RESEARCH ARTICLE

Diagnostic Evaluation of Pregnancy Detection Methods in Dairy Cattle

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ABSTRACT

The objective of the present study was to evaluate the diagnostic potential of pregnancy detection methods at an early stage in cattle using transrectal ultrasonography, per-rectal palpation and commercially available PAG based ELISA kit. The study was conducted on 116 crossbred cyclic cows, aged 3-8 years. The cows at estrus were artificially inseminated using 2 doses of quality semen at 10-12 h interval. They were subjected to trans-rectal ultrasonography and PAG test in randomized animals at around 28-35 days' post-insemination. The animals were further examined by second trans-rectal ultrasonography and per-rectal palpation on day 45-50 post-AI. Ultrasonography was found to be more effective in diagnosing early pregnancy followed by PAG test around day 28 and per-rectal examination by day 45-50 post-insemination. However, PAG test was more accurate in diagnosing open cows at the earliest.
Key words: Cattle, ELISA, PAG, Per-rectal palpation, Pregnancy diagnosis, Ultrasound.
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INTRODUCTION

The most integral part of efficient farm animal management is the early identification of pregnancy as it is the key in optimizing reproductive efficiency in the farm animals, which not only enables the identification of open (non-pregnant) animals and their treatment further but also to rebreed them in the next estrous cycle. There are several methods for pregnancy detection which are in practice, *viz.,* direct (visual and clinical) and indirect (laboratory) methods. Pregnancy diagnosis is traditionally done by the visual observations and/ or clinical methods like rectal palpation and ultrasonography.

Transrectal palpation is one of the oldest and most commonly used methods of pregnancy diagnosis in large animals and has been practiced since long. In per-rectal examination, palpation of fetal amniotic vesicle and slipping of fetal membranes are considered as positive signs of pregnancy from day 30 onwards till 50 days post-AI (Zemjanis, 1970). Pregnancy is generally detected by 45-60 days through rectal palpation in bovine, though it does not detect the exact viability of the fetus resulting in variable accuracy. With the advent of trans-rectal ultrasonography in the 1980s, the real time B-mode transrectal ultrasonography has been considered as "gold standard" for early pregnancy diagnosis in cows owing to its ability to detect pregnancy by 28 days of gestation onwards envisaging the early embryonic structures and viability of the embryo (fetal heart beat) as early as 19-24 days post-gestation (Ginther, 1995; Bagley *et al*., 2023). In USG, at gestation day 20-22, the embryo appears as a short line that becomes C-shaped at around day 22-30 and by day 30-32 of gestation converts to L shape (Broaddus and DeVries, 2005).

On the other hand, many laboratory methods have been reported for diagnosis of pregnancy at an early stage in bovine, such as detection of progesterone hormone

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levels at around day 21±4 (Pohler *et al*., 2015), of course with poor sensitivity. Subsequently, pregnancy biomarker called Pregnancy-associated glycoproteins (PAGs) or Pregnancy-Specific Protein B (PSP-B) secreted from mono-nucleate as well as bi-nucleate trophoblastic cells of placentomes at around 3-4 weeks of pregnancy has been proven to play a major role in early pregnancy detection in bovine (Humblot *et al*., 1988). Several PAG molecules identical to PSPB exist in ruminant species (Gajewski *et al*., 2014) and are being used in the development of various assays to determine pregnancy in different farm animal species. This study was aimed to use commercially available visual PAG test for detection of pregnancy and to compare it with the transrectal ultrasonography and per-rectal palpation in dairy cattle.

MATERIALS AND METHODS

A total of 116 crossbred cows maintained at Cattle & Buffalo Farm, IVRI, Izatnagar (India) aged 3-8 years old with good body condition score were considered for the study following

approval from IAEC. The animals were kept in iso-managerial conditions and subjected for evaluation of pregnancy using different diagnostic methods. The cows observed at estrus were artificially inseminated using 2 doses of quality semen at 10-12 h interval. The cows were subjected further to trans-rectal ultrasonography (TRUS) using B-mode ultrasound scanner (Exago-ECM, IMV) with 7.5 MHz curved transducer, and commercially available PAG test at around day 28-35 post-insemination. The animals were further examined by second trans-rectal ultrasonography and per-rectal palpation on day 45-50 post-AI. The animal was considered pregnant upon visualization of anechoic (allantoic) fluid and/or the embryo proper. The findings of the ultrasonography obtained on 45-50 days post-insemination were considered as gold standard for diagnostic evaluation of the pregnancy status for all other methods (PAG test and per-rectal palpation). Approximately, 2-4 mL of blood from each selected cattle was collected by jugular venipuncture using aseptic procedures in EDTA vacutainer. This was stored at -20°C until used for PAG test.

PAG test, an ELISA based test, was performed on 58 selected whole blood (EDTA) samples (in duplicate) of 28-35 days post-AI using Alertys* Rapid Visual Pregnancy Test Kit (IDEXX Laboratories, Inc., USA). For rapid visual assay, randomized blood sampling was done involving almost equal proportion of pregnant (n=30) and non-pregnant (28) samples in duplicate from 28-42 days post-AI. Pregnancy status of the cow was determined by comparing the color development in the sample well as compared to the negative (control) well. The sample wells with blue color development greater than that of the negative well were considered pregnant (Plate 1). Per-rectal palpation of the genitalia on day 45-50 post-insemination was also done targeting structures like palpation of amniotic vesicle and slipping of fetal membranes considered as positive signs of pregnancy.
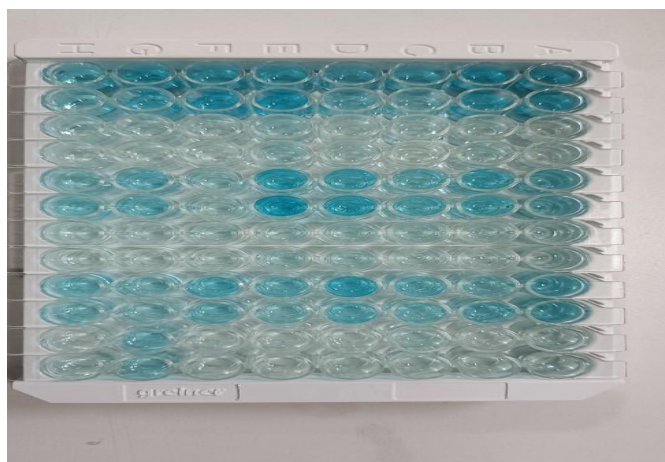
The data generated was analyzed by one-way ANOVA using GraphPad Prism version 8.0.2 to compare the sensitivity and specificity of these pregnancy tests.

RESULTS AND DISCUSSION

The comparative diagnostic efficacies of the pregnancy diagnostic methods used in this study are shown in Table 1. The findings of the transrectal ultrasonography (TRUS) performed during the days 28-35 post-insemination in the targeted cows (n=116) revealed that out of 116 cows, 66 cows were diagnosed as pregnant with 97.06% sensitivity primarily based on the presence of anechoic (allantoic) fluid and the embryo proper, whereas 50 cows were diagnosed as non-pregnant with 91.67% specificity based on the absence of these characteristics (Plate 2, 3). Eight out of 116 animals were re-examined for ultrasonography in order to confirm their pregnancy status. The animals (n=116) were further subjected to second ultrasonography on day 45-50 post-AI for further confirmation of pregnancy and 68 cows were found pregnant and 48 were diagnosed as non-pregnant with 100% each sensitivity and specificity. Similar to our findings, Pieterse *et al.* (1990) reported sensitivity and specificity of 97.7% and 87.7%, respectively, when transrectal ultrasonography was performed 26 to 33 days post-insemination. Also, Nation *et al.* (2003) performed TRUS at 28 and 35 days after AI and correctly identified 96% of the pregnant cows and 97% of the non-pregnant cows, resulting in an overall accuracy of 96.5%. Comparing other findings when TRUS was performed at 40 days or later, it was reported to be 100% accurate and ranged around 94% accuracy when used between 26 and 33 days of gestation (Romano *et al.*, 2006; Brownlie *et al.*, 2016; Fricke and Lamb, 2022).

Plate 1: ELISA based PAG test using Alertys IDEXX rapid visual pregnancy test kit

Plate 2: Presence of embryo proper in embryonic vesicle

Plate 3: Allantoic fluid containing embryo proper

Inability to demonstrate embryo in the enlarged uterine horns resulted in a false positive diagnosis. Badtram *et al.* (1991) reported that the false positive results might result due to the fluid accumulation at estrus or due to pyometra and mucometra. However, 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 anechoic vesicle (Braganca *et al.*, 2018).

Twenty-eight (28) out of the 30 pregnant animals from 28-42 days post-AI were diagnosed as pregnant in PAG ELISA test with sensitivity of 93.33%, based on the development of blue color in the sample wells. However, 26 out of 28 non-pregnant animals tested were diagnosed as non-pregnant with the specificity of 92.86% based on no blue color development (Plate 1). False negative diagnosis may be due to delay in the increase of PAG concentration in peripheral circulation. Low concentration of PAGs is due to either delay in increase in PAGs concentration or its decline 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).

The results of the present PAG study on whole blood showed comparable high sensitivity, but less specificity and accuracy than that (97-99%, 64-88%, 84-94% resp) reported by Northrop *et al.* (2019) using similar kit, at day 28 post-breeding. A lower sensitivity, specificity and accuracy has also been reported by Kacar *et al.* (2017) and Karakus *et al.* (2021) in blood samples tested for PAGs >30 days post-breeding. Lower accuracy and specificity can be attributed to delay in decline in the PAG concentrations due to early embryonic loss. In an earlier study, the sensitivity of IDEXX bovine pregnancy test was observed very high for both plasma and serum samples on 28 days after breeding, and concomitantly high specificity, *i.e.,* 95.1% for plasma and 93.8% for serum of cows or heifers (Velek *et al.*, 2011). Some other workers also recorded higher sensitivity of PAG ELISA (Commun *et al.*, 2016; Braganca *et al.*, 2018; Northrop *et al.*, 2019). However, higher accuracy has been documented by Piechotta *et al.* (2011) and Commun *et al.* (2016). High accuracy of PAG ELISA is due to increased immunoreactivity of proteins across pregnancy (Braganca *et al.*, 2018).

Cows with palpable slipping of fetal membranes with amniotic vesicle during per-rectal palpation on day 45-50 post-AI were considered pregnant. Out of 68 cows diagnosed as pregnant from second USG, 59 cows were diagnosed as pregnant on per-rectal palpation with sensitivity of 86.67%. However, 44 out of 48 cows were diagnosed as non-pregnant by palpation with specificity of 91.67%. False positive obtained in per-rectal palpation results due to presence of intraluminal fluid or pathological fluid in the uterus (chronic endometritis) leading to slight asymmetry of uterine horns. However, false negative results may be attributed to false tactile perception of double membrane slip during pregnancy diagnosis. Kastelic *et al.* (1989) mentioned that chorioallantoic fluid at day 18-21 and uterine fluid accumulated during estrus are difficult to differentiate.

In line with our results, Gowan *et al.* (1982) reported sensitivity and specificity of 83% and 95%, respectively, when per-rectal palpation was performed between 36 and 42 days post-breeding. Day *et al.* (1995) found that sensitivity varied among palpators (29-64%), with less variation in the specificity (99-100%) and overall accuracy rates for veterinarian-palpators (95 to 97%). Experienced veterinarians can achieve around an accuracy of 96% in predicting pregnancy via per-rectal palpation (Mathews and Morton, 2012). So, per-rectal palpation should be employed after 45 days post-breeding to obtain maximum accuracy in detection.

Table 1: Comparative diagnostic efficacy of pregnancy diagnostic methods at an early stage in dairy cattle

Parameters	Transrectal ultrasonography		PAG test	Per-rectal palpation
Days post-AI	28-35	45-50	28-35	45-50
Sensitivity (%)	97.06	100	93.33	86.76
Specificity (%)	91.67	100	92.86	91.67
PPV (%)	94.29	100	93.33	93.65
NPV (%)	95.65	100	96.30	83.02
Accuracy (%)	94.83	100	93.10	88.79

PPV = positive predictive value; NPV =Negative predictive value.

CONCLUSION

It can be concluded that TRUS, PAG test and per-rectal palpation are accurate and safe techniques to evaluate pregnancy at an early stage in cattle. TRUS and PAG tests are expensive methods but detect pregnancy earlier in the breeding cycle, which can assist in maintaining the reproductive efficiency. There are more benefits to TRUS as compared to PAG tests and per-rectal palpation as it is able to detect fetal structures and viability. Based on the above findings, in general two-time diagnosis should be done first at 28-35 days using either TRUS, PAG or rectal palpation followed by second examination by per-rectal palpation on 45-60 days post-AI for confirmation.

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