

## Comparative Evaluation of Early Pregnancy Diagnostic Methods in Dairy Cows

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### Abstract

*A study was conducted on 50 crossbred cows aged between 1½ to 8 years with an aim of early pregnancy diagnosis and to record embryonic mortality in cows by transrectal ultrasonography, rectal palpation, serum progesterone assay and pregnancy associated glycoprotein (PAG). In the present study, 6.41% embryonic mortality was observed between 28-42 days post-insemination. Ultrasonography was found to be more effective in diagnosing early pregnancy followed by PAG test by day 28, progesterone assay by day 36. However, PAG test was more accurate in diagnosing open cows at the earliest.*

**Keywords:** Accuracy, Dairy Cows, Early Pregnancy Diagnosis, Embryonic Loss

## Introduction

Reproductive efficiency is a prerequisite to realize the maximum life-time production of dairy cows. Dairy cows need to become pregnant after insemination, carry the conceptus till term, deliver healthy calves and produce milk regularly in order to make dairy farming economically viable and profitable. A novel method to reduce service period is accomplished by pregnancy diagnosis at early stages in the dairy cows (Pieterse *et al.*, 1990). Hence, the early pregnancy diagnosis becomes crucial to shorten the service period in cows, which need to be treated and/or rebred at the earliest opportunity. Direct method of pregnancy diagnosis involves direct detection of the tissues and/or associated fluids of the conceptus either manually (rectal palpation) or visualization using electronic instrumentation (ultrasonography). Secretions of the conceptus and corpus luteum such as early pregnancy factor, interferon-tau, estrone sulphate, pregnancy associated proteins (pregnancy-associated glycoproteins or early pregnancy factor) and progesterone are measured qualitatively or quantitatively at specific stages for early pregnancy diagnosis (Balhara *et al.*, 2013). An ideal early pregnancy test for dairy cattle should be accurate, sensitive, specific, cost effective and simple to perform under field conditions (Fricke *et al.*, 2016).

Pregnancy diagnosis is an important reproductive management tool to optimize the reproductive efficiency of dairy cattle (DeVries, 2006). Early detection of pregnant and non-pregnant cows with accuracy is essential for profitable dairy farming. The most common and oldest method of pregnancy diagnosis is transrectal palpation and was practiced in the 19<sup>th</sup> century (Cowie, 1948). Per-rectal palpation of the amniotic vesicle (Wisnicky and Cassida, 1948) and slipping of the chorioallantoic membranes (Zemjanis, 1970) are considered as positive signs of pregnancy at day 30 onwards. The transrectal real time B-mode ultrasonography for pregnancy diagnosis in cows has been considered as “gold standard” since it can detect vitality of the embryo (fetal heartbeat) as early as 19-24 days of gestation (Ginther, 1995). At day 20-22, the embryo appears as a short line, later becomes C-shaped (day 22-30), and by day 30-32 of gestation assumes an L shape (Broaddus and DeVries, 2005). Progesterone concentration increases up to 14 days post estrus and continues to elevate up to day 21 after successful fertilization with the specificity of 98.00 per cent (Nebel *et al.*, 1987; Waldman, 1993).

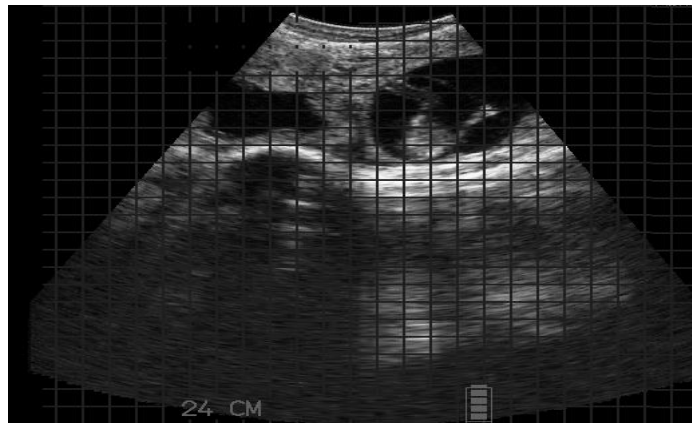
Pregnancy-associated glycoproteins (PAGs) are considered to be pregnancy specific markers. The PAGs are present in the blood serum soon after implantation and pregnancy specific protein B (PSPB) was the first identified PAG proteins; PAGs secreted from mono-nucleated and bi-nucleated trophoblastic cells of the outer epithelial layer in the placenta (Butler *et al.*, 1982). Wooding (1992) reported that PAGs enter the maternal circulation as early as day 22 to 24 and reach levels required for accurate pregnancy diagnosis at day 28. A substantial increase occurs in few weeks preceding parturition and peaks at calving and negligible level of PAGs could be found in maternal circulation at eight weeks postpartum (Sousa *et al.*, 2003; Green *et al.*, 2005). Green *et al.* (2005) developed an ELISA to reduce the false positives even for PAGs with shorter half-life of 4.3-8.4 days (early in gestation) and longer half-life of 7-9 days (later in gestation) in cows (Sousa *et al.*, 2003).

## Materials and Methods

Crossbred cows maintained in two organised farms in Bangalore were utilized for the study. About 50 cows out of 78 cows aged between 1½ - 8 years with body condition score of 2.5 to 3.0 which did not return to estrus within 20-24 days of insemination, were considered and subjected for evaluation of different pregnancy diagnostic methods in the study. The date of insemination was considered to calculate the gestational age. The cows were subjected to pregnancy associated glycoproteins estimation on day 28. The cows were further subjected to transrectal ultrasonography, rectal palpation and serum progesterone assay at certain days post-insemination.

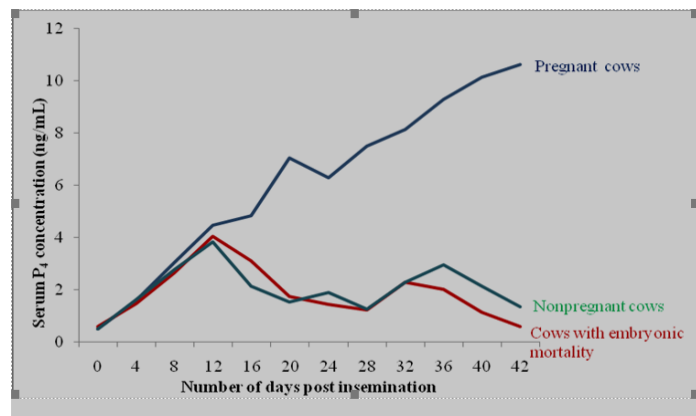
## Experimental Design

Transrectal ultrasonography using B-mode ultrasound scanner (Easi-Scan<sup>TM</sup>, BCF Technology Limited, UK) equipped with a 3-7 MHz curved transducer was carried out on days 20-24, 28, 35 and 42 post-insemination. The presence of anechoic (allantoic) fluid and/or the embryo proper was considered as a positive sign for pregnancy (Plate 1). Rectal palpation on day 35 and 42 post insemination was carried out and distended uterine lumen with allantoic fluid and slipping of membranes were considered as positive signs of pregnancy.



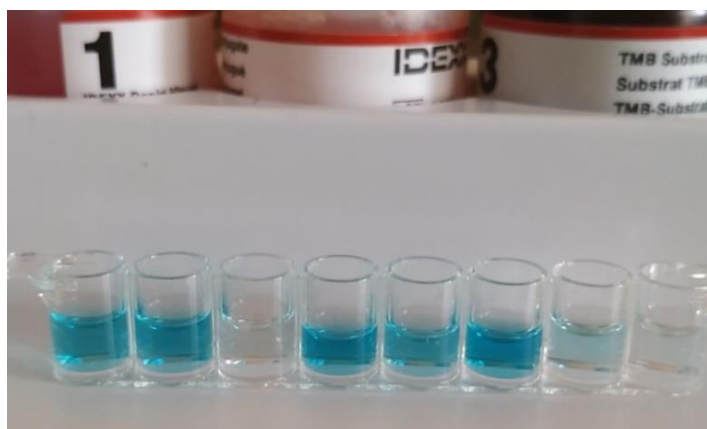
**Plate 1:** Ultrasonographic image of embryo

Estimation of serum progesterone was performed using radioimmunoassay at four days interval from the day of insemination to day 42 post insemination. The blood samples (2 mL) collected through jugular venipuncture were allowed to clot for 15-20 minutes at room temperature and then were centrifuged at 1500 rpm for 20 minutes. The separated serum was transferred to labelled eppendorf tubes and stored at  $-20^{\circ}\text{C}$  until estimation. Serum progesterone level of  $\geq 2.00$  ng/mL on days 20-24 and  $\geq 3.00$  ng/mL on days 30-36 was taken as criteria for determination of pregnancy (Figure 1).



**Figure 1:** Serum progesterone level in pregnant, nonpregnant and cows with embryonic mortality at various days post insemination (n=50)

Pregnancy associated glycoprotein test was performed on day 28 and 42 using IDEXX Rapid Visual Pregnancy Test Kit (IDEXX Laboratories, Inc., USA), a qualitative test using ELISA. The development of blue color of the sample wells were considered positive for pregnancy (Plate 2).



**Plate 2:** Result of PAG using IDEXX Rapid Visual Pregnancy Test Kit

Chi-square test was used to compare the sensitivities and specificities of the two pregnancy tests as per Steel and Torrie (1980). Difference between pregnant and non-pregnant cows in the level of progesterone was analyzed using a Student's t-test as per Petrie and Watson (1999).

## Results and Discussion

Of the 50 cows subjected to ultrasonography, 38, 36, 36 and 35 cows were diagnosed as pregnant on day 20-24, 28, 35 and 42 respectively. The overall diagnostic accuracy of 82.00 per cent obtained at day 20-24 is comparable with Badtram *et al.* (1991) and Beal *et al.* (1992). Comparative accuracy of different early pregnancy diagnostic methods is portrayed in Table 1. False positive result was observed due to incorrect pregnancy diagnosis. The presence of anechoic allantoic fluid only without an embryo proper was considered as false negative, which might be due to embryonic mortality. Low sensitivity might be due to difficulty in visualizing the gestational vesicle (Braganca *et al.*, 2018). Inability to demonstrate embryo in cases of embryonic death resulted in a false positive diagnosis. Badtram *et al.* (1991) reported that even the false positive results might be attributable to the fluid accumulation at estrus or due to pyometra and mucometra.

**Table 1:** Relative efficacy of different early pregnancy diagnostic methods in cows (n=50)

Method of early pregnancy diagnosis		Accuracy (%)	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)
USG	Day 20-24	82	81.57	83.33	93.93	58.82
	Day 28	86	88.88	78.57	91.42	73.33
	Day 35	98	97.22	100	100	93.33
	Day 42	100	100	100	100	100
Transrectal palpation	Day 35	88	84.84	94.11	96.55	76.19
	Day 42	100	100	100	100	100
Serum progesterone assay	Day 20-24	80	79.48	81.81	93.93	52.94
	Day 28	90	87.5	100	100	66.66
	Day 36	100	100	100	100	100
	Day 42	100	100	100	100	100
PAG test	Day 28	90	87.5	100	100	66.66
	Day 42	100	100	100	100	100

Cows with slipping of membranes, and distended uterine lumen with allantoic fluid during transrectal palpation on day 35 and 42 were considered to be pregnant. Out of 50 cows, 33 and 35 cows were diagnosed to be pregnant at day 35 and 42, respectively through transrectal palpation. The accuracy obtained in the present study at day 35 is higher than the result obtained by Karen *et al.* (2011). The results reported at day 42 by Karen *et al.* (2011) in buffaloes were lower than the results obtained in the present study. False positive result obtained in the present study may be due to presence of intraluminal fluid and slight asymmetry of uterine horns. Furthermore, false negative results may also be attributed to false tactile perception of double membrane slip during pregnancy diagnosis. Kastelic *et al.* (1989) emphasized that chorioallantoic fluid at day 18-21 and uterine fluid accumulated during estrus are difficult to differentiate. False positive diagnosis was also noted and this may be due to pathological fluid in the uterus (chronic endometritis).

39, 40, 35 and 35 cows were diagnosed pregnant based on serum progesterone assay on days 20-24, 28, 36 and 42, respectively. Serum progesterone level of  $\geq 2.00$  ng/mL on days 20-24 and  $\geq 3.00$  ng/mL on days 30-36 were considered as pregnant. The accuracy reported by Wishart *et al.* (1975) and Otava *et al.* (2007) were similar with our study. Muhammd *et al.* (2000) reported lower sensitivity and higher specificity than the result obtained in the study. Muhammd *et al.* (2000) reported lower sensitivity at day 28 than that obtained in the present study (Table 1). The accuracy, sensitivity and specificity at day 36 and 42 were 100.00 per cent which is in agreement with the result reported by Muhammd *et al.* (2000). False positive results may be due to embryonic mortality, luteal cysts, and infections of the genital system and artificial insemination of females not in estrus. Abdullah *et al.* (2014) suggested that at day 20 post-insemination, the minimum plasma progesterone concentration of 3.00 ng/mL is required for

pregnancy maintenance. The serum progesterone level in pregnant, nonpregnant and cows with embryonic mortality at various days post-insemination is represented in Figure 1. Fertilisation failure or early regression of the corpus luteum despite a successful fertilisation may be the causes for low progesterone level at day 10 post AI. Higher P4 concentration at AI and lower P4 concentration at days 10, 21 and 30 after AI compared to pregnant cows have been reported to be associated with associated with pregnancy loss (Nyman *et al.*, 2018).

The presence of PAG in the blood serum was detected at day 28 and 42 and the development of blue color of the sample wells were considered as positive and no color development as negative. Based on PAG test in 50 cows, 40 and 35 cows were found pregnant on day 28 and 42, respectively. The accuracy of PAG at day 28 in the present study is comparable with the report by Sinedino *et al.* (2014). Lower accuracy and specificity have been recorded by Northrop *et al.* (2019). However, higher accuracy was reported by Piechotta *et al.*, 2011 and Commun *et al.*, 2016. Some authors have recorded higher sensitivity of PAG ELISA (Sinedino *et al.*, 2014; Commun *et al.*, 2016; Braganca *et al.*, 2018; Northrop *et al.*, 2019). Friedrich and Holtz (2010) reported similar sensitivity but lower specificity at day 42 using serum PAG ELISA compared to the present study. High accuracy of PAG ELISA is due to increased immunoreactivity of proteins across pregnancy (Braganca *et al.*, 2018). Higher specificity than sensitivity of serum PAG ELISA was due to the possibility of pregnancy loss and the relatively long half-life of PAGs in the maternal circulation varying between 2.7-8.0 days after induction of embryonic mortality. False negative diagnosis may be due to delay in the PAG increase in peripheral circulation. Low concentration of PAGs is due to either delay in increase or a decline due to early embryonic loss (Piechotta *et al.*, 2011).

In the present study, embryonic mortality was detected based on the presence of allantoic fluid alone without embryo proper by USG at day 20-24 and 28. The mean serum progesterone level of  $1.56 \pm 0.05$  ng/mL and  $1.21 \pm 0.07$  ng/mL at day 20-24 and 28, respectively were considered as indicator for embryonic mortality in the present study, which concurs with the findings of Beal *et al.* (1992), Prvanovic *et al.* (2009) and Rani *et al.* (2018). Out of 78 cows inseminated, 35 (44.87%) were confirmed pregnant and 6.41 per cent embryonic mortality was recorded between day 20-24 and 42 post insemination using various diagnostic methods. In the present study, 15 cows which did not return to estrus within 24 days post insemination were diagnosed as open. It was concluded that USG to be highly effective method for diagnosing early pregnancy as it aids in direct visualisation of conceptus and PAG test could be used more accurately at the earliest to diagnose open cows.

## Conclusion

We conclude from the findings of the study that ultrasonography was more effective in diagnosing early pregnancy followed by PAG test by day 28, progesterone assay by day 36. However, PAG test was more accurate in diagnosing open cows at the earliest. Embryonic mortality of 6.41% was observed between 28-42 days post-insemination.

## Conflict of Interests

There is no conflict of interest.

## Publisher Disclaimer

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