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# Diagnosis of Late Embryonic Mortality in Dairy Cows by Measuring Pregnancy-Associated Glycoprotein and Pregnancy-Specific Protein B Levels

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**OE, ID, SS and SA conceived the study. OE, ID and SA performed TRUS examinations. SS performed ELISA assays. OE, ID, SS and SA interpreted the data and wrote the manuscript.**

**Key words**
**Embryonic mortality, Ultrasonography, Pregnancy specific proteins, Pregnancy specific protein-B, Cow.**

## ABSTRACT

In the study, blood and milk samples were taken from fifty-eight cows at 28, 30, 32 and 40 days after artificial insemination (AI). Transrectal ultrasonography (TRUS) was performed on all cows on days 30 and 40 after AI. Measurements were carried out on 9 cows whose pregnancy was confirmed and 9 cows whose embryonic mortality (EM) was determined. In PAG-Serum, there was a statistically significant difference ($P < 0.05$, $P < 0.01$) between the measurements on 40th day when EM was detected and on the 28th and 32nd days of pregnancy. In the PAG-Milk and PSPB-serum tests, there was a difference ($P < 0.05$) in terms of blood values between the 32nd day and the 40th day when EM was detected. Statistically significant differences in PAG-Serum test ($P < 0.001$) and PSPB test ($P < 0.01$) was obtained between animals that were pregnant on the 40th day and animals that had EM on the 40th day according to TRUS observations. Sensitivity and specificity rates were determined in the tests as 22.2% vs 44.4% and 57.1% vs 77.8%, respectively, on the 40th day when embryonic mortality was detected. In the cows determined to be pregnant on the 30th day, the test results obtained on the day when EM was determined by the TRUS method on the 40th day and the diagnosis of EM were not individually reliable in every animal. However, it is thought that in between 28th day and 40th day, statistically significant difference ($P < 0.01$; $P < 0.001$ between the pregnant and EM animals can be predicted.

## INTRODUCTION

Factors causing embryonic mortalities (EM) may be summarized as oocyte maturation at different oocyte stages, secretion of oestradiol in inadequate concentration during preovulatory period, progesterone hormone insufficiency, inability of uterine environment for embryo, placental development insufficiency and physiological, genetical, morphological and developmental factors of the embryo itself (Pohler *et al*., 2016; Pytlewski *et al*., 2018).

The embryonic period is defined as the period from conception to the period of differentiation, and this process continues until the 42nd day (Committee on Bovine Reproductive Nomenclature, 1972). EMs during pregnancy can occur early (before day 28) or late (after day 28). It has been reported that 20-30% of embryonic mortalities were on 28th day of pregnancy and between 3.2% and 42.7% of mortalities were after 28th day. However, there is a paucity of information regarding EMs in the period of placental formation, which corresponds to about 35-40 days of gestation (Pohler *et al*., 2016). EM in this period causes economic loss due to luteal regression in oestrus cycle and prolongation of number of days from calving to first oestrus interval.

Trophoblastic proteins such as bovine pregnancy-associated glycoprotein-1 (bPAG-1) and bovine pregnancy-specific protein B (PSP-B), are secreted by the binucleate cells (Zoli *et al*., 1992; Wooding *et al*., 2005).

PAG and PSPB tests have shown that a high accuracy (93-98%) in early pregnancy diagnosis could be determined (Silva *et al*., 2007; Romano and Larson, 2010; Ergene *et al*., 2018).

In early EMs, it has been reported that these proteins have fallen to an unmeasurable level in blood serum when measurements are performed 24-30 days after insemination (AI). In contrast, in late EMs, the measured values of these glycoproteins in both heifers and cows were found to be 20-30% lower than those of pregnant animals,

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and individual differences were obtained at the time EMs were observed. A study conducted with PSB-60, reported that this protein was an important method for determining gestation between 28-90$^{th}$ days, but no difference could be obtained between embryonic mortality and unfertilized animals (Mialon *et al*., 1993).

In this study, we aimed to determine the late EM in time of detection of EM using transrectal ultrasonography by PAG-Serum, PAG-Milk and PSPB-serum tests and these procedures were performed in animals previously determined to be pregnant with transrectal ultrasonographic (TRUS) controls.

## MATERIALS AND METHODS

*Animals*

After TRUS examinations of the cows, a total of 100 animals with no ovarian and uterine pathological problems and at postpartum 70-90 days were artificial inseminated. Initial control of animals after insemination was performed on day 28 with the TRUS method (B-Mode, 5.0 to 7.5 MHz, linear probe, ECM IMAGO®Veterinary; Angouleme/France). 58 cows whose were detected pregnant with TRUS were enrolled into the study. On the 30th day, second TRUS control was performed and it was determined that pregnancies were continued. On the 40th day, it was determined that 49 pregnant cows were still pregnant and 9 pregnant cows were not being continued. In the TRUS examination, despite the detection of embryos and gestational sac at day 30, these structures had completely disappeared by the 40th day, and embryos were not seen. Those animals that were found to be pregnant at day 30, but not on 40th day were evaluated as embryonic mortality (EM) group (n = 9). The sera of EM cows, whose insemination and conception times (78.4 ± 7.4, 79.6 ± 7.7 days, first gestation) were same, were compared with the sera of pregnant cows whose were pregnant corresponding to the same period. Lactating cows were housed in a covered freestall barn. The cows milked twice daily and were fed a total mixed ration consisting of grain sorghum, soybean meal, alfalfa, and corn silage. Water were offered *ad libitum* and the cows were milked 2 times daily.

*Collection of blood and milk samples*

Blood and milk samples (28, 30, 32 and 40 days) were taken from the jugular vein of the pregnant animals (n = 49) and from the animals identified as embryonic mortality (n = 9). Pregnancies were controlled via TRUS control procedures. Serum was obtained by centrifugation of blood samples at 1500 X g for 10 min. Milk samples were centrifuged at 4800 X g for 20 min to obtain skimmed milk. Blood samples were collected into vacuum tubes (Vacutainer® SST™ 9 mL, BD, New Jersey, USA) and milk samples were collected into sterile 10 mL plastic tubes. The collected serum and skimmed milk samples were stored at -20 ºC until analyses. PAG-serum, PAG-milk and PSPB-serum tests were then performed on the 18 samples that were determined as EM (n=9) and as pregnant (n = 9). The analyses were performed at Diagnostic Laboratory, Animal Hospital Near East University. The laboratory staff was blinded to the results of the TRUS exams.

*PAG-serum and milk analysis*

Sandwich ELISA tests (IDEXX Bovine Pregnancy Test Ref 99-41169 Lot. E171 and IDEXX Milk Pregnancy Test Ref 99-41209 Lot. E881, One IDEXX Drive, Westbrook, ME, USA) was used for the measurement of PAG in serum and milk samples. Samples were assayed and results were calculated as described by manufacturer. Test results were expressed as optical density (OD) (sample OD-negative OD; S-N). For serum samples ≥ 0.300 optical density (S-N) was assessed as pregnancy positive (pregnant), and <0.300 optical density as pregnancy negative (not pregnant). For milk samples ≥0.150 OD was evaluated as pregnancy positive (pregnant), and <0.100 OD as pregnancy negative (not pregnant). The results, which ranged between 0.100 and 0.150, were considered uncertain.

*PSPB serum analysis*

PSPB serum were assayed with a sandwich ELISA test and result were calculated as described (BioPRYN test Lot No: 5P106, Biotracking LLC, Moscow, ID, USA). The test results were evaluated as >0.210 OD, pregnancy positive (pregnant); OD between 0.135 and 0.210, uncertain; and <0.135 OD, pregnancy negative (not pregnant).

*Statistical analyses*

All statistical analyses were performed using the IBM SPSS Statistic 21 (version 21 for Windows, SPSS Ltd, Hong Kong) software. Normality and homogeneity of groups were determined by the Shapiro-Wilk test. Data are given as means ± standard deviations (x ± SD). To test for differences between groups over the time period, the "Repeated Measures Define Factors" test was performed. The "Kruskal-Wallis' test was used to test for normal distribution and in case there was not, comparison between groups was performed using the Mann-Whitney-U test. Crosstabs were used for specificity, sensitivity calculations. Estimates of the agreement between TRUS and PSP-B results were determined by Kappa values and 95% confidence intervals (95% CI) were also determined. Differences were considered statistically significant when $P < 0.05$.

**Table I.- PAG-Serum (S-N), PAG-Milk (S-N) and PSPB-Serum (S-N) in cows on different days after artificial insemination.**

| Days after AI | PAG-Serum (n = 9) | | | PAG-Milk (n = 9) | | | PSPB-Serum (n = 9) | | |
|---|---|---|---|---|---|---|---|---|---|
| | X±SE | Min | Max | X±SE | Min | Max | X±SE | Min | Max |
| 28 | 1.93±0.12 (a) | 0.0 | 2.74 | 1.02±0.24 (a) | -0.02 | 1.84 | 0.43±0.07(a) | 0.05 | 0.65 |
| 30 | 1.81±0.42 (a) | 0.0 | 2.98 | 1.04±0.26 (a) | 0.02 | 2.13 | 0.41±0.06 (a) | 0.05 | 0.65 |
| 32 | 2.09±0.33 (a,b) | 0.44 | 2.96 | 1.13±0.23 (a,b) | 0.19 | 2.20 | 0.48±0.06 (a,b) | 0.13 | 0.66 |
| 40 | 0.96±0.34 (c) | 0.07 | 2.64 | 0.68±0.23 (a.c) | 0.02 | 1.51 | 0.29±0.07(a,c) | 0.06 | 0.63 |
| P* | a: P < 0.05; b:c P < 0.001 | | | b:c P < 0.05 | | | b:c P < 0.05 | | |

*Means with different letters in the same column are statistically significant; optical density (S-N). AI, artificial insemination.

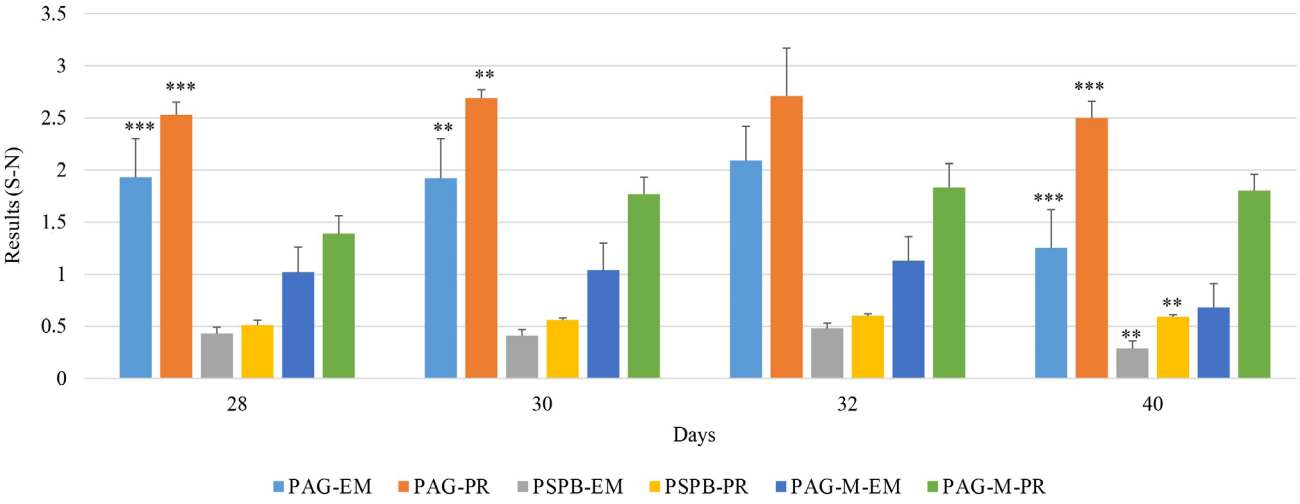


Fig. 1. Levels of PAG and PSPB in cows on different days (28 and 40 days) of artificial insemination when embryonic mortality was detected. ***, P<0.0001; **, P< 0.001; EM, embryonic mortality; PR, pregnant; M, milk.

## RESULTS

On the 30th day after insemination, 58 cows used in study were found to have a positive pregnancy from TRUS examinations. However, using the same TRUS procedure it was found that 9 of these animals (15.5%) had not continued their pregnancy until day 40.

According to PAG-serum test results, the difference between the mean value obtained on day 28 (1.93±0.12 S-N) and the mean value obtained on day 40 (0.96±0.34 S-N) of the EM was found to be statistically significant (P <0.05). In addition, the difference between the day 30 (1.81±0.42 S-N) and day 40 (0.96±0.34 S-N) was statistically significant (P < 0.01). In PAG-milk and PSPB-serum tests, it was determined that the difference between results obtained on day and 40 were significant (P <0.05) (Table I).

A statistically significant difference was found only in PAG-serum results between day 28 and day 30 (P <0.001; P <0.01) between pregnant (n = 9) and EM-detected animals (n = 9). The results of PAG- and PSPB-serum among the animals with positive pregnancy and EM on the 40th day when EM was detected by ultrasound were statistically significant (Fig. 1, Table II).

Sensitivity and specificity values between the findings obtained at day 30 with TRUS and the serum and milk test results at day 28 showed 100% agreement. On the other hand, the sensitivity of the test was 22.2% to 44.4% according to comparison of the blood serum values and the TRUS findings at day 40 when the EM was determined by TRUS (Table III).

On the 28th day of gestation, the results of kappa statistic obtained between ultrasound findings and test results of pregnant animals were in 100% to 88.9% agreement. Reliability of test results was confirmed by Kappa value found to be 1.000 in all tests at day 28. Alternatively, at day 40, the EM and TRUS results and the ability of the tests to detect the EM directly were found to be almost undetectable (value 0.0) (Table IV).

**Table II.- Levels of PAG and PSPB in serum and milk of cows on different days after artificial insemination when embryonic mortality was detected.**

| Tests | Days | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 28 | | | 30 | | | 32 | | | 40 | | |
| | X±SE | Min | Max | X±SE | Min | Max | X±SE | Min | Max | X±SE | Min | Max |
| PAG-Serum EM (n=9) | 1.93±0.37 | 0.0 | 2.74 | 1.92±0.38 | 0.0 | 2.98 | 2.09±0.33 | 0.44 | 2.96 | 1.25±0.37 | 0.07 | 2.65 |
| PAG-Serum PR (n = 9) | 2.53±0.12 | 1.70 | 2.92 | 2.69±0.008 | 2.25 | 3.09 | 2.71±0.46 | 2.51 | 2.87 | 2.50±0.16 | 1.28 | 2.87 |
| | P < 0.001 | | | P < 0.01 | | | P > 0.05 | | | P < 0.001 | | |
| PAG-Milk EM (n = 9) | 1.02±0.24 | -0.02 | 1.84 | 1.04±0.26 | -0.02 | 2.13 | 1.13±0.23 | 0.19 | 2.20 | 0.68±0.23 | 0.02 | 1.51 |
| PAG-Milk PR (n = 9) | 1.39±0.17 | 0.63 | 2.33 | 1.77±0.16 | 0.94 | 2.44 | 1.83±0.16 | 1.12 | 2.70 | 1.80±0.23 | 0.35 | 2.61 |
| | P > 0.05 | | | P > 0.05 | | | P > 0.05 | | | P > 0.05 | | |
| PSPB-Serum EM (n=9) | 0.43±0.69 | 0.05 | 0.65 | 0.41±0.06 | 0.05 | 0.65 | 0.48±0.05 | 0.13 | 0.66 | 0.29±0.07 | 0.06 | 0.63 |
| PSPB-Serum PR (n=9) | 0.51±0.05 | 0.23 | 0.65 | 0.56±0.02 | 0.40 | 0.65 | 0.60±0.016 | 0.52 | 0.69 | 0.59±0.03 | 0.41 | 0.69 |
| | P > 0.05 | | | P > 0.05 | | | P > 0.05 | | | P < 0.01 | | |

EM, embryonic mortality; PR, pregnancy.

**Table III.- Sensitivity and specificity of PAG and PSPB in serum and milk of cows on day 28 and 40 when embryonic mortality was detected.**

| Tests | Sensitivity # | Specificity ## |
|---|---|---|
| PAG-Serum (28 d) (n = 9) | 100.0% | 100.0% |
| PAG-Serum (40 d) (n = 9) | 22.2% | 77.8% |
| PAG-Milk (28 d) (n = 9) | 100.0% | 100.0% |
| PAG-Milk(40 d) (n = 9) | 44.4% | 55.6% |
| PSPB* (28 d) (n = 9) | 100.0% | 100.0% |
| PSPB** (40d) (n = 9) | 42.9% (n = 3) | 57.1% (n = 4) |

#Sensitivity, true positive, true diagnostic power of the test; ##Specificity, non-EM determination.

**Table IV.- Relationship between tests for PAG and PSPB in serum and milk of cows and ultrasonography with Kappa Test for PAG and PSPB (1.00 highest agreement).**

| Tests | D28* (TEST/ TRUS) | D40** (TEST/ TRUS) | D28 (KAPPA) | D40 (KAPPA) |
|---|---|---|---|---|
| PAG-Serum | 9/9 (100%) | 2/9 (22.2%) | 1.000 (P<0.001) | 0.0 (P=1) |
| PAG-Milk | ##8/9 (88.9%) | 5/9 (55.6%) | 0.609 (P<0.05) | 0.0 (P=1) |
| PSPB | #8/9 (88.9%) | ###5/9 (55.6%) | 1.000 (P<0.01) | 0.0 (P=1) |

*TRUS was done on day 30 (Results: 1 Negative; 8 Positive). ** TRUS was done on day 40 (Results: 9 EM). #One of them suspicious. ##One case: TRUS result positive; PAG-milk result negative. ###Three negative; two suspicious.

## DISCUSSION

Fertility decline has become a global problem and causes economic losses and an increased culling rate in dairy cattle (Khatib *et al*., 2009). After initial seeding, embryonic mortality approximates to 40% and causes repeat breeding in cows with 65% rate (Inskeep and Dailey, 2005). Since the progesterone test cannot differentiate between the presence of the offspring and embryonic mortality (Humblot *et al*., 1988), various tests have been performed in current research to detect EM. Late embryonic mortalities are deaths after day 28 of gestation (Silke *et al*., 2002) and the incident rate in cows varies between 3% and 40% (Lamb, 2001; Ribeiro *et al*., 2011, 2013). In this study, the controls performed (15.5%) on 58 cows with embryos detected at day 30, 9 of the 58 cows demonstrated a non-redefined uterus at day 40.

Some studies have reported that the level of blood circulation of bPAG-1 is correlated with the EM (Humblot, 2001; Breukelman *et al*., 2012; Pohler *et al*., 2012, 2016), while in others any correlation was not obtained (Ricci *et al*., 2015).

In this study, it was determined that sensitivity and specificity were 100% according to the results of all three tests on pregnant cows diagnosed at day 28$^{th}$ (TRUS control on the 30$^{th}$ and 40$^{th}$ day) and were not pregnant at day 40. This rate varies between 62.5% and 95% from different sources (Prvanovic *et al*., 2009; Breukelman *et al*., 2012; Gábor *et al*., 2007, 2016; Pohler *et al*., 2016). At day 40 of gestation when embryonic mortality was detected with TRUS, the specificity (between 22.2% and 44.4%) and sensitivity (between 55.6% and 77.8%) rates were found to be very low in all tests. The relationship between PAG concentration and pregnancy status in dairy cows has been investigated in detail (Thompson *et al*., 2010; Breukelman *et al*., 2012). However, there was no correlation between circulating PAG concentration and embryonic viability during the early gestation period between the 28$^{th}$-32$^{nd}$

days in dairy cows (Ricci *et al*., 2015).

It was found that TRUS findings and the three test results at day 40, cannot be reliably determined using the kappa statistical test in this study. On the contrary, the PAG-Serum results in cows showed a significant decrease from day 28 to 40 ($P<0.05$), whilst the PAG-Milk and PSPB-Serum tests showed a significant statistical decrease especially between day 32 and 40 ($P<0.05$). This finding indicates that there is a difference between the days of gestation and the days of EM. When late EM was detected, the PSPB concentration reached or fell below this cut-off value 0-20 days later. In cases of late embryonic or foetal mortality, bPAG values in four of ten cows were found to reach cut-off values at 58$^{th}$ days after insemination (Whitlock and Maxwell, 2008).

Here we found PAG milk and serum and PSPB serum values to be significantly lower than their baseline values. In this study, EM was not detected with the test results from the same day (day 40) in which EM was detected in many animals by TRUS. Based on the large individual variations in the values, late-stage EM could not be early predicted with b-PSBP and bPAG1 (Humblot *et al*., 1988; Szenci *et al*., 2000). Serum laboratory ELISA values revealed that PAG concentrations were below 1.4-1.8 ng/ml at 31 days post-seeding, resulting in 95% embryonic mortality at day 60 (Pohler *et al*., 2015). However, in this study, the course of PAG-serum values was observed in the direction of decrease rather than in the point where the EM was determined in TRUS.

When the pregnant and EM animals were compared, there was a difference between $P<0.001$ and $P<0.01$ on day 30 and 40 of the PAG serum test and day 40 of the PSPB Serum test. This result shows that, except for the PAG-Milk test on day 40, the EM values of the two tests were significantly lower than those of the pregnant cows. One study found that circulating concentrations of bPAGs at day 28 of pregnant cows was higher than that of cows determined to be EM at day 100 (Pohler *et al*., 2016). In the same way, it was revealed by Humblot *et al*. (1988) that there is a statistically significant difference in mean values between days 24 and 30-35 days post insemination in pregnant and late EM cows. Parallel to our findings, many animals have indicated that embryonic mortality cannot be determined in advance, since values in pregnant animals are also obtained in late embryonic mortalities.

The Kappa statistical test was conducted in various publications and was applied for consistency of the TRUS findings and serum or milk test findings (Silva *et al*., 2007; Whitlock and Maxwell, 2008; Romano and Larson, 2010; Karen *et al*., 2015). In the 40$^{th}$ day of the detection of embryonic mortalities, the laboratory test results showed that the highest rate of compliance with TRUS (55.6%, $P>0.005$), suggesting these tests could are not sufficient in determining EMs.

In this study, the comparison of test serum or milk data on the day that EM was detected by TRUS, results suggested that these tests were inadequate in determining EM.

## CONCLUSION

In conclusion, a new control (rectal palpation or TRUS) is required due to possibility of pregnancy-related embryonic mortality on day 40, even though there is a very high level of compliance (specificity, sensitivity and kappa) between test results and TRUS at 28th day of gestation. A significant decrease ($P<0.001$) in PAG-Serum values, especially between day 30 and 40, can be seen as a marker for EM. In this study, cows determined to be pregnant on the day 30, it has been determined that the EM test (PAG-serum, PAG-milk, PSPB-serum) obtained at day 40 when EM was detected by TRUS method cannot be reliably performed individually for each animal but it is thought that a significant decrease ($P<0.001$) in mean values between day 28 and 40, and a significant statistical difference ($P<0.01$ and $P<0.001$) between the pregnant and EM animals may indicate the likelihood of EM. Therefore, it is considered beneficial to perform rectal palpation or TRUS control in terms of embryonic mortality at day 40 in cows considered to be pregnant by these tests at day 28.

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*Statement of conflict of interest*

The authors declare that there is no conflict of interests regarding the publication of this article.

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