RESEARCH ARTICLE

Evaluation of Efficacy of BovEasy Bovine Rapid Pregnancy Detection Kits in Crossbred Cattle

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Abstract

Bovine early pregnancy detection is a boon to optimize reproductive rhythm and economic dairy entrepreneur. This study evaluated the efficacy of different early pregnancy detection methods in crossbred cows on the University Farm. In all 80 freshly inseminated crossbred cattle, which did not return to estrus within 22 days post-mid-estrus AI were included in the study. Blood samples were collected in EDTA/clot activator vaccutainers on days 21, 28, 32, 36, and 45 post-AI for various tests. Transrectal ultrasonography was performed on day 28 post-AI at random on 45 animals. BovEasy bovine rapid PD kits were used on fresh whole blood on days 28, 32 and 36. Blood plasma samples were analyzed for progesterone by RIA technique on all days, and for pregnancy associated glycoproteins (PAGs) on day 28 and 36 post-AI by using ELISA kits. Final pregnancy was confirmed by actual per rectal palpation on day 45 post-AI. Out of 80 cows, 38 (57.50 %) were confirmed to be pregnant by day 45 post-AI. However, by early PD kits, the animals declared pregnant on days 28, 32 and 36 were 41, 40 and 40, respectively, with suspicious results in 5, 2 and 2 animals. The accuracy, specificity and sensitivity of rapid test kits recorded were 93.75, 95.24 and 92.00% on day 28 post-AI, and 97.50, 97.62 and 97.00% on day 32 as well as 36 post-AI, respectively. The plasma progesterone concentrations showed a significant ($p<0.05$) increase in the levels from day 21 to day 45 in conceived subgroup with significantly higher values than in non-conceived subgroup. The plasma PAG in conceived subgroup was significantly ($p<0.001$) higher than in non-conceived subgroup on both the days of testing, with around 1.5 to 1.7-fold increase on day 36 compared to day 28 in conceived subgroup. The accuracy of positive pregnancy on day 28 post-breeding was 100% by both plasma progesterone and USG, and 97% by plasma PAGs. The study also recorded 10.00% early embryonic mortality in crossbred cattle.

Keywords: BovEasy bovine rapid PD kit, Crossbred cattle, Efficacy, Plasma progesterone, Pregnancy associated glycoproteins (PAGs), Ultrasonography.

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Introduction

Early pregnancy diagnosis is crucial in dairy animals to shorten the calving interval through enabling the farmers to identify open animals and treat and/or rebreed them at the earliest opportunity. Ideally a 60-90 day post-parturient barren interval is recommended for bovine breeding. Rectal palpation is most widely utilized technique for diagnosing pregnancy under field conditions; however, it is practiced mostly around 45 to 60 days or later in the field. Use of transrectal ultrasonography at around day 28-30 and again at day 45 post-breeding is considered gold standard for early pregnancy diagnosis, but under filed condition it is not possible due to cost and mobility issue of USG machine.

Estrus identification, successful fertilization and minimum embryonic loss are crucial for achieving high pregnancy rates, and reducing the time between calving and conception through early pregnancy diagnosis as one of the methods of enhancing reproductive performance (Suthar and Dhami, 2010). There are management strategies that can be employed to lessen the financial harm that embryonic death causes, but these heavily rely on new technology to detect fetal mortality early after AI (Suthar and Dhami, 2010, Balhara *et al.,* 2013, Szenci, 2021). The criteria that characterize the perfect early pregnancy test for dairy animals include high specificity, sensitivity, accuracy, low cost of testing, ease of use in the field, and capacity to identify early pregnancy. Blood or milk progesterone is the test of choice, may be by RIA or ELISA kits, mainly to diagnose non-pregnant rather than pregnant animals by 100% accuracy, but the assay kit

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available currently is for bulk samples and requires specialized lab facilities, hence is out of reach for the farmers.

Determining pregnancy-associated glycoproteins (PAGs) is another test of choice. The first pregnancy-specific marker - a pregnancy-specific protein-B (PSPB) discovered in cattle (Barbato *et al.,* 2022) shares the identical N-terminal amino acid sequence with bovine PAG-1 (Ricci *et al.,* 2015). Recently, the researchers have developed PAG-based on farm early pregnancy detection kits/strips with accuracy ranging from 95 to 98%. This study was planned to evaluate the efficacy of such a commercially available rapid farm test kit under controlled conditions on crossbred cattle between days 28 and 36 along with plasma PAGs, plasma progesterone on day 21 through 45, and actual per rectal palpation on day 45 post-AI.

Materials and Methods

The work was carried out at Livestock Research Station, Kamdhenu University, Anand (India) on crossbred (HF x Kankrej) cattle from December 2023 to June, 2024 following approval of IAEC. In all 80 breedable freshly inseminated crossbred cattle, which did not return to estrus within 22 days post-mid-estrus AI were included in the study. The mature animals with clinically sound reproductive tract and standing estrus were artificially inseminated using frozen-thawed semen during later half of estrus by the professionals engaged on the farm. Pregnancy was confirmed by actual per rectal palpation in all animals on day 45 post-AI. Transrectal ultrasonography was also used on day 28 post-AI at random on 45 animals.

Blood samples (5 mL) were collected by jugular venipuncture in K_3EDTA/clot activator vaccutainers on days 21, 28, 32, 36, and 45 for various pregnancy detection tests. Fresh whole blood was used for detecting early pregnancy through BovEasy bovine rapid PD kits (Prompt Equipments Pvt Ltd, Ahmedabad) on days 28, 32 and 36 as per the instructions provided by the manufacturer on the kit. Blood plasma samples of days 21, 28, 36 and 45 were stored at -20°C with a drop of merthiolate until further analysis. Estimation of plasma progesterone (P_4) was carried out on days 21, 28, 36 and 45 post-AI by employing Radio Immuno Assay technique and kits procured from Beckman & Coulter / Immunotech, France as per the manufacturer's instructions. The plasma levels of pregnancy associated glycoproteins (PAGs) or pregnancy specific protein B (PSPB) were determined on day 28 and 36 post-AI in non-returned animals by using ELISA kits procured from MyBiosource Inc, USA as per the manufacturer's instructions.

The data on plasma P_4 and PAGs were expressed as Means and Standard Errors, and analyzed by one way ANOVA. Duncan's NMRT or unpaired Student's 't' test was used to compare the results of test days, and methods. Conception rates and embryonic losses were also analyzed. Sensitivity, specificity, accuracy and reliability of rapid test kits in relation to actual pregnancy on day 45 post-AI were worked out.

Results and Discussion

Validation of BovEasy Early PD Kits

The findings of BovEasy early PD kits used on 80 crossbred cows for validation of its accuracy on days 28, 32 and 36 post-AI with reference to 45 days actual pregnancy are presented in Table 1. Out of 80 cows that did not return to estrus by day 22 post-AI, 38 (57.50 %) were confirmed to be pregnant by day 45 post-insemination. However, by early PD kits, the animals declared pregnant on days 28, 32 and 36 were 41, 40 and 40, with suspicious or doubtful results in 5, 2 and 2 animals, respectively.

Table 1: Efficacy of BovEasy early pregnancy detection kits on days 28, 32 and 36 post-AI in non-return crossbred cows against actual per rectal palpation on day 45

Sr. No.	Measure	Day 28	Day 32	Day 36
1	Total animal tested	80	80	80
2	Total animal found positive	41	40	40
3	Total animal found negative	34	38	38
4	Suspicious result	5	2	2
5	Actual pregnant (day 45)		38	
6	Actual non-pregnant		42	
7	True positive	40	41	40
8	True negative	35	37	38
9	False positive	3	1	1
10	False negative	2	1	1
11	Total	80	80	80
12	Accuracy (%)	93.75	97.50	97.50
13	Sensitivity (%)	95.24	97.62	97.56
14	Specificity (%)	92.00	97.00	97.00

Accuracy = (True positive + True negative) / (True positive + True negative + False positive + False negative)
Sensitivity = True positive / (True positive + False negative)
Specificity = True negative / (True negative + False positive)

Based on the number of cases found true positive, true negative, false positive and false negative from the total tested, the accuracy, specificity and sensitivity of test kits recorded were 93.75, 95.24 and 92.00 % on day 28 post-AI; and 97.50, 97.62 and 97.00 % on day 32 as well as day 36 post-AI, respectively (Table 1). The crossbred animals tested were all high yielders, under production stress which may be probable reason for little higher suspicious results with early embryonic mortality resulting in more false positive in PD test kit results. It is very clear that testing blood samples on day 32 post-AI rather than day 28 gives more accurate results, at par with day 36 testing, and therefore day 32 may be recommended for using these kits by the farmers to obtain almost 97 % accuracy, specificity and sensitivity. The confusion matrix analysis on day 32 and 36 further revealed only one case each either false positive or false

negative from the actual pregnant and actual non-pregnant animals compared to 3 false positive and 2 false negative cases on day 28. This was mainly due to early embryonic mortality or low production of PAG by the retarded growth of early embryos before day 32 in some animals and even individual variations in positive prediction of color bands in the test kits used. These findings were very close to the report of Devmurari *et al.* (2025), who reported comparable results of 99-100% accuracy, specificity and sensitivity in Gir cows and Jaffarabadi buffaloes with 5-8% embryonic mortality between days 28 and 45 in these breeds. Akköse (2023) evaluated the IDEXX on-farm pregnancy test (OFPT) and rapid visual pregnancy test (RVPT) for early pregnancy diagnosis between 28 to 31 days post-AI in 317 Holstein cows, and recorded comparable results to our findings with BovEasy rapid farm test kit. Moussafir *et al*. (2018) and Dana *et al*. (2021) found higher sensitivity specificity and accuracy of the bPAGs ELISA test for pregnancy detection compared to IDEXX rapid farm test kits.

Plasma Profile of Progesterone and PAGs

The findings on plasma profile of hormone progesterone (P_4) determined by RIA kits on days 21, 28, 36 and 45 post-AI in conceived and non-conceived subgroups of cows are presented in Table 2. The plasma P_4 concentrations recorded on all 4 days post-breeding showed a gradual and significant ($p<0.05$) increase in the levels from day 21 to day 45 in conceived subgroup including the overall means. In non-conceived subgroup, the values on these days were significantly lower ($p<0.001$) than in conceived subgroup, and also fluctuated significantly ($p<0.05$) between days with higher levels on days 28 and 45 over days 21 and 36. This trend of mean values of P_4 suggested that some of the non-conceived animals were cyclic and in luteal phase of repeat cycles around days 28 and 45, but these cycles were either missed or gone unobserved by the persons involved in heat detection. Among them a few animals also had shown somewhat higher PAGs (> 1.5 ng/mL) on day 28 suggesting retarded growth or death of early embryos, which however needs confirmation by early detection of PAGs between days 15 and 28, which we have not done in this study. These observations concurred well with the findings of Ghanem and Nishibori (2015), Thanh *et al*. (2023), and Devmurari (2025), who recorded that plasma progesterone levels were significantly higher in pregnant animals with advancing pregnancy compared to their non-pregnant counterparts.

The mean plasma concentration of PAGs recorded on day 36 was significantly ($p<0.001$) higher than that at day 28 in conceived subgroup of cows. However, the difference was statistically non-significant between days in non-conceived group with almost identical and less than 1 ng/mL value. Moreover, the mean value of plasma PAG in conceived subgroup was significantly ($p<0.001$) higher than in non-conceived subgroup on both the days of testing, with around 1.5 to 1.7-fold increase on day 36 compared to day 28. Comparable findings were also observed by Devmurari (2025) in Gir cows and Jaffarabadi buffaloes. The observed rise in PAG levels in conceived cows aligned with similar patterns reported by Shahin *et al.* (2014), Barbato *et al.* (2017), and Borakhatariya *et al.* (2024).

Table 2: Average peripheral plasma levels of PAG and progesterone (ng/mL) assessed in conceived and non-conceived cows on different days after AI (Mean ± SEM)

Parameter	Days post-AI	Conceived (n=42)	Non-conceived (n=38)	Overall (n=80)
Plasma progesterone (ng/mL)	21	4.53 ± 0.70^{Aa}	1.07 ± 0.19^{Ab}	2.89 ± 0.22^{A}
	28	6.47 ± 1.0^{Ba}	2.04 ± 0.36^{Cb}	4.36 ± 0.26^{B}
	36	10.29 ± 1.59^{Ca}	1.97 ± 0.35^{Cb}	6.34 ± 0.47^{C}
	45	14.09 ± 2.17^{Da}	1.29 ± 0.23^{Bb}	8.01 ± 0.78^{D}
	Overall	8.85 ± 1.37^{a}	1.59 ± 0.28^{b}	5.40 ± 0.43
PAG (ng/mL)	28	5.69 ± 0.88^{Aa}	0.77 ± 0.14^{b}	3.35 ± 0.32^{A}
	36	7.15 ± 1.10^{Ba}	0.80 ± 0.14^{b}	4.14 ± 0.41^{B}
	Overall	6.42 ± 0.99^{a}	0.79 ± 0.14^{b}	3.75 ± 0.53

Values with different superscripts (A, B) within column and (a, b) within the row differ significantly ($p<0.05$)

Detection of PAGs, which come only from the mononucleate and binucleate cells of the embryonic trophoblast, suggest that the pregnancy has already established in a particular animal. However, the mean plasma PAG concentrations in cattle rises between day 15 and 35 of gestation; but until roughly 26 to 30 days following AI, PAG testing cannot be used as a reliable pregnancy indicator due to variations in cows' plasma PAG levels (Zoli *et al.*, 1992, Humblot, 2001) for early embyonic mortality or lower secretion from trophoblasts of some of the growth retarged embryos. The current findings also support this view looking to PAG profile on days 28 and 36, and these results very well correlated with the early PD test kits outcomes on day 28, 32 and 36. This implies that the BovEasy bovine early PD kits can be used most efficiently on day 32 rather than day 28 in dairy animals for accurately detecting early pregnancy as an economic reproductive management strategy.

In the present study, the accuracy of positive pregnancy on day 28 post-breeding was 100 % by both plasma progesterone and USG performed in 45 animals, and 97 % by plasma PAGs. However, PAG test was more accurate in diagnosing open cows at the earliest with sensitivity of 93.33%, and specificity of 92.86%. False negative diagnosis may be due to delay in the increase of PAG concentration

in peripheral circulation or due to early embryonic loss. Individual variation in placental production of PAG molecules could also be a reason for these variations (Piechotta *et al.*, 2011). Northrop *et al.* (2019) however reported high sensitivity of plasma PAG (97-99%), but less specificity (64-88%) and accuracy (84-94%) by using similar ELISA based kit at day 28 post-AI.

Embryonic Mortality

In the present study the early embryonic mortality recorded between day 28 and 45 based on USG findings, P_4 and PAG status was 10.0 (8/80) % in crossbreds. The major losses were before day 36 or so. Thus, the major cause of false positive found following use of early PD kits was early embryonic death, which is unavoidable for several reasons. That is why the accuracy of early PD kits results around day 28-32 post-AI if found beyond 95-99%, the test can be considered reliable provided re-confirmation of pregnancy is done around day 45-60 by per rectal palpation to detect and manage some non-pregnant animals those might have suffered from early embryonic losses. Borakhatariya *et al.* (2024) and Devmurari *et al.* (2025) however recorded somewhat lower (5-8%) early embryonic mortality in Gir cattle and Jaffarabadi buffaloes, with little better accuracy, specificity and sensitivity of early bovine PD kits on day 28 and 32 post-AI.

Correlations of PD Kit Results with Plasma PAG and Progesterone

The correlation findings of early PD kits results' arbitrary scores on day 32 post-AI (assigned as 7 for pregnant, 5 for doubtful/suspicious and 3 for non-pregnant) with plasma PAG and P_4 profile on different days post-AI (Table 3) revealed highly significant ($p<0.001$) and positive association of all these parameters on different days tested, further substantiating the validation of accuracy of early PD kits results in declaring the animal either pregnant or non-pregnant. These correlations concurred well with the report of Devmurari *et al.* (2025) in cattle and buffaloes.

Table 3: Pearson's correlations of PD kits results with plasma PAG and P_4 on different days post-AI in crossbred cattle

	PD Score	PAG28	PAG36	$P_4$21	$P_4$28	$P_4$36	$P_4$45
PD Score	1						
PAG, Day 28	0.844**	1					
PAG, Day 36	0.854**	0.842**	1				
P_4, Day 21	0.905**	0.760**	0.800**	1			
P_4, Day 28	0.929**	0.818**	0.810**	0.867**	1		
P_4, Day 36	0.955**	0.832**	0.878**	0.885**	0.934**	1	
P_4, Day 45	0.882**	0.771**	0.896**	0.843**	0.869**	0.935**	1

**Significant at $p<0.001$ level (2-tailed)

Plasma P_4 and PAG concentrations increased significantly from day 21/28 with advancing pregnancy by day 45/36, and the results of PD kit score on day 32 post-breeding, and the plasma levels of P_4 and PAG on different days were significantly and positively correlated, suggesting that the use of an easy simple cost effective cow-side BovEasy early PD kit is more advantageous to the farmers with 97 % accuracy of positive pregnancy, rather than going for lab oriented clumsy, laborious, costly and time consuming P_4 or PAG tests requiring some special lab facilities.

Conclusion

Based on the results of the current investigations, it can be concluded that the accuracy of positive pregnancy diagnosis on day 28 post-AI was 100 % by both plasma progesterone and USG, and 97 % by plasma PAGs. The accuracy, specificity and sensitivity of BovEasy early bovine PD test kits recorded on day 32 and 36 were better (around 97%) compared to day 28 (93%), suggesting that for better accuracy blood samples of cows should be tested on day 32 rather than day 28 post-AI by BovEasy early PD kit, to reduce chances of false positive. Detection of non-pregnant animals at an early stage of 30-32 day post-AI by practical cost-effective simple PD kits would save at least one month's maintenance cost by adopting reproductive management strategies, rebreeding and making the animals again early pregnant, thus reducing the calving interval in the herd and earning additional income from milk yield, making the dairy entrepreneur economically viable.

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