin®, Vetaş, Türkiye) were administered intramuscularly (Fig. 1) (Uyanık et al., 2024). From the 24th hour (h) after the removal of the sponges, fertile rams ($n = 10$) were added to the flock twice a day, and matings were followed. The time of mating was considered day 0. Mating monitoring was terminated at 72 h, and rams were isolated from the flock and not reintroduced to ewes during the study period.

Diagnosis of pregnancy from whole blood samples

Blood samples were collected 21, 28, and 35 days after mating by puncture of the jugular vein, and samples were collected in 6 mL vacutainer tubes (Vacusera®, Disera, Türkiye) containing tripotassium ethylenediaminetetraacetic acid (K_3EDTA) (Fig. 1). A commercial lateral flow assay (Alertys OnFarm Pregnancy Test, IDEXX Laboratories, Westbrook, ME, USA, Cat. No. 98–26441–00) developed for pregnancy detection from whole blood was used. According to the manufacturer's instructions, four drops of whole blood and six drops of wash solution were added to the well of the test cartridge. Then it was incubated for approximately 20 min at room temperature (20°C-25°C). Following incubation, a double line (C and T) on the test cartridge was considered positive for pregnancy, and a single line (C) was considered negative

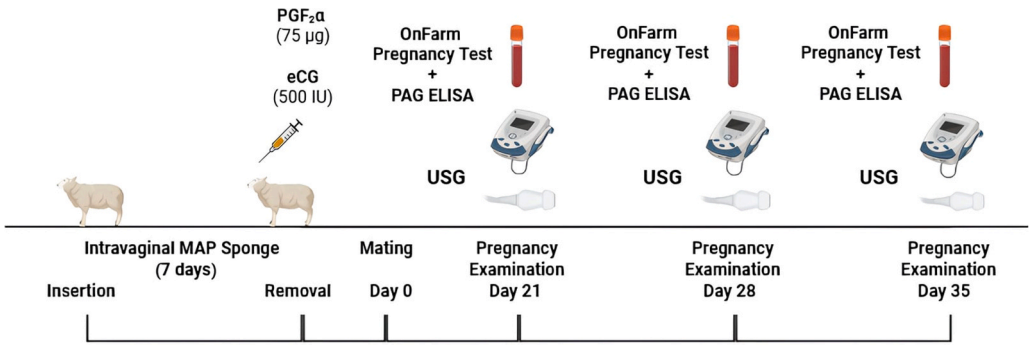


Fig. 1. Experimental timeline illustrating the synchronization protocol and pregnancy diagnosis methods used in ewes. Intravaginal MAP sponge was inserted for 7 days, followed by administration of 75 µg $PGF_2\alpha$ and 500 IU eCG upon sponge removal to induce estrus. Natural mating was performed on Day 0. Pregnancy examinations were conducted on Days 21, 28, and 35 post-mating using an Alertys OnFarm Pregnancy Test (AOPT), PAG ELISA, and transrectal ultrasonography (USG).

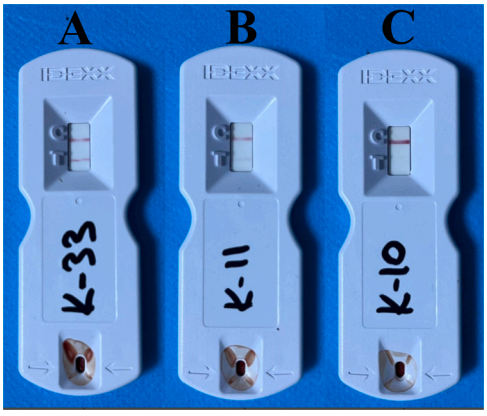


Fig. 2. Photograph of lateral flow pregnancy test results from three ewes. The test line (T) intensity may vary from dark to faint. The presence of a red or pink test line of any intensity is interpreted as a positive pregnancy, while the absence of a test line indicates a negative result. (A) Positive pregnancy; dark test line, (B) Positive pregnancy; faint test line, (C) Negative pregnancy; no test line visible.

(Fig. 2). Immediately after the completion of whole blood pregnancy tests, blood samples were centrifuged at 3000 rpm for 10 min, and plasma was separated to determine individual plasma PAG concentrations by ELISA. Plasma samples were stored at −20°C until the time of analysis.

Semiquantitative plasma PAG analysis

PAG levels in plasma samples were measured using a commercial ELISA kit (Alertys Ruminant Pregnancy Test Kit, IDEXX Laboratories, Westbrook, ME, USA, Cat. No. 99–41169) according to the manufacturer's instructions and as described in the literature (Roberts et al., 2017). For analysis, 100 µL of plasma sample was taken onto anti-PAG monoclonal antibody-coated microtiter plates, diluted with 25 µL of sample diluent, and incubated at 37°C for 60 min. Post-incubation, the plates were washed four times with washing solution, then 100 µL of detection solution (anti-PAG antibody) was added and incubated at room temperature (20°C-25°C) for 30 min. The washing procedure (4X) was repeated after incubation, and 100 µL of conjugate solution (anti--IgG horseradish peroxidase) was added, followed by incubation at room temperature for another 30 min. The plate was then washed again (4X), and 100 µL of substrate solution (3,3′,5,5′-tetramethylbenzidine) was pipetted into all wells, followed by incubation at room temperature in the dark for 15 min. Finally, 100 µL of stop solution was added, and the absorbance values were measured at 450 nm using a microplate reader (Erba Mannheim, Lisascan EM, Czech Republic). The optical density (OD) value of each sample was calculated by subtracting the average OD value of the negative control group from the sample OD value (S-N method). Since the colour intensity obtained in the test was proportional to the PAG concentration in the sample, the OD values were used as the plasma PAG levels of the samples. For the positive controls, the intra-plate and interplate coefficient of variation (CV) were 2.7 % and 6.3 %, respectively.

Diagnosis of pregnancy by ultrasonography

Pregnancy diagnosis was performed by the single veterinarian using transrectal USG on days 21, 28, and 35 post-mating. A portable, real-time B-Mode ultrasonography device (SonoSite M-Turbo, Fujifilm, USA) with a 5–10 MHz linear rectal probe was used for transrectal examination. The diagnosis of a positive pregnancy was based primarily on ultrasonographic visualization of the corpus luteum (CL), embryonic vesicle, amniotic fluid, embryo, and heartbeat (Fig. 3) (Crilly et al., 2017). In addition to the presence of an embryo and anechoic fluid in the uterus, especially on the 21st day after mating, special attention was paid to the evaluation of the CL to increase the reliability of the diagnosis. First, the presence of CL was detected by scanning the ovaries with B-mode ultrasonography; then, color Doppler was activated for a complete CL assessment. The degree of luteal vascularization was scored from 1 to 4 as 1 (0–25 %), 2 (26–50 %), 3 (51–75 %), and 4 (76–100 %) according to the ratio of the luteal area containing colored pixels to the CL, as described by Arashiro et al. (2018). Accordingly, ewes with at least one CL with a vascularization score of ≥ 2 were considered pregnancy positive, while those with no embryo and no CL were classified as pregnancy negative. In addition, impaired integrity of the embryonic membranes and absence of a heartbeat were considered embryonic death (Kaulfuss et al., 1997; Rickard et al., 2017) and classified as negative pregnancy.

Statistical analysis

The sample size of the study was calculated with G*Power software version 3.1. It had resulted that the minimum number of ewes was 100, considering a medium effect size of 0.57, an alpha value of 0.05, and a power of 0.80.

The pregnancy results obtained from transrectal USG, which was used as a reference test, were compared with the results of the AOPT kit. The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and accuracy of the test were calculated. The results of the test were determined as correct positive (Cp; USG result pregnant, test result pregnant), false positive (Fp; USG result not pregnant, test result pregnant), false negative (Fn; USG result pregnant, test result not pregnant) and correct negative (Cn; USG result not pregnant, test result not pregnant). Using these data, test sensitivity [Cp/ (Cp + Fn) × 100], specificity [Cn/ (Cn + Fp) × 100], PPV [Cp/ (Cp + Fp) × 100],

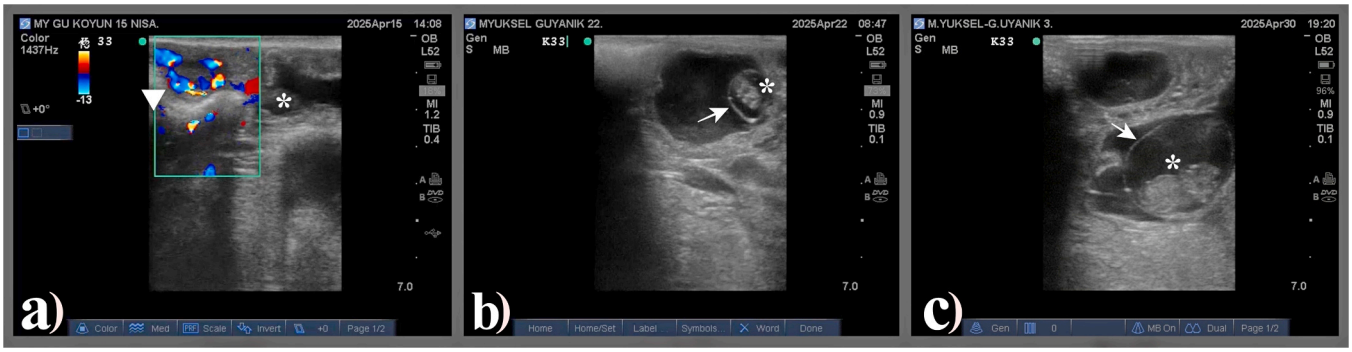


Fig. 3. Transrectal ultrasonographic images of pregnancy obtained on Days 21 (a), 28 (b), and 35 (c) after mating. The vascularized corpus luteum is marked with an arrow (→), the embryo with an asterisk (*), and the embryonic vesicle with an arrowhead (▲).

and NPV [Cn/ (Cn + Fn) × 100] were calculated. The effectiveness of the test in diagnosing pregnancy was compared by calculating the accuracy rates [(Cp + Cn)/ (Cp + Fp + Fn + Cn) × 100] (Safak et al., 2025). In addition, the agreement between the reference method, transrectal USG, and AOPT test was evaluated using the kappa test. Kappa coefficients were classified as minimal (≤0.20), weak (0.21–0.40), moderate (0.41–0.60), strong (0.61–0.80), and excellent (0.81–1.00) based on the level of agreement (McHugh, 2012).

Differences between PAG levels in groups determined according to pregnancy status were determined using the Mann-Whitney *U* test for each sampling day. After that, the receiver operator characteristics (ROC) analysis was used to determine a predictive threshold with PAG levels at Day 21, 28 and 35 for differentiation of the pregnancy status. ROC curves for detection of pregnancy status were obtained for the PAG levels at Day 21, 28 and 35. Sensitivity, specificity, 95 % confidence interval (CI) and area under the curve (AUC) were calculated. If the calculated *P*-values were less than 0.05, this was considered statistically significant. Statistical analyses were performed using Stata 15.1 statistical software (StataCorp, College Station, TX, USA).

Results

Diagnostic performance of AOPT

The diagnostic performance values of AOPT on days 21, 28, and 35 post-mating are summarized in Table 1.

Transrectal USG performed on the 21st day after mating revealed that 49 ewes (49.00 %) were pregnant and 51 ewes (51.00 %) were not pregnant. Forty-two positive pregnancies were detected with AOPT; 38 of them were correct and four were false. Of the 58 negative test results, 47 were correct and 11 were false. These results showed that the sensitivity of AOPT on the 21st day was 77.55 %, specificity was 92.15 %, accuracy was 85.00 %, PPV was 90.47 % and NPV was 81.03 %. According to these results, there was a strong agreement between transrectal USG and AOPT on day 21 (Kappa= 0.699; $P < 0.001$).

On the 28th day after mating, embryonic death was detected in four ewes (8.16 %) (between days 21 and 28) by transrectal ultrasonography, and as a result, the number of pregnant ewes decreased to 45 (45.00 %). AOPT analysis revealed that 48 ewes were pregnant and 52 were not. When the test results were compared with the transrectal USG findings, it was determined that five of the positive pregnancies (48) were false positives, and two of the negative pregnancies (52) were false negatives. AOPT results were positive in all ewes (n = 4) that experienced embryonic death, and this was the main cause of the false positives. According to these results, on day 28, the sensitivity of AOPT was 95.55 %, specificity was 90.90 %, accuracy was 93.00 %, PPV was 89.58 %, NPV was 96.15 %, and excellent agreement was found with transrectal USG (Kappa= 0.859; $P < 0.001$).

By day 35 post-mating, two additional embryonic deaths (4.44 %) (between days 28 and 35) were detected by transrectal USG, the number of pregnant ewes reduced to 43 (43.00 %). AOPT analysis revealed that 45 ewes were pregnant, while 55 were not. Comparison of the test results with the transrectal USG findings revealed only two false positive results, both corresponding to ewes experiencing embryonic death. Thus, on day 35, the sensitivity of AOPT was noticed to be 100.0 %, specificity was 96.49 %, accuracy was 98.00 %, PPV was 95.55 % and NPV was 100.0 %. As a result of these values, excellent agreement was found with transrectal USG (Kappa= 0.959; $P < 0.001$).

Table 1
The contingency, validity and reliability table for the evaluation of the diagnostic test characteristics of the Alertys OnFarm Pregnancy Test (AOPT) in determining pregnancy status 21, 28 and 35 days after mating, considering transrectal ultrasonography (USG) as the gold standard.

Parameters	Day of pregnancy		
	21st	28th	35th
Correct positive (Cp)	38	43	43
False positive (Fp)	4	5	2
Correct negative (Cn)	47	50	55
False negative (Fn)	11	2	0
Sensitivity (%)	77.55	95.55	100.00
Specificity (%)	92.15	90.90	96.49
Positive predictive value (%)	90.47	89.58	95.55
Negative predictive value (%)	81.03	96.15	100.00
Accuracy (%)	85.00	93.00	98.00

Sensitivity [Cp/(Cp + Fn)]. **Specificity** [Cn/(Cn + Fp)]. **PPV** [Cp/(Cp + Fp)]. **NPV** [Cn/(Cn + Fn)].
Accuracy [(Cp + Cn)/(Cp + Cn + Fp + Fn)]. **CI**: 95 % Confidence interval.

Plasma PAG concentrations

Plasma PAG (OD) levels were significantly higher in pregnant than in non-pregnant ewes at all time points ($P < 0.001$). On day 21 post-mating, mean PAG values in pregnant ewes were 0.43 ± 0.02 compared with 0.25 ± 0.01 in non-pregnant ewes. Levels increased to 0.85 ± 0.06 on day 28 and peaked at 1.65 ± 0.10 on day 35, while non-pregnant ewes remained consistently low (≤ 0.22) (Fig. 4). These mean PAG values were calculated after excluding ewes that experienced embryonic death to determine optimal diagnostic conditions.

The sensitivity, specificity, and AUC values for the diagnostic performance of plasma PAG concentrations in detecting pregnancy in ewes on days 21, 28, and 35 after mating are shown in Table 2 and Fig. 5. The optimal cut-off values for distinguishing between pregnant and non-pregnant ewes were determined to be > 0.28 ng/mL (day 21), > 0.48 ng/mL (day 28), and > 0.43 ng/mL (day 35). The corresponding sensitivity and specificity values were 91.84 % and 86.27 % on day 21, 93.33 % and 94.12 % on day 28, and 100.00 % for both on day 35. The respective AUC values were 0.941, 0.986, and 1.000, respectively, and were statistically significant ($P < 0.001$ for days 21 and 28; $P = 0.998$

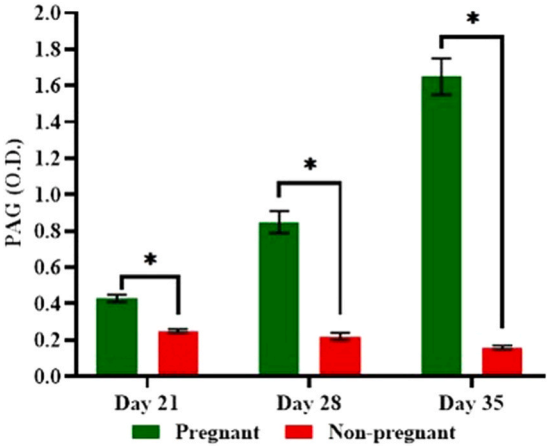


Fig. 4. The mean plasma concentration (O.D.) of pregnancy-associated glycoprotein (PAG) in pregnant and non-pregnant ewes 21, 28 and 35 days after mating. **There is a significant difference between the means of columns marked with an asterisk (*) ($P < 0.001$).*

Table 2
Receiver operating characteristic (ROC) curve analysis for the determination of optimal cut-off values and corresponding area under the curve (AUC) with 95 % confidence intervals (CI) of plasma pregnancy-associated glycoprotein (PAG) concentrations (OD) as a predictor of pregnancy status in ewes on days 21, 28, and 35 post-mating.

Days	Parameters				
	Threshold	Sensitivity (%95 CI)	Specificity (%95 CI)	AUC	*P*-value
21st	> 0.28	91.84 (80.4–97.7)	86.27 (73.7–94.3)	0.941	< 0.001
28th	> 0.48	93.33 (81.7–98.5)	94.12 (83.7–98.7)	0.986	< 0.001
35th	> 0.43	100.0 (91.7–100.0)	100.0 (93.4–100.0)	1	0.998

for day 35).

Comparison between plasma PAG levels and AOPT results

According to the AOPT results, individual plasma PAG levels (OD, mean ± SEM) of ewes were evaluated descriptively according to pregnancy day and diagnostic outcome, and are presented in Table 3. These values are shown to illustrate how correct and false test results relate to the underlying circulating PAG concentrations measured by ELISA, rather than to perform statistical comparisons between subgroups. On Day 21 of pregnancy, ewes yielding false positive results had PAG values (0.386 ± 0.031 OD) within the same general range as those of correctly diagnosed pregnant ewes (0.428 ± 0.023 OD), whereas animals with false negative results (0.295 ± 0.010 OD) showed PAG levels within the range observed in non-pregnant ewes (0.254 ± 0.007 OD). Similarly, on Day 28 after mating, false positive ewes exhibited PAG concentrations (0.567 ± 0.069 OD) that fell within the lower range of pregnant ewes (0.855 ± 0.058 OD), while remaining above the typical values observed in non-pregnant ewes (0.223 ± 0.021 OD). Likewise, on the 35th day of pregnancy, ewes with false positive results (0.663 ± 0.008 OD) had PAG levels higher than those of non-pregnant ewes (0.157 ± 0.015 OD).

Discussion

The present study evaluated the efficacy and diagnostic accuracy of AOPT, a PAG-based lateral flow test for early pregnancy detection in ewes under field conditions. Our findings showed that the diagnostic performance of AOPT increased progressively as pregnancy progressed from day 21 to day 35 after mating. AOPT achieved an accuracy rate of 85.00 % by demonstrating moderate sensitivity (77.55 %) and high specificity (92.15 %) on the 21st day after mating in ewes (Table 1). These findings reveal that there is strong agreement between AOPT and transrectal ultrasonography on day 21 post-mating (Kappa= 0.699; $P < 0.001$) and that pregnancy status can be accurately determined in the vast majority of ewes. However, 11 false negative results indicate

Table 3
The results of the Alertys OnFarm Pregnancy Test (AOPT) test administered on different days of pregnancy (21st day, 28th day, and 35th day) showed that, when compared to transrectal ultrasonography, the mean ± standard error of the mean (SEM) optical density (OD) of plasma pregnancy-associated glycoprotein (PAG) levels in the correct (Cp: correct positive, Cn: correct negative) and false (Fp: false positive, Fn: false negative) diagnosis groups obtained by transrectal ultrasonography (USG).

PAG level (Mean±SEM, OD)	AOPT Results			
	Cp	Cn	Fp	Fn
21st Day	0.428 ± 0.023 *(n = 38)*	0.254 ± 0.007 *(n = 47)*	0.386 ± 0.031 *(n = 4)*	0.295 ± 0.010 *(n = 11)*
28th Day	0.855 ± 0.058 *(n = 43)*	0.223 ± 0.021 *(n = 50)*	0.567 ± 0.069 *(n = 5)*	0.367 ± 0.015 *(n = 2)*
35th Day	1.652 ± 0.096 *(n = 43)*	0.157 ± 0.015 (n = 55)	0.663 ± 0.008 (n = 2)	-

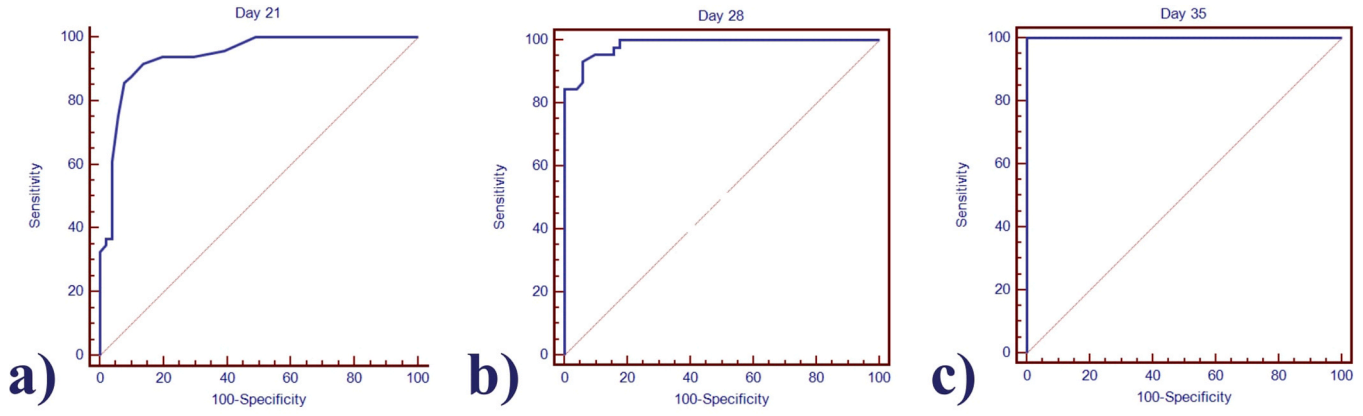


Fig. 5. Receiver operating characteristic (ROC) curves illustrating the diagnostic performance of plasma PAG concentrations for pregnancy detection in ewes on (a) Day 21, (b) Day 28, and (c) Day 35 after mating.

that some pregnant ewes cannot yet be reliably detected during this period. This situation may be due to the fact that low plasma PAG concentrations in the early stages of pregnancy in some ewes in our study population cannot be detected by AOPT. Indeed, in our study, the average plasma PAG level (0.295 ± 0.010 OD) of ewes with false negative results was remarkably lower than the level (0.428 ± 0.023 OD) of ewes with correct positive results, which supports this possibility (Table 3). On the other hand, Barbato et al. (2009) reported that pregnancy diagnosis is possible from the 18th day based on PAG concentrations in ewes; however, due to individual differences in PAG concentrations in pregnant ewes during this period, the highest accuracy can be achieved from the 24th day onwards. In line with our results, Steckeler et al. (2019) reported that the highest number of false negative results in PAG-based pregnancy test in ewes was observed on the 21st day after mating, but that these false negative results were minimised by the 35th day. The researchers explained this situation by the low PAG concentrations exhibited by pregnant ewes in the early stages, which increase as pregnancy progresses and thus reach detectable levels in test kits.

The sensitivity of AOPT increased significantly 28 days after mating, reaching 95.55 % and providing an overall accuracy of 93.00 %. During this period, only two false negative results were detected, which provided a significant improvement in NPV compared to day 21 (Table 1). This finding indicates that by the fourth week of pregnancy, the majority of pregnant ewes have reached a sufficient level of PAG concentration in the circulation (Rovani et al., 2015) and only a limited number of pregnancies cannot be detected at this stage. Consistent with our results, previous studies have reported that PAG-based pregnancy tests can detect pregnancy in ewes on day 28 post-mating with a sensitivity of 92.68–100.0 % (da Costa et al., 2017; Karen et al., 2003; Steckeler et al., 2019). However, although there was an increase in the sensitivity and NPV values of AOPT on day 28 compared to day 21, a slight decrease was observed in the specificity and PPV rates. This decrease was due to the five false positive results obtained by AOPT on day 28. When the five ewes with false positives were examined individually, it was determined that four of them were ewes that had experienced embryonic death between days 21 and 28. When the plasma PAG concentrations of these ewes were evaluated on day 28, the mean value was found to be 0.567 ± 0.069 OD, which was similar to that of pregnant ewes (0.855 ± 0.058 OD) (Table 3). This situation reveals that false positive results are caused by PAG levels remaining in maternal circulation after embryonic death. Indeed, it has been reported in numerous studies that PAGs secreted by trophoblasts can remain in the maternal circulation for approximately one week after embryonic death and may temporarily cause positive results despite pregnancy failure (Akköse, 2023; Andrade et al., 2025; Garbayo et al., 2008; Safak et al., 2025; Sousa et al., 2006). This finding reveals a known limitation of PAG-based tests and emphasises the need for careful interpretation of test results, especially in individuals who have recently experienced embryonic loss.

When the 35th day after mating was reached, AOPT achieved an almost perfect accuracy rate of 98.00 %, with 100.0 % sensitivity and 96.49 % specificity (Table 1). During this period, all previously undetected pregnancies were correctly identified, with only two false positive results observed. It was determined that all of these false positive results were due to embryonic deaths occurring between days 28 and 35. The findings indicate that AOPT can reliably confirm pregnancy in ewes approximately four weeks after mating and that its performance between days 28 and 35 was in excellent agreement with transrectal USG (Kappa= 0.859; 0.959; $P < 0.001$). The increase in NPV from 81.03 % on day 21–100.0 % on day 35 is particularly important in terms of reproductive management. This means that a negative AOPT result after approximately five weeks can indicate with almost complete certainty that the ewe is not pregnant.

Overall, our results are largely consistent with those reported by the test kit manufacturer and previous reports in cattle. In dairy cattle, Akköse et al. (2023) reported that AOPT provided 100.0 % sensitivity and 93.10 % specificity with 95.60 % accuracy between days 28 and 31 after insemination and showed excellent agreement with USG (Kappa= 0.907; $P < 0.001$). Similarly, Safak et al. (2025) found that USG showed significant agreement with AOPT in cows four weeks after insemination, with high sensitivity (92.40 %) and acceptable specificity (80.00 %) (Kappa= 0.735; $P < 0.001$). While these studies show that AOPT is effective in early pregnancy detection in cattle, to our knowledge, no study has been reported on its application in ewes. Therefore, it is not possible to directly compare our results. However, when compared with the results of similar PAG-based pregnancy tests used in ewes, our results show high consistency. The findings show that plasma PAG levels of pregnant ewes increase significantly from the 3rd week after mating, and the difference with non-pregnant ewes expands as pregnancy progresses (Fig. 4). This trend has been similarly reported in many previous studies, where PAG concentrations have been reported to differ significantly between pregnant and non-pregnant ewes as pregnancy progresses (Akköse et al., 2022; Akköse et al., 2024; De Carolis et al., 2020; El Amiri et al., 2007; El Amiri et al., 2015; Rovani et al., 2015; Steckeler et al., 2019). In the current study, the accuracy rate of pregnancy diagnosis based on PAG levels increased significantly as pregnancy day progressed (Table 2). Using the optimal cut-off value determined on day 21 (>0.28 OD), the test sensitivity was 91.8 % and its specificity was 86.3 %; On the 28th day (>0.48 OD), sensitivity increased to 93.3 % and specificity to 94.1 %. A more pronounced differentiation was observed on the 35th day of pregnancy, with a cut-off value of > 0.43 OD, resulting in both sensitivity and specificity of 100 % (Fig. 5). This suggests that PAG-based tests can be used for screening in the early stages, but the highest accuracy is achieved in the later weeks of pregnancy. Similarly, Steckeler et al. (2019) reported that pregnancies in ewes can be detected with high accuracy on day 35 after mating using the PAG-ELISA test. On the other hand, Rovani et al. (2015) reported that serum PAG concentrations in ewes exceeded the cut-off value from day 28 after mating and reached continuously detectable levels in all pregnant ewes on day 33. In addition, many researchers have reported that PAG concentrations clearly distinguish pregnant animals from those in the 4th to 5th weeks of pregnancy, and that diagnostic performance reaches its highest level during this period (Akköse et al., 2024; Barbato et al., 2009; Karen et al., 2003; Milisits-Németh et al., 2018; Rovani et al., 2015; Steckeler et al., 2019). da Costa et al. (2017) found that the visual PAG-ELISA test provided 100.0 % sensitivity, 93.80 % specificity, and 98.46 % accuracy on day 30, which closely matches our findings. Similarly, Rovani et al. (2015) reported that the PAG-ELISA test could detect pregnancies with 96.10 % accuracy, with 93.50 % sensitivity and 98.90 % specificity on day 33 of pregnancy, in a study evaluating the effectiveness of the PAG-ELISA test in ewes. Therefore, this study demonstrates once again that PAG can be detected early in the maternal circulation, but its diagnostic value increases significantly with the progression of pregnancy.

In addition to diagnostic accuracy, the real impact of any pregnancy diagnosis method in small ruminant production systems is determined by its economic feasibility, logistical requirements, and compatibility with routine farm conditions (Satıcı and Özyurtlu, 2024). In this context, the AOPT offers a highly practical alternative to USG, particularly in rural or resource-limited areas. While USG requires specialized equipment, trained personnel, and additional labor, the AOPT can be applied using only a few drops of whole blood with minimal operator training, providing important practical advantages. The main innovation of this test lies in its ability to work directly with whole blood, thereby eliminating the serum or plasma preparation and laboratory analysis steps required for conventional PAG-ELISA tests (Mayo et al., 2016).

Numerous alternative approaches have been investigated for early pregnancy diagnosis in small ruminants based on currently available biomarkers, including P_4, interferon-stimulated genes (ISG), miRNAs, and various metabolomic and proteomic markers (Karen et al., 2003; Zhai et al., 2023; Lazim et al., 2025; Gözer et al., 2025). However, each of these approaches has important limitations. P_4-based early pregnancy

diagnosis may yield false-positive results in cases such as persistent corpus luteum or luteal cysts (Ganaie et al., 2009). Moreover, the requirement to measure P_4 in serum or plasma rather than whole blood markedly reduces the practical applicability of this method under field conditions (Karen et al., 2003; Roberts et al., 2017). Similarly, ISG, miRNA, and metabolomic/proteomic-based approaches are not yet suitable for routine field application because they require advanced laboratory infrastructure and have not been sufficiently validated (Zhai et al., 2023; Lazim et al., 2025; Gözer et al., 2025). In contrast, PAGs, owing to their trophoblast origin and pregnancy-specific nature in ruminants, provide high diagnostic reliability; furthermore, their structural similarity among species allows the same tests to be used across different ruminant species (Xie et al., 1991; Mayo et al., 2016; Steckeler et al., 2019). In addition, the commercial availability of PAG-based tests and their validation in numerous field studies (Karen et al., 2015; Steckeler et al., 2019; Akköse, 2023; Safak et al., 2025) make these biomarkers a practical and reliable option for field applications.

From an economic point of view, the unit cost of transrectal USG in Türkiye is approximately 6–8 USD per ewe. However, this value may vary regionally depending on travel expenses, operator availability, and equipment depreciation. In contrast, the unit cost of the AOPT used in this study is approximately 10 USD per animal. At first glance, this suggests a modest direct cost advantage in favor of USG. Nevertheless, this simple comparison does not fully reflect the true economic context. USG requires expensive equipment, trained personnel, and longer examination times per animal (Gonzalez-Bulnes et al., 2010). All of these factors substantially increase indirect and fixed costs, particularly in extensive flocks or in rural areas. By contrast, the AOPT represents a method that requires minimal labor and capital investment, provides high throughput, offers a fixed and predictable cost per test, and allows a large number of animals to be screened rapidly under farm conditions (Safak et al., 2025). PAG-ELISA and other laboratory-based biomarker tests (P_4, miRNA etc.) may appear cheaper per test when only reagent costs are considered. However, these methods generate additional costs and logistical burdens related to sample collection, transportation, laboratory infrastructure, and the time required to obtain results. This considerably reduces their practicality and competitiveness under field conditions (Mayo et al., 2016; Akköse, 2023; Safak et al., 2025). In contrast, although the unit cost of the AOPT is higher than that of USG, the operational simplicity, time savings, and reduced labor requirements it provides under farm conditions make it, in many cases, a more rational option in terms of total application cost. Nevertheless, it should be noted that the AOPT has certain limitations. At very early stages of pregnancy, low circulating PAG concentrations may lead to false-negative results. Conversely, embryonic losses occurring at later stages may result in residual PAG remaining in the maternal circulation and potentially cause false-positive results. These limitations indicate that AOPT results, especially in early pregnancy, should be interpreted with caution.

Finally, the present study has several limitations that should be considered. First, the animals were not followed until parturition; therefore, final birth outcomes could not be used as a reference to confirm the early pregnancy diagnosis results. Although transrectal USG is a well-known and reliable reference method (Ganaie et al., 2009; Yadav et al., 2019; Satıcı and Özyurtlu, 2024), the lack of follow-up until parturition prevented the evaluation of possible fetal losses that may have occurred after the last examination. Second, the number of fetuses was not recorded in this study. Since it is known that PAG concentrations are influenced by fetal number (Wallace et al., 2015; Alkan et al., 2020), this prevented an assessment of whether the diagnostic performance of the AOPT varied according to litter size. On the other hand, a detailed cost-benefit analysis to reveal the true economic impact of the methods under field conditions was not performed in this study. Therefore, future studies are recommended to be designed not only to include follow-up of animals until parturition, recording of litter size, and direct comparison of diagnostic results with actual birth outcomes, but also to incorporate comprehensive economic analyses in which total application costs and operational benefits of diagnostic methods are evaluated together across different production systems. Such studies are expected to provide more comprehensive and applicable data regarding the field use of the AOPT in the early pregnancy diagnosis of ewes.

Conclusion

In conclusion, commercial AOPT developed for cattle appears to be an effective and practical tool for early pregnancy detection in ewes. AOPT, which enables fast and reliable pregnancy testing on the farm, especially from 28 days after mating, can contribute to producers making timely and informed reproductive management decisions. However, since false negatives due to insufficient PAG concentrations during early pregnancy and false positives due to embryonic deaths in later stages may occur, test results should be interpreted with caution. Therefore, repeated testing when necessary and the use of USG to confirm embryonic viability may be appropriate. Finally, further studies are needed to investigate the efficacy of AOPT in different production systems and larger study populations.

CRediT authorship contribution statement

Filiz Kara: Investigation. **Ahmet Gözer:** Writing – review & editing, Investigation. **Gökçek Ishak:** Writing – review & editing, Validation, Investigation. **Ebru Arslanhan:** Investigation. **Murat Abay:** Writing – review & editing, Resources, Methodology. **Murat Yüksel:** Writing – review & editing, Methodology, Investigation. **Gökhan Uyanık:** Writing – review & editing, Writing – original draft, Visualization, Resources, Methodology, Investigation, Data curation, Conceptualization. **Ufuk Kaya:** Writing – review & editing, Validation, Formal analysis.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this manuscript, the authors used machine-assisted translation tools (Google Translate and DeepL) solely to enhance linguistic clarity and readability. All content generated through these tools was carefully reviewed, revised, and validated by the authors. The scientific ideas, study design, data interpretation, and conclusions presented in this article were conceived and developed entirely by the authors. The authors assume full responsibility for the accuracy, integrity, and originality of the published work.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We would like to thank the students of the Faculty of Veterinary Medicine at Hatay Mustafa Kemal University and Mahmut Yayar, owner of the livestock farm, for their generous assistance during the fieldwork. We are also grateful to Diagen Biotechnological Systems Co. for their valuable support and assistance throughout the analysis processes.

Data availability

The data supporting the findings of this study are contained within the article. Additional information related to the datasets is available

from the corresponding author upon reasonable request.

References

Adeyeye, A.A., Abubakar, Yu.U., Leigh, O.O., Ate, I.U., Stephen, J., Raheem, K.A., Ubah, S.A., 2021. Pregnancy-specific protein B in Yankasa ewes during pregnancy and postpartum periods. Macedonian Veterinary Review 44, 55–62. https://doi.org/10.2478/macvetrev-2021-0010.

Akbulut, N.K., Çelik, H.A., 2021. Differences in mean grey levels of uterine ultrasonographic images between non-pregnant and pregnant ewes may serve as a tool for early pregnancy diagnosis. *Animal Reproduction Science* 226, 106716. https://doi.org/10.1016/j.anireprosci.2021.106716.

Akköse, M., 2020. Evaluation of a bovine rapid visual PAG ELISA test and transabdominal ultrasonography for early pregnancy diagnosis in Awassi sheep. *KSU Journal of Agriculture* and Nature 23, 1366–1372. https://doi.org/10.18016/ksutarimdoga.vi.668707.

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.

Akköse, M., Çebi-Şen, C., Polat, Y., 2022. The prediction of lambing rate in Awassi sheep using a rapid visual PAG ELISA test. Journal of the Hellenic Veterinary Medical Society 73, 4303–4308. https://doi.org/10.12681/jhvms.26024.

Akköse, M., Çınar, E.M., Yazlık, M.O., Kaya, U., Polat, Y., Çebi, Ç., Özbeyaz, C., Vural, M. R., 2024. Serum pregnancy–associated glycoprotein profiles during early gestation in Karya and Konya Merino sheep. *Veterinary Medicine* and *Science* 10, e1345. https://doi.org/10.1002/vms3.1345.

Ali, A., Derar, D.R., Abdel-Razek, A.-R.K., 2024. Ultrasonography for the detection of pregnancy and study of embryonic and fetal development in camels, buffaloes, and sheep: techniques, equations, and limitations. *Animal Reproduction Science* 268, 107566. https://doi.org/10.1016/j.anireprosci.2024.107566.

Alkan, H., Kivrak, M., Satilmis, F., Tekindal, M., Dinc, D., 2020. Detection of twin pregnancies in ewes by pregnancy-associated glycoprotein assay and transabdominal ultrasonography. *Domestic Animal Endocrinology* 72, 106399. https://doi.org/10.1016/j.domaniend.2019.106399.

Allabban, M., Erdem, H., 2020. Koyunlarda real-time ultrasonografik muayene ile gebelik tanısı. Journal of Bahri Dagdas Animal Research 9, 47–55.

Andrade, J.P.N., Domingues, R.R., Gomez-León, V.E., Madureira, G., Sartori, L.C., Grillo, G.F., Fosado, M., Sala, R.V., Wiltbank, M.C., 2025. Delayed embryonic attachment, circulating pregnancy-associated glycoproteins, and pregnancy loss in heifer embryo recipients. Theriogenology 247, 117561. https://doi.org/10.1016/j.theriogenology.2025.117561.

Arashiro, E.K.N., Ungerfeld, R., Clariget, R.P., Pinto, P.H.N., Balaro, M.F.A., Bragança, G. M., Ribeiro, L.S., da, Fonseca, J.F., Brandão, F.Z., 2018. Early pregnancy diagnosis in ewes by subjective assessment of luteal vascularisation using colour Doppler ultrasonography. Theriogenology 106, 247–252. https://doi.org/10.1016/j.theriogenology.2017.10.029.

Barbato, O., Sousa, N., Debenedetti, A., Canali, C., Todini, L., Beckers, J.-F., 2009. Validation of a new pregnancy-associated glycoprotein radioimmunoassay method for the detection of early pregnancy in ewes. Theriogenology 72, 993–1000. https://doi.org/10.1016/j.theriogenology.2009.06.020.

Bossuyt, P.M., Reitsma, J.B., Bruns, D.E., Gatsonis, C.A., Glasziou, P.P., Irwig, L., Lijmer, J.G., Moher, D., Rennie, D., De Vet, H.C., 2015. STARD 2015: an updated list of essential items for reporting diagnostic accuracy studies. Radiology 277, 826–832. https://doi.org/10.1148/radiol.2015151516.

Crilly, J., Politis, A., Hamer, K., 2017. Use of ultrasonographic examination in sheep veterinary practice. *Small Ruminant* Research 152, 166–173. https://doi.org/10.1016/j.smallrumres.2016.12.021.

da Costa, R.L.D., Duarte, K.M.R., Machado, D.C., de Paz, C.C.P., Beltrame, R.T., 2017. Visual ELISA for detection of pregnancy-associated glycoproteins (PAGs) in ewe serum. Theriogenology 97, 78–82. https://doi.org/10.1016/j.theriogenology.2017.04.026.

De Carolis, M., Barbato, O., Acuti, G., Trabalza-Marinucci, M., Melo de Sousa, N., Canali, C., Moscati, L., 2020. Plasmatic profile of pregnancy-associated glycoprotein (PAG) during gestation and postpartum in sarda and lacaune sheep determined with two radioimmunoassay systems. Animals 10, 1502. https://doi.org/10.3390/ani10091502.

El Amiri, B., Karen, A., Sulon, J., Melo de Sousa, N., Alvarez-Oxiley, A., Cognie, Y., Szenci, O., Beckers, J.-F., 2007. Measurement of ovine pregnancy-associated glycoprotein (PAG) during early pregnancy in Lacaune sheep. *Reproduction in Domestic Animals* 42, 257–262. https://doi.org/10.1111/j.1439-0531.2006.00761.x.

El Amiri, B., Sousa, N., Oxiley, A.A., Hadarbach, D., Beckers, J.-F., 2015. Pregnancy-associated glycoprotein (PAG) concentration in plasma and milk samples for early pregnancy diagnosis in Lacaune dairy sheep. *Research in Veterinary Science* 99, 30–36. https://doi.org/10.1016/j.rvsc.2014.12.016.

Erdem, H., Alkan, H., Kıvrak, M.B., Satılmış, F., Alabban, M., 2020. İvesi irkı koyunlarda gebelik muayenesi amacıyla dorsal transrektal ve transabdominal yolla yapılan ultrasonografik muayenenin etkinliği. Journal of Bahri Dagdas Animal Research 9, 7–12.

Ganaie, B.A., Khan, M.Z., Islam, R., Makhdoomi, D.M., Qureshi, S., Wani, G.M., 2009. Evaluation of different techniques for pregnancy diagnosis in sheep. *Small Ruminant* Research 85, 135–141. https://doi.org/10.1016/j.smallrumres.2009.09.003.

Garbayo, J.M., Serrano, B., Lopez-Gatius, F., 2008. Identification of novel pregnancy-associated glycoproteins (PAG) expressed by the peri-implantation conceptus of domestic ruminants. *Animal Reproduction Science* 103, 120–134. https://doi.org/10.1016/j.anireprosci.2006.12.002.

Goldansaz, S.A., Markus, S., Plastow, G., Wishart, D.S., 2022. Predictive blood biomarkers of sheep pregnancy and litter size. Scientific Reports 12, 10307. https://doi.org/10.1038/s41598-022-14141-w.

Gonzalez-Bulnes, A., Pallares, P., Vázquez, M.I., 2010. Ultrasonographic imaging in small ruminant reproduction. *Reproduction in Domestic Animals* 45, 9–20. https://doi.org/10.1111/j.1439-0531.2010.01640.x.

Gözer, A., Uyanık, G., Keçeli, H.H., Kaya, U., Özkan, H., 2025. Upregulation of miR-125b and miR-23b in plasma might be related with the early pregnancy in Awassi Ewes. *Small Ruminant* Research 252, 107579. https://doi.org/10.1016/j.smallrumres.2025.107579.

Jones, A.K., Reed, S.A., 2017. Benefits of ultrasound scanning during gestation in the small ruminant. *Small Ruminant* Research 149, 163–171. https://doi.org/10.1016/j.smallrumres.2017.02.008.

Karen, A., Beckers, J.-F., Sulon, J., de Sousa, N.M., Szabados, K., Reczigel, J., Szenci, O., 2003. Early pregnancy diagnosis in sheep by progesterone and pregnancy-associated glycoprotein tests. Theriogenology 59, 1941–1948. https://doi.org/10.1016/S0093-691X(02)01289-X.

Karen, A., De Sousa, N.M., Beckers, J.-F., Bajcsy, Á.C., Tibold, J., Mádl, I., Szenci, O., 2015. Comparison of a commercial bovine pregnancy-associated glycoprotein ELISA test and a pregnancy-associated glycoprotein radiomimmunoassay test for early pregnancy diagnosis in dairy cattle. *Animal Reproduction Science* 159, 31–37. https://doi.org/10.1016/j.anireprosci.2015.05.005.

Karen, A., Samir, H., Ashmawy, T., El-Sayed, M., 2014. Accuracy of B-mode ultrasonography for diagnosing pregnancy and determination of fetal numbers in different breeds of goats. *Animal Reproduction Science* 147, 25–31. https://doi.org/10.1016/j.anireprosci.2014.03.014.

Kaulfuss, K.-H., May, J., Süss, R., Moog, U., 1997. In vivo diagnosis of embryo mortality in sheep by real-time ultrasound. *Small Ruminant* Research 24, 141–145. https://doi.org/10.1016/S0921-4488(96)00939-X.

Kline, A.C., Menegatti Zoca, S., Epperson, K.M., Quail, L.K., Ketchum, J.N., Andrews, T. N., Rich, J.J., Rhoades, J.R., Walker, J.A., Perry, G.A., 2024. Evaluation of pregnancy associated glycoproteins assays for on farm determination of pregnancy status in beef cattle. Plos One 19, e0306325. https://doi.org/10.1371/journal.pone.0306325.

Lazim, E.H., Hussein, K.A., Aziz, D.M., 2025. The efficiency of interferon stimulated gene 15 expression in peripheral blood for early pregnancy prediction in Awassi ewes. *Iraqi Journal of Veterinary Sciences* 39, 169–176. https://doi.org/10.33899/ijvs.2025.155210.4021.

Mayo, L., Moore, S., Poock, S., Silvia, W., Lucy, M., 2016. Validation of a chemical pregnancy test in dairy cows that uses whole blood, shortened incubation times, and visual readout. *The Journal of Dairy Science* 99, 7634–7641. https://doi.org/10.3168/jds.2016-11224.

McHugh, M.L., 2012. Interrater reliability: the kappa statistic. Biochemia Medica 22 276–282.

Milisits-Németh, T., Balogh, O.G., Egerszegi, I., Kern, L., Sasser, R.G., Gábor, G., 2018. Detection of pregnancy in sheep using an ELISA for pregnancy-specific protein B. Acta Veterinaria Hungarica 66, 329–336. https://doi.org/10.1556/004.2018.029.

Moussafir, Z., Allai, L., El Khalil, K., Essamadi, A., El Amiri, B., 2018. Could a bovine pregnancy rapid test be an alternative to a commercial pregnancy-associated glycoprotein ELISA test in dairy cattle? *Animal Reproduction Science* 192, 78–83. https://doi.org/10.1016/j.anireprosci.2018.02.016.

Northrop, E.J., Rich, J.J., Rhoades, J.R., Perry, G.A., 2019. Comparison of two bovine serum pregnancy tests in detection of artificial insemination pregnancies and pregnancy loss in beef cattle. PloS One 14, e0211179. https://doi.org/10.1371/journal.pone.0211179.

Ott, T., Dechow, C., O'connor, M., 2018. Advances in reproductive management: pregnancy diagnosis in ruminants. *Animal Reproduction* 11, 207–216.

Pickworth, C.L., Sheffield, J., Poole, R.K., 2020. Pregnancy specific protein B concentration for the prediction of lambing rate in sheep. *Small Ruminant* Research 184, 106052. https://doi.org/10.1016/j.smallrumres.2020.106052.

Redden, R., Passavant, C., 2013. Efficacy of pregnancy-specific protein B assay to detect pregnancy and lambing rates in sheep. *Sheep* and *Goat Research Journal* 23, 21–24.

Rial, C., Hussain, I., Hoff, R., Tompkins, S., Erickson, D., Branen, J., Giordano, J., 2024. Development and evaluation of a lateral flow-based portable optical system for determination of the pregnancy status of dairy cows. *The Journal of Dairy Science* 107, 8642–8653. https://doi.org/10.3168/jds.2024-24899.

Rickard, J.P., Ryan, G., Hall, E., de Graaf, S.P., Hermes, R., 2017. Using transrectal ultrasound to examine the effect of exogenous progesterone on early embryonic loss in sheep. PloS One 12, e0183659. https://doi.org/10.1371/journal.pone.0183659.

Roberts, J., May, K., Ajani, O., Kaneene, J., 2019. A comparison of pregnancy diagnosis methods in commercial sheep using lambing as a gold standard. Clinical Theriogenology 11, 107–113.

Roberts, J., May, K., Veiga-Lopez, A., 2017. Time-dependent changes in pregnancy-associated glycoproteins and progesterone in commercial crossbred sheep. Theriogenology 89, 271–279. https://doi.org/10.1016/j.theriogenology.2016.10.029.

Romano, J., Christians, C., 2008. Early pregnancy diagnosis by transrectal ultrasonography in ewes. *Small Ruminant* Research 77, 51–57. https://doi.org/10.1016/j.smallrumres.2008.02.004.

Rovani, M.T., Cezar, A.S., Rigo, M.L., Gasperin, B.G., Nóbrega, J.Ed, Torres, F.D., Gonçalves, P.B.D., Ferreira, R., 2015. Evaluation of a bovine pregnancy-associated glycoprotein enzyme-linked immunosorbent assay kit for serological diagnosis of pregnancy in sheep. Ciência Rural 46, 362–367. https://doi.org/10.1590/0103-8478cr20150270.

Safak, T., Tümer, K.Ç., Alp, Y.İ., Özen, T.Y., Yilmaz-Koc, O., 2025. Evaluation of the accuracy and performance of two commercial pregnancy-associated glycoprotein

tests for early pregnancy detection in cows. *Veterinary Medicine* and *Science* 11, e70226. https://doi.org/10.1002/vms3.70226.

Satıcı, E., Özyurtlu, N., 2024. Koyunlarda Gebelik Tanı Yöntemleri ve Gebelik Sürecinin Düzenlenmesinde Rol Oynayan miRNA'ların Fonksiyonları. *ISPEC Journal of Science Institute* 3, 53–59. https://doi.org/10.5281/zenodo.13373622.

Sousa, N., Ayad, A., Beckers, J., Gajewski, Z., 2006. Pregnancy-associated glycoproteins (PAG) as pregnancy markers in the ruminants. *Journal of Physiology* and *Pharmacology* 57, 153–171.

Steckeler, P., Weber, F., Zerbe, H., Rieger, A., Voigt, K., 2019. Evaluation of a bovine visual pregnancy test for the detection of pregnancy-associated glycoproteins in sheep. *Reproduction in Domestic Animals* 54, 280–288. https://doi.org/10.1111/rda.13356.

Szenci, O., 2021. Recent possibilities for the diagnosis of early pregnancy and embryonic mortality in dairy cows. Animals 11, 1666. https://doi.org/10.3390/ani11061666.

Uyanık, G., Abay, M., Demiral, Ö.O., 2024. Effect of post-mating GnRH or TAK-683 (Kisspeptin analog) treatment on the reproductive performance and serum progesterone concentration in ewes during the non-breeding season. *Small Ruminant* Research 238, 107331. https://doi.org/10.1016/j.smallrumres.2024.107331.

Wallace, R.M., Pohler, K.G., Smith, M.F., Green, J.A., 2015. Placental PAGs: gene origins, expression patterns, and use as markers of pregnancy. Reproduction 149 (3), R115–R126. https://doi.org/10.1530/REP-14-0485.

Xie, S., Low, B.G., Nagel, R.J., Kramer, K.K., Anthony, R.V., Zoli, A.P., Beckers, J.-F., Roberts, R.M., 1991. Identification of the major pregnancy-specific antigens of cattle and sheep as inactive members of the aspartic proteinase family. Proceedings of the National Academy of Sciences 88, 10247–10251. https://doi.org/10.1073/pnas.88.22.10247.

Yadav, V., Chandolia, R., Bisla, A., Saini, G., Dutt, R., Ranga, L., 2019. Accuracy of real-time B-mode trans-rectal ultrasonography for early pregnancy diagnosis in crossbred ewes. *International Journal of Livestock Research* 9, 170–178. https://doi.org/10.5455/ijlr.20190821041723.

Zhai, Y., Xia, F., Shi, L., Ma, W., Lv, X., Sun, W., Zhang, L., 2023. Early pregnancy markers in the serum of ewes identified via proteomic and metabolomic analyses. *International Journal of Molecular* Sciences 24, 14054. https://doi.org/10.3390/ijms241814054.