Comparative assessment of two tests for early pregnancy detection in dairy cattle

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Abstract

The objective of this study is to determine and compare the accuracy of two tests for early pregnancy diagnosis in dairy cattle by measuring pregnancy-associated glycoproteins (PAG), the Rapid Visual Pregnancy Test (Ubio quickVET; VPRT) and the commercial ELISA-PAG test (Bovine Pregnancy Test DG29®). Blood samples were collected from 180 cows between 28 and 35 days after artificial insemination (AI) to quantify the concentrations of PAG in each sample. Both tests were performed on plasma within two days after blood collection. Transrectal ultrasonography (TRUS) was performed for pregnancy diagnosis after 40 ± 3 days post-AI as a gold standard. Results indicated that the sensitivity (Se) of the VPRT and DG29 for diagnosing pregnant cattle were 90% and 100%, respectively. The specificity (Sp) of the two tests for diagnosing non-pregnant animals was 90.2% and 82%, respectively. The accuracy of both tests was 94% and 90%, for DG29 and VPRT respectively. The ability of both tests to distinguish between pregnant and non-pregnant cows was excellent. This implies that the VPRT test could be considered more accurate than the PAG-ELISA test and TRUS on days 28–35 after AI. The VPRT test, therefore, can be used as an alternative to the PAG-ELISA test with some constraints that need to be considered.

Keywords: Dairy Cattle, Pregnancy diagnosis, Pregnancy-associated glycoprotein, Rapid visual pregnancy test, ELISA.

Évaluation comparative de deux tests pour la détection précoce de la gestation chez les bovins laitiers

Résumé

Le but de cette étude est de déterminer et à comparer la précision diagnostique de deux tests des glycoprotéines associées à la gestation (PAG) ; Test rapide de gestation visuel (Ubio quickVET ; VPRT) et un test ELISA-PAG commercial (Bovine Pregnancy Test DG29®) pour le diagnostic précoce de la gestation chez les bovins laitiers. Des échantillons de sang ont été prélevés sur 180 vaches entre 28 et 35 jours après l'insémination artificielle (IA) pour quantifier les concentrations de PAG dans chaque échantillon. Les deux tests ont été effectués dans les deux jours suivant le prélèvement sanguin. Une échographie transrectale (TRUS) a été réalisée pour le diagnostic de gestation après 40 ± 3 jours post-AI comme référence. Les résultats ont indiqué que la sensibilité (Se) du VPRT et du DG29 pour le diagnostic des vaches gestantes était de 90 % et 100 %, respectivement. La spécificité (Sp) des deux tests pour le diagnostic des animaux non gravides était de 90,2 % et 82 %, respectivement. La précision des deux tests était de 94 % et 90 %, pour DG29 et VPRT respectivement. La capacité des deux tests à distinguer les vaches gestantes et non gestantes était excellente. Cela implique que le test VPRT pourrait être considéré comme plus précis que le test PAG-ELISA et TRUS les jours 28 à 35 après IA. Le test VPRT peut donc être utilisé comme une alternative au test PAG-ELISA avec certaines contraintes qui doivent être prises en compte lors de son utilisation.

Mots clés : Bovins laitiers, Diagnostic de gestation, Glycoprotéine associée à la gestation, Test de gestation visuel rapide, ELISA.

تقييم مقارن لإختبارين للكشف المبكر عن الحمل في الأبقار الحلوب

زينب مسافر، أنس بن مولى، بوشرى العيمري

ملخص

هدفت هذه الدراسة إلى تحديد ومقارنة الدقة التشخيصية لاثنين من اختبارات تشخيص الحمل المبكر لدى الأبقار الحلوب المعتمدة على البروتينات السكرية المرتبطة بالحمل؛ اختبار الحمل البصري السريع (VPRT ؛Ubio quickVET) واختبار ELISA-PAG التجاري (اختبار حمل الأبقارDG29 ®). تم جمع عينات الدم من 180 بقرة بين 28 و35 يومًا بعد التلقيح الاصطناعي (AI) لتحديد تركيزات PAG في كل عينة. تم إجراء كلا الاختبارين في البلازما بعد غضون يومين بعد جمع الدم. تم إجراء التصوير بالموجات فوق الصوتية (TRUS) لتشخيص الحمل بعد 40 ± 3 أيام بعد التلقيح الاصطناعي كمعيار. أشارت النتائج إلى أن حساسية (Se) لـ VPRT وDG29 لتشخيص الأبقار الحامل كانت 90٪ و100٪ على التوالي. كانت خصوصية (Sp) الاختبارين لتشخيص الحيوانات غير الحوامل 90.2٪ و82٪ على التوالي. كانت دقة كلا الاختبارين 94٪ و90٪ على التوالي لـ DG29 وVPRT. كانت قدرة كلا الاختبارين على التمييز بين الأبقار الحوامل والغيرحاملة ممتازة. هذا يعني أن اختبار VPRT يمكن اعتباره أكثر دقة من اختبار PAG-ELISA وTRUS في الأيام 28-35 بعد التلقيح الاصطناعي. لذلك، يمكن استخدام اختبار VPRT كبديل لاختبار PAG-ELISA مع بعض القيود التي يجب مراعاتها عند استخدامه.

الكلمات المفتاحية: الأبقار الحلوب، تشخيص الحمل، البروتين السكري المرتبط بالحمل، اختبار الحمل البصري السريع، .ELISA

Introduction

Early and reliable pregnancy detection in dairy cows is essential in reproductive monitoring (Inchaisri et *al.,* 2010). The challenge is diagnosing non-pregnant cows as early as possible to monitor them better or treat them to allow a re-insemination (Karen *et al.,* 2015). This early diagnosis makes it possible to reduce the calving-calving interval and thus optimize milk production over a cow's career (Al-Hassan and Al-Samawi, 2017). Various diagnostic methods presently utilized in veterinary practice to confirm pregnancy in cows include rectal palpation, transrectal ultrasound, and the determination of progesterone or pregnancy-associated proteins (PAGs) in blood and milk as estrone sulfate. Each method has advantages and disadvantages (Rockström *et al.,* 2017; Chang *et al.,* 2021). To ensure swift detection, a diagnostic procedure must possess specific attributes such as reliability, accuracy, practicality, swiftness, cost-effectiveness, and simplicity to perform under field conditions. This is crucial to avoid any diagnostic errors, as highlighted by Lucy *et al*. (2011). It is approved that PAGs are valuable biomarkers for determining early pregnancy in ruminant animals and can be measured in blood or milk samples, especially from days 26 to 30 after AI, as maternal blood levels before this time are highly variable and may compromise the accuracy of test results (Fricke *et al.,* 2016). Pregnancy-associated glycoprotein is a type of protein produced by the placenta and secreted into the maternal bloodstream of ruminant animals during pregnancy, where it can be detected as early as 28 days after conception (Mayo *et al.,* 2016). The levels of PAGs increase as pregnancy progresses, and different PAG isoforms are expressed at various stages of gestation (Northrop *et al.,* 2018).

Recently, commercial tests have been developed for detecting PAG in maternal cow plasma, serum, and whole blood and have provided rapid results by staining the samples, thereby allowing the analysis of many pieces simultaneously (Friedrich and Holtz, 2010; Karen *et al.,* 2015). More recently, a novel commercial lateral flow test has been developed for use in the dairy industry to detect PAG in plasma, serum, and whole blood samples (Mayo *et al.,* 2016; Moussafir *et al.,* 2018). This test does not require a specialized laboratory to detect PAG in a qualitative assessment, and the procedures to conduct the ELISA can be performed in field conditions (Akköse, 2023). Still, they should be used with other diagnostic tools and veterinary expertise to ensure the best possible outcomes for the animals and their owners.

The current study aims to assay pregnancy-associated proteins and compare the reliability of a Visual Pregnancy Rapid Test (VPRT) with a commercial PAG-ELISA test (DG29) in determining the gestation status of cows within 28 to 35 days after insemination. The study measures "early" PAGs in cows' blood during the initial pregnancy stage.

Material and methods

Experimental design

The study was conducted regarding the approval of the local ethics committee for the National Institute of Agricultural Research animal experiments. The study was carried out according to the standards of diagnostic accuracy test study reports (Bossuyt *et al.,* 2015). The study population consisted of 180 dairy cattle of varying ages, parity, and breeds from the same farm from January to October 2018. The herd managers chose the semen used for AI as part of the routine herd management.

Blood sampling

Blood samples were collected from all cows (180) at the time of pregnancy determination (28–35 days after AI) by jugular venipuncture into 10 mL heparinized vacutainer tubes. The day of AI was considered Day 0 for calculating the gestation period. Blood samples were transported to the laboratory and centrifuged at 2000 × g for 10 min at 4˚C. Plasma was collected and stored at -20 °C until pregnancy tests were performed.

Bovine pregnancy tests

Plasma samples were examined in duplicate using the DG29 test (Bovine Preg Test DG29®, by CONCEPTION, Beaumont, Québec, CANADA) and a Visual Pregnancy Rapid Test VPRT (Ubio quickVET, Rubio Biotechnology Systems Pvt Ltd. Kalamassery, Cochin, Kerala, India) according to the manufacturer's instructions.

The DG29 test is a laboratory enzyme-linked immunosorbent assay (ELISA) type test, a competitive two-step immune enzymatic assay. Two antibodies specific to two different epitopes on the target antigen are used. The capture antibody is bound to the bottom of the microplate well and binds to an epitope of the antigen. The detection antibody binds to the antigen at a different epitope and is conjugated to an enzyme that allows detection (If the detection antibody is not conjugated, then a detection antibody conjugated to a second enzyme is required). The kit includes the modular ELISA plates (12 × 8 modules) and the necessary reagents for blood sample analysis in cattle. The standard curve ranges from 0 to 3500 pg/ml. A Stat Fax Microplate Reader (Stat Fax ® 4200 Microplate Reader) was used to measure the absorbance at 450 nm. The results were calculated on a standard curve plotted using the simple linear regression equation (Fig.1).

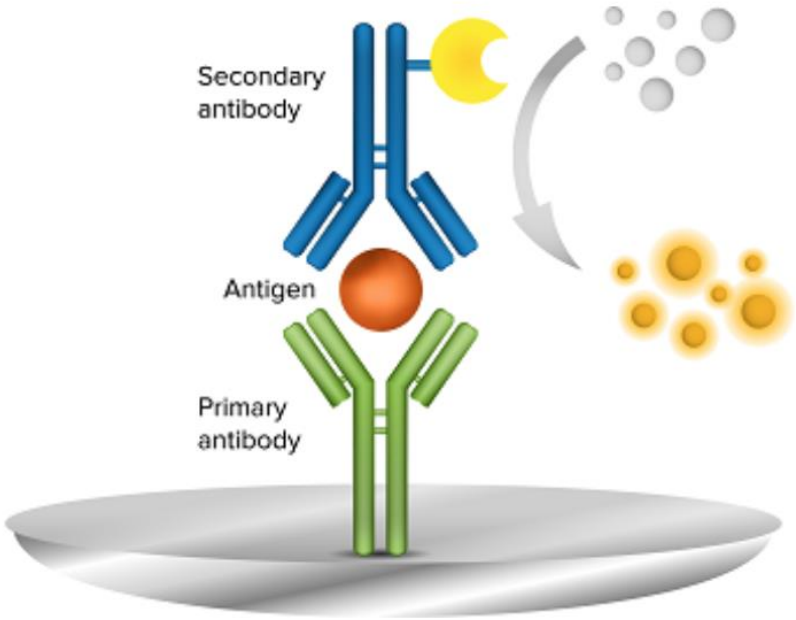

Figure 1. Principle of the PAG-ELISA test of (Bovine Pregnancy Test DG29®)

For the VPRT, the test is a one-step immune-chromatographic assay that detects PAGs in the serum of cattle, plasma, or whole blood. The device must be kept horizontal throughout the process to ensure accurate results. Before use, the plasma samples should be mixed. Squeeze the upper bulb of the pipette to entirely dispense the sample into the sample port of the device. One drop of whole blood or serum samples and two drops of a washing solution were added to the test wells, do not exceed 2 minutes between adding blood and wash solution, and incubated for 15 min. If both test, T, and control, C, lines appear before 15 minutes, the animal is pregnant, and the test is complete. If there is blood in the result window, allow 5 to 10 minutes for the window to clear.

Transrectal ultrasonography

Pregnancy status was determined via transrectal ultrasonography of 180 cattle for pregnancy after 40 ± 3 days following AI, which was used as the gold standard reference test. It serves to compare its sensitivity with that of VPRT and PAG-ELISA tests. The same operator performed transrectal ultrasonography examinations using a real-time B-mode ultrasonic device (Falco Vet, Esaote/Pie Medical, Maastricht, Netherlands) with a 6–8 MHz linear endo-rectal transducer.

Statistical analysis

All assays were performed according to the manufacturer's instructions. The results were presented as figures and compared between VPRT and PAG ELISA tests ultrasonography based on plasma collected between 28 and 35 days after AI. The results are using transrectal ultrasound after 40 ± 3 days after AI. The results were ranked according to the method of Barbry *et al.* (2012) as follows:

- Sensitivity (Se): number of true-positive results / (number of true-positive results + number of false-negative results).
- Specificity (Sp): number of true-negative results / (number of true-negative results + number of false-positive results).
- Positive predictive value (PPV): number of true-positive results / (number of true-positive results + number of false-positive results).
- Negative predictive value (NPV): number of true-negative results / (number of true-negative results + number of false-negative results).
- Accuracy (AC): (number of true-positive results + number of true-negative results) / (number of true-positive results + number of true-negative results + number of false-positive results + number of false-negative results).

The results were calculated based on the correct/incorrect positive or negative diagnoses.

Results

The VPRT and PAG-ELISA tests were used to assess the pregnancy status of a group of cows. During the diagnosis tests involving the assay of PAGs, VPRT, and TRUS, 180 cows were examined. The PAG-ELISA test has confirmed that 119 cows were pregnant and 48 were non-pregnant. The VPRT test diagnosed 107 cows as pregnant and 64 as non-pregnant. Furthermore, 115 cows were diagnosed as pregnant based on ultrasonography performed on Day 40 ± 3.

Of the 180 cows examined, nine were classified as having non-differentiating results by the VPRT test, while 13 were classified as such by the PAG-ELISA test. These data were excluded from the statistical analysis presented in Table 1.

Table1. Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy (%) for the Pregnancy Associated Glycoproteins test (PAG ELISA), Visual Pregnancy Rapid Test (VPRT) test using plasma, results interpreted by using transrectal ultrasonography (TRUS) as the reference standard for pregnancy diagnosis in dairy cows between 28 and 35 days after AI.

	PAG-ELISA (DG 29) (%)	**VPRT (%)**
Sensitivity (Se)	100	90
Specificity (Sp)	82	90.2
Positive predictive value (PPV)	92	95
Negative predictive value (NPV)	100	82
Accuracy (AC)	93	91

Regarding false diagnoses, the VPRT test yielded six false positives and 12 false negatives, while the PAG-ELISA test resulted in 10 false positives but no false negatives (Fig. 2).

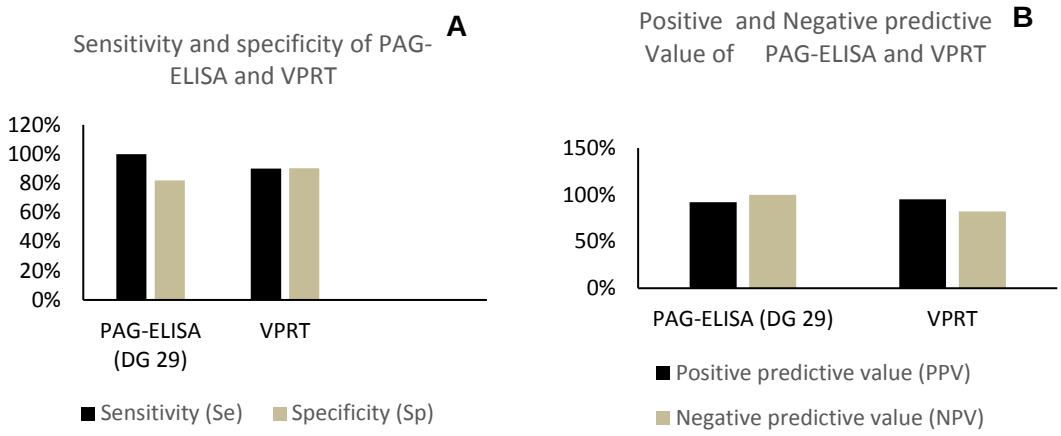

Figure 2. A. Comparison of the Sensitivity, Specificity of both tests PAG-ELISA and VPRT in dairy cattle between 28 and 35 days after AI. B. Comparison of the positive and negative predictive values of both tests PAG-ELISA and VPRT in dairy cattle between 28 and 35 days after AI.

To evaluate pregnancy diagnosis between 28 and 35 days after AI, the results obtained from PAG-ELISA and VPRT tests were compared to those based on TRUS assessments 40 ± 3 days after AI. There was no difference in the sensitivity (pregnant, correctly diagnosed pregnant) and specificity (non-pregnant, correctly diagnosed non-pregnant) of the VPRT test (90 % and 90.2 %, respectively). However, there was a difference in the sensitivity and specificity of the PAG-ELISA (100% and 82 %, respectively). In the positive predictive value (PPV), results obtained in the PAG-ELISA and the VPRT tests were 92 % and 95 %, respectively. Both trials' negative predictive value (NPV) was 100% and 82 %, respectively. The overall accuracy of the PAG-ELISA and VPRT tests between 28 and 35 days after AI was 93% and 91% (Fig. 3).

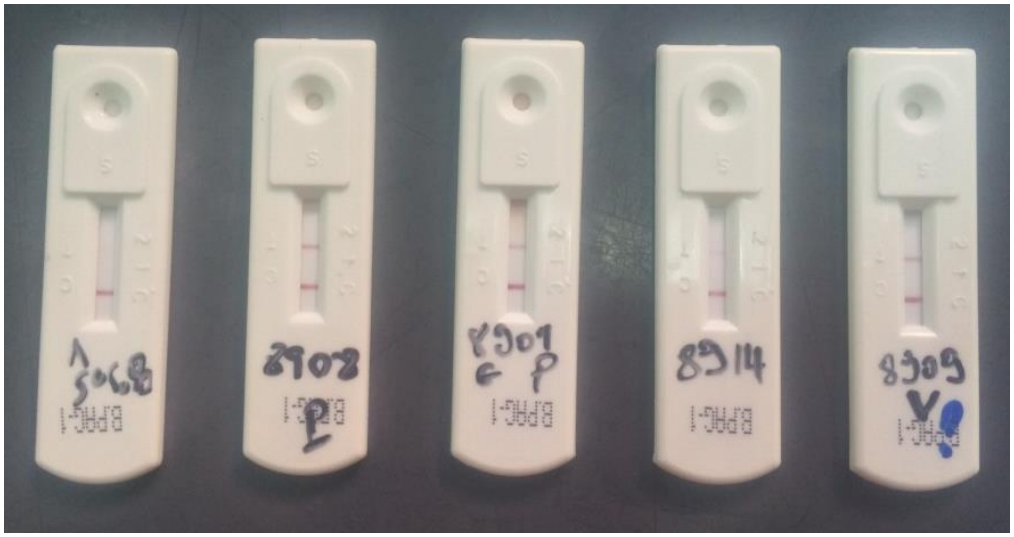

Figure 3. Photograph of the VPRT pregnancy test of five cows. The test line may range from dark to very light. The presence of a red test line T is considered Pregnant. The absence of a test line indicates that the cow is Open.

Discussion

It is essential in reproduction monitoring to quickly identify empty cows to save time between the previous calving and the mating. For this, the test must be specified by giving as few false positives as possible. Transrectal ultrasonography is the most common method for pregnancy detection in cattle, although a more accessible and cheaper way is needed, such as chemical pregnancy detection (Ott *et al.,* 2014). In the past, PAG detection was conducted using the radioimmunoassay (RIA) method in early studies. However, due to the potential risks posed by radioactive substances to human and environmental health, PAG-ELISA tests were developed and can be used on milk and blood samples (Green *et al.,* 2005). Despite this development, previous studies have shown that PAG-based pregnancy diagnosis can still achieve high accuracy rates as early as day 28 post-artificial insemination (AI) (Reese *et al.,* 2018; Szenci *et al*., 2021). The present study showed that the PAG-ELISA and VPRT, which determined PAG concentrations, produced very accurate results for early pregnancy diagnosis 28–30 days after AI (93% and 91%, respectively). Using similar tests, M. Akköse (2023) reported the sensitivity, specificity, PPV, NPV, and accuracy for the RVPT on day 28 post-AI as 98%, 85%, 87%, 98%, and 92%, respectively. Northrop *et al.* (2019) found that the RVPTOD (lateral flow test) was less sensitive, with decreased percent correct and reduced positive predictive values compared to the BPT, RVPT (two bovine pregnancy test) score, and RVPTY/N. The difference between the accuracies of the two tests (93% and 91%) could have resulted because of samples being collected on different days after AI (28–35) and because accuracy usually increases as the stage of pregnancy advances (Humblot *et al.,* 1988).

Based on sensitivity, the PAG-ELISA was an excellent test (100%). However, the VPRT was less sensitive (90%) with decreased positive predictive values (95%) compared to the PAG-ELISA test (92%). Results of 96.6% and 97.8% for sensitivity were less than those reported in other studies using different commercial ELISA tests between days 26 and 58 after AI (Silva *et al.,* 2007; Piechotta *et al.,* 2011). In contrast, other studies which used different commercial PAG-ELISA tests for pregnancy detection when samples were collected between days 25 and 29 after AI indicate the sensitivity for the assay ranged from 93.9% to 98.8% (Silva *et al.,* 2007; Green *et al.,* 2009; Romano and Larson, 2010; Piechotta *et al.,* 2011; Sinedino *et al.,* 2014). This difference can be attributed to less than the results obtained in the present study using different antisera to detect PAG in various commercial tests. More than 22 transcribed coding genes for bovine pregnancy-associated glycoproteins (bo-PAG) are expressed on day 45 of pregnancy, highlighting the importance of recognizing their significance (Green *et al.,* 2000). The outcomes of the PAG-ELISA and VPRT tests employed in the present study align with previous research findings. However, the VPRT has some limitations in this study, including the requirement to perform the test after 35 days from the artificial insemination date (AI).

False positive results produced by PAG tests may be caused by several reasons previously discussed by Bragança *et al*. (2018). One of the main factors that influence the profitability of beef and dairy herds is embryonic mortality occurring before 30 days. Early embryonic losses analyzed as occurring prior between days 24-27 in dairy cattle account for approximately 57% of all pregnancy losses (Lopez-Gatius *et al.,* 2007), and these animals that lose an embryo will either conceive late in the breeding season or fail to conceive during a defined breeding season. A significant decrease may be

reported in maternal circulation due to PAG having a long half-life of approximately eight days (Mialon *et al.,* 1993).

Compared with ultrasonography, both test results showed a desirable accuracy (93% and 91%, respectively) for identifying pregnant cows 28 to 35 days after AI. A small number of cows may explain the slightly lesser specificity when using the PAG-ELISA test with a weak signal and a false positive result (Piechotta *et al.,* 2011; Karen *et al.,* 2015; Mayo *et al.,* 2016). Moreover, the VPRT test has somewhat less sensitivity (90%), and the lack of PAG needs to be clarified. Still, it may be due to differences in herds' numbers of primiparous and multiparous cows (Ricci *et al.,* 2015). Thus, the results of the present study indicate that the VPRT test can be used in place of the PAG-ELISA test with some constraints to consider.

Conclusion

Both pregnancies test, the VPRT and the PAG-ELISA were accurate at determining pregnancies in plasma samples collected between 28- and 35-days post-AI. Moreover, The VPRT test can be used as an alternative to transrectal ultrasonography. Nevertheless, it should be noted that the need to be confirmed by a supplemental assay when detecting early pregnancy among cows on day 25 after AI.

Conflict of interest

None of the authors have any conflict of interest to declare.

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