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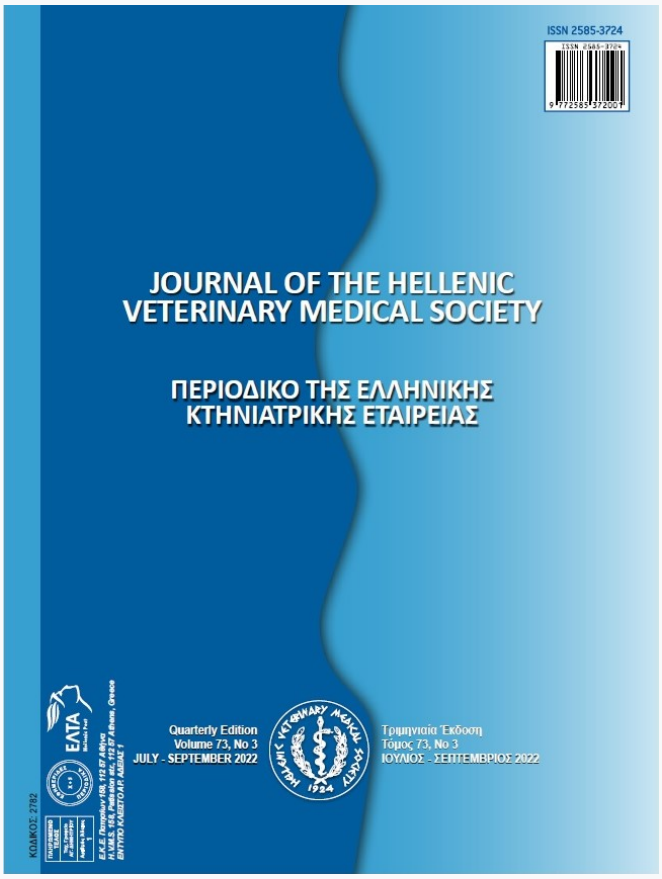


# The prediction of lambing rate in Awassi sheep using a rapid visual PAG ELISA test

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Research article
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# The prediction of lambing rate in Awassi sheep using a rapid visual PAG ELISA test

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**ABSTRACT:** The accurrate diagnosis of pregnancy and prediction of lambing rate are important tools that ensure the proper nutrition of pregnant ewes, optimize the birth weight of offspring and decrease the occurrence of pregnancy toxemia in sheep production.The aim of this study was to evaluate the reliability of the rapid visual PAG ELISA test in predicting the lambing rate, when performed in two different stages of pregnancy in Awassi sheep. A total of 210 ewes, belonging to a commercial flock, were assigned to two groups according to their mating date. Group 1 comprised of 120 ewes, which were in the period from 28 to 38 days post-mating, and Group 2 comprised of 90 ewes, which were in the period from 40 to 59 days post-mating. The rapid visual PAG ELISA test was performed on serum samples of the ewes, according to the manufacturer's instructions. The diagnostic test characteristics of the rapid visual PAG ELISA test were assessed for the prediction of lambing rate, using the lambing records as a gold standard.The sensitivity, specificity, positive predictive value, negative predictive value, and accuracy of the rapid visual PAG ELISA test were 95.79%, 52.38%, 90.10%, 73.33%, and 87.93%, respectively, in G1 and 92.75%, 70.00%, 91.43%, 73.68%, and 87.64%, respectively, in G2.While the rapid visual PAG ELISA test predicted (falce positive + true positive) that 87.1% of the ewes exposed to rams in G1 would lamb, in reality it achieved (true positive) that 78.5% of them lambed. The rapid visual PAG ELISA test predicted that 77.5% of the ewes exposed to rams in G2 would lamb, in reality it achieved that 71.9% of them lambed. The McNemar analysis showed that there was no statistically significant difference between the rapid visual PAG ELISA test and lambing records in the two groups.The differences between predicted and achieved lambing rate by the rapid visual PAG ELISA indicate embryonic mortality. The results of this study indicated that the lambing rate could be predicted with a high sensitivity from the 28$^{th}$ day of pregnancy, using the rapid visual PAG ELISA test.

**Keywords:** lambing rate, pregnancy, pregnancy-associated glycoproteins, sheep.

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## INTRODUCTION

The accurate determination of the pregnancy status and lambing rate of ewes offers various management options and increases reproductive success and profitability. The culling of non-pregnant ewes and proper feeding of pregnant ewes reduce feeding costs, the risk of pregnancy toxemia and lamb losses due to dystocia, and optimize the birth weight of the offspring (Pickworth et al., 2020; Redden and Passavant, 2013).

Pregnancy-associated glycoproteins (PAG) aresynthesized in the mono- and binucleate cells of the ruminant's trophectoderm (Sousa et al., 2006). PAG can be used for pregnancy diagnosis in ruminants by detecting their presence or concentrations in the maternal blood circulation (Akköse and Cebi-Sen, 2019; Chaves et al., 2017; Silva et al., 2007; Singh et al., 2021). de Carolis et al. (2020), reported that the cut-off value of PAG concentrations (radioimmuno assay, RIA-706 and RIA-srPool) are ≥ 1.0 ng/mL in order to distinguish between pregnant and nonpregnant Sarda and Lacaune ewes. While the blood and milk concentrations of PAG were initially determined by the RIA method, in the last two decades, practical and reliable PAG ELISA (enzyme-linked immunosorbent assay) pregnancy test kits have been developed, which can be used from the 28$^{th}$ day post-artificial insemination (AI) in cattle (El Amiri et al., 2014; Green et al., 2009; Moussafir et al., 2018; Mayo et al., 2016). It has been reported that PAG ELISA tests developed for cattle can also be used in sheep owing to the high similarity of the molecular structure of PAG in cattle and sheep (Chaves et al., 2020; Roberts et al., 2019). In a recent study, it has been reported that the rapid visual PAG ELISA test could be used with 100% sensitivity and specificity from the 26$^{th}$ day of pregnancy onwards (Chaves et al., 2020). Furthermore, it has been indicated that the rapid visual PAG ELISA test provides a diagnostic performance similar to that of ultrasonography in sheep (Akköse, 2020; Roberts et al., 2019).

The objective of this study was to determine the usability of the bovine rapid visual PAG ELISA test in estimating the lambing rate in sheep, when performed at different stages of pregnancy (28-38 days and 40-59 days).

## MATERIALS AND METHODS

This study was approved by the Local Ethics Committee for Animal Experiments of Harran University.

### Animals

This study was carried out between June-December 2019, in 210 Awassi sheep,which were aged 2-5 years, weighed 40-60 kg, and belonged to a commercial flock raised in the Sanliurfa province in the Southeastern Anatolia Region. The sheep were assigned to two groups. The first group (G1) included 120 ewes, which were in the period between 28-38 days post-mating, and the second group (G2) comprised 90 ewes, which were in the period between 40-59 days post-mating.The stages of pregnancy were choosed based on the PAG or pregnancy specific protein B (PSPB) profiles reported in previous studies for other breeds because of the serum PAG profile in pregnant Awassi sheep was not known at the time of methodology design in the current study (Roberts et al., 2017; Redden and Passavant, 2013). All ewes were grazed on pasture (wheat stubble) during summer.

Estrus detection was performed twice a day (in the morning and evening) using teaser rams. Sheep were mated by hand-mating system. The sheep enrolled in this study were separated from rams during the anoestrus season (from lambing to breeding season, approximately 6 months), and had not mated before the breeding season. The mating and lambing records of all ewes were maintained in the herd management system of the holding.

### Blood collection and implementation of the rapid visual PAG ELISA test

Blood samples were collected by jugular venipuncture into 9-ml plain vacutainer tubes and allowed to clot at room temperature. Sera were extracted by centrifugation at 3000 x g for 15 minutes.

The rapid visual PAG ELISA test (IDEXX Rapid Visual Pregnancy Test, Westbrook, ME) was performed in accordance with the manufacturer's instructions. Briefly, 100-µl serum samples and assay controls (positive and negative) were loaded into anti-PAG-coated wells. Incubations were performed at room temperature and each incubation period lasted 7 minutes after three washings in distilled water. After the test was completed, the pregnancy status of the sheep was determined by comparing the color of the test wells with that of the positive and negative controls. When the test is complete, the positive control turns blue, and the negative control remains transparent. Accordingly, test wells bluer than the negative control were assessed as "positive (pregnant)", and wells of the same color with or more transparent

than the negative control were assessed as "negative (non-pregnant)".

**Statistical analysis**

Lambing results were used as a gold standard to test the accuracy of the rapid visual PAG ELISA test in pregnancy diagnosis ( Roberts et al., 2019). The lambing rate was calculated as the percentage ratio of the number of ewes that lambed to the number of ewes exposed to rams (Lambing rate: [number of lambed ewes/total number of mated ewes]x100) (Kridli et al., 2006; Zarkawi and Al Daker, 2018). The truenegative [a], false positive [b], false negative [c], and truepositive [d] results of the rapid visual PAG ELISA test were tabulated on a 2X2 contingency table.Subsequently, the diagnostic test characteristics of the rapid visual PAG ELISA test were calculated for each pregnancy period. These include sensitivity [d/(d+c)x100], specificity [a/(a+b)x100], positive predictive value (PPV) [d/(d+b)x100], negative predictive value (NPV) [a/(a+c)x100], accuracy [(a+d)/(a+b+d+c)x100], positive likelihood ratio [sensitivity]/(1-specificity)], negative likelihood ratio [(1-sensitivity)/specificity], and odds ratio [sensitivity/(1-sensitivity)]/[(1-specificity)/specificity)] (Timsit et al., 2018).Predicted lambing rate with PAG ELISA =(b+d)/(a+b+c+d) x 100; achieved lambing rate with PAG ELISA = (d/(a+b+c+d) x 100). McNemar test was used to compare the rapid visual PAG ELISA test with the lambing results (Roberts et al., 2019). Statistical calculations were performed using version 24.0 of the IBM Statistical Package for Social Sciences (SPSS) software. The statistical significance level was set at $p < 0.05$.

## RESULTS

**Lambing records and performance of rapid visual PAG ELISA**

During the course of the study, four ewes from the first group and one ewe from the second group were excluded from the flock due to health problems, and data were analysed over the remaining animals.

According to the lambing results, in the first group (G1), 95 ewes lambed (81.9%) and 21 ewes did not lamb (18.1%). In the second group (G2), 69 ewes lambed (77.5%), and 20 ewes did not lamb (22.5%). Furthermore, while 164 out of the 205 ewes that were exposed to rams lambed (80%), 41 did not lamb (20%).A bortion did not occur in the flock.To evaluate the pregnancy outcomes of each period of pregnancy diagnosis in the ewes, a 2X2 contingency table was constructed (Table 1) and the test characteristics were calculated (Table 2). In G1, 10 ewes were diagnosed false positive and four ewes were diagnosed false negative, whilst in G2, six ewes were diagnosed false positive and five ewes were diagnosed false negative.

In G1, 81.9% of the ewes exposed to rams lambed. While the rapid visual PAG ELISAtest predicted that 87.1% of the ewes exposed to rams would lamb, in reality 78.5% of them lambed. In G2, 77.5% of the ewes exposed to rams lambed. While the rapid visual PAG ELISA test predicted that 78.7% of the ewes exposed to rams would lamb, in reality 71.9% of them lambed. Overall (G1+G2), 80%of the ewes exposed to rams lambed. While the rapid visual PAG ELISA test predicted that 83.4% of the ewes exposed to rams would lamb, in reality 75.6% of them lambed.These results are presented in Table 3. The McNemar test revealed that no difference was determined between the results of the rapid visual PAG ELISA test and the lambing results in G1 and G2.

**Table 1.** A 2x2 contingency table for the rapid visual PAG ELISA test performed in two different periods of pregnancy

| | | Rapid Visual PAG ELISA | | | | | |
|---|---|---|---|---|---|---|---|
| | | 28 - 38 days | | | 40 - 59 days | | |
| | | Non-pregnant (will not lamb) | Pregnant (will lamb) | Total | Non-pregnant (will not lamb) | Pregnant (will lamb) | Total |
| Lambing records | Did not lamb | 11 (tn) | 10 (fp) | 21 | 14 (tn) | 6 (fp) | 20 |
| | Lambed | 4 (fn) | 91 (tp) | 95 | 5 (fn) | 64 (tp) | 69 |
| | Total | 15 | 101 | 116 | 19 | 70 | 89 |

tn: truenegative; tp: truepositive; fn: false negative; fp: false positive

**Table 2.** Sensitivity, specificity, positive predictive value, negative predictive value, accuracy, positive likelihood ratio, and negative likelihood ratio of the rapid visual PAG ELISA test performed in two different periods of pregnancy

| | Rapid Visual PAG ELISA | | |
|---|---|---|---|
| | 28 - 38 d | 40 - 59 d | Overall |
| Sensitivity (%) | 95.79 | 92.75 | 94.51 |
| Specificity (%) | 52.38 | 70.00 | 60,98 |
| Positive predictive value (%) | 90.10 | 91.43 | 90.64 |
| Negative predictive value (%) | 73.33 | 73.68 | 73.52 |
| Accuracy (%) | 87,93 | 87.64 | 87.80 |
| Likelihood ratio + (LR+) | 2.01 | 3.09 | 2.42 |
| Likelihood ratio - (LR-) | 0.08 | 0.10 | 0.09 |
| Odds ratio | 1.030 | 1.026 | 1.028 |

**Table 3.** The lambing rates predicted and achieved with the rapid visual PAG ELISA test

| | Breeding ewes | Lambing rate | Predicted lambing rate with PAG ELISA | Achieved lambing rate with PAG ELISA | Difference between the lambing rate and the achieved lambing rate with PAG ELISA | Difference between the lambing rates predicted and achieved with PAG ELISA |
|---|---|---|---|---|---|---|
| | n | % | % | % | % | % |
| G1 | 116 | 81.9 (n=95) | 87.1 (n=101) | 78.5 (n=91) | 3.4 (n=4) | 8.6 (n=10) |
| G2 | 89 | 77.5 (n=69) | 78.7 (n=70) | 71.9 (n=64) | 5.6 (n=5) | 6.8 (n=6) |
| Overall | 205 | 80 (n=164) | 83.4 (n=171) | 75.6 (n=155) | 4.4 (n=9) | 7.8 (n=16) |

Predicted lambing rate with PAG ELISA = (b+d) / (a+b+c+d) x 100
Achieved lambing rate with PAG ELISA = (d / (a+b+c+d) x 100
a: true negative, b: false positive, c: false negative, and d: true positive

## DISCUSSION

In the present study, the reliability of the rapid visual PAG ELISA test in predicting the lambing rate was compared for two different periods of pregnancy. Identifying pregnant and non-pregnant ewes is important in terms of predicting the lambing rate and providing various management options. Nutritional requirements are shown significantly different among the non pregnant and pregnant ewes (National Research Council, 2007). Thereby, pregnant ewes can be fed properly and problems caused by metabolic diseases during pregnancy can be reduced (Pickworth et al., 2020; Redden and Passavant, 2013).

Sensitivity refers to the probability of a pregnancy diagnostic test identifying pregnant animals.In other words, it refers to how many of the actually pregnant animals the pregnancy diagnostic test is able to determine. In the present study, the sensitivity of the visual PAG ELISA test referred to the probability of the test detecting the ewes that would lamb. The sensitivity of the rapid visual PAG ELISA test was found to be similar in both periods of pregnancy (28-38 days: 95.79% versus 40-59 days: 92.75%). The sensitivity results obtained in the present study are similar to those obtained with other PAG assays (rapid visual pregnancy test, visual pregnancy test, bovine pregnancy test) investigated in previous studies (Chaves et al., 2017; Chaves et al., 2020; Roberts et al., 2019; Rovani et al., 2016; Steckeler et al., 2018). It could be said that the concentration of circulating PAG reached a detectable level in ewes defined as pregnant by the assay, but circulating PAG concentrations did not reach a detectable level in ewes that could not be identified as pregnant (but were actually pregnant).

Specificity refers to the possibility of a pregnancy diagnostic test identifying non-pregnant animals. In other words, it refers to how many of the actually non-pregnant animals the pregnancy diagnosis test is able to determine. In the present study, the specificity of the rapid visual PAG ELISA test referred to the probability of the test detecting the sheep that would not lamb. The specificity of the rapid visual PAG ELISA test was lower than that reported in previous studies ( Chaves et al., 2017; Chaves et al., 2020; Roberts et al., 2019; Rovani et al., 2016; Steckeler et al., 2018).An interesting result obtained in the present study is that the specificity of the visual PAG ELISA test was lower in G1 compared to G2 (52.38% and 70%, respectively). The majority of embryonic deaths occur in early pregnancy (Ataman et al., 2013; Chun-

dekkad et al., 2020) and 8.1% of embryos have been documented to be lost from 25 to 65 days of pregnancy (3.7% from 25 to 45 days; 4.3% from 45 to 65 days) (Dixon et al., 2007). This may cause false positive results for the rapid visual PAG ELISA test. Embryonic deaths could be the most important reason of the high number of non-lambing ewes the rapid visual PAG ELISA test could not identify in this study. The half-life of PAG is 6.3 days in Sarda ewes, 6.9 days in Lacaune ewes (de Carolis et al., 2020), 4.5 days in Texel x Norwegian crossbred ewes (Haugejorden et al., 2006). These differences could be due to different PAG produced by local sheep breeds, and assays used. Plasma PAG concentrations decrease within around 4 weeks postpartum (Haugejorden et al., 2006). The detection of PAG in the blood circulation of ewes that have mated for the first time during the breeding period definitely indicates pregnancy. According to data obtained from cows, it has been reported that PAG circulate the in blood for a while after embryonic death (Szenci et al., 2003). While lower serum PAG concentrations have been reported in sheep suffering embryonic death (Hussein et al., 2017), no data is available on the clearance time of PAG from the bloodstream in sheep with embryonic death.

The positive predictive value (PPV) of the rapid visual PAG ELISA test was similar in both periods of pregnancy (G1: 90.10%; G2: 91.43%), but was slightly lower than the values reported in previous studies (Chaves et al., 2017; Chaves et al., 2020;Roberts et al., 2019).The cause of the lower PPV could be embryonic death, as is the case with low Sp. The negative predictive value (NPV) of the rapid visual PAG ELISA test (G1: 73.33%, G2: 73.68%) in both periods of pregnancy were found to be lower than those reported in previous studies (Chaves et al., 2017; Chaves et al., 2020;Roberts et al., 2019).The reason for this may be that the circulating PAG concentration of the pregnant ewes did not reach the level that the pregnancy test could detect. Although the accuracy of the rapid visual PAG ELISA test (G1: 87.93%, G2: 87.64%) is slightly lower (about 10%) than that reported in previous studies (Chaves et al., 2017; Chaves et al., 2020; Roberts et al., 2019), it was found to be satisfactory in both periods of pregnancy.

The difference between the lambing rates predicted and achieved by the rapid visual PAG ELISA test (means false positive; G1: 8.6%, G2: 6.8%, overall: 7.8%; Table 3) indicates the rate of ewes with embryonic death. The difference between the lambing rate and the lambing rate achieved by the rapid visual PAG ELISA test (means false negative; G1: 3.4%, G2: 5.6, overall: 4.4%) indicates the rate of ewes which insufficient release of PAG from the placenta into the bloodstream at pregnancy diagnosis time (Table 3).

According to the McNemar analysis, there was no difference between the results of the rapid visual PAG ELISA test and the lambing results in the two groups. The LR+, LR- and odds ratio calculated in the present study indicate that the visual PAG ELISA test shows a high diagnostic performance in predicting ewes that will or will not lamb. The results show that the lambing rate can be predicted from the 28$^{th}$ day of gestation with the rapid visual PAG ELISA test

## CONCLUSION

The current study indicate that the results of rapid visual PAG ELISA test may have been influenced by the pattern of release of PAG from the placenta into the bloodstream as well as by embryonic/fetal death. If deemed necessary, the ewes predicted to be non-lambing with the rapid visual PAG ELISA test can be checked for pregnancy status at a later date, and thereby, false negative ewes can be detected. In conclusion, using the rapid visual PAG ELISA test, the lambing rate can be predicted successfully from the 28$^{th}$ day of pregnancy with a high sensitivity, and non-lambing ewes can be predicted with a higher specificity as of the 40$^{th}$ day of pregnancy.

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## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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