ACCURACY OF PREGNANCY-ASSOCIATED GLYCOPROTEIN-ENZYME-LINKED IMMUNOSORBENT ASSAY (PAG-ELISA) IN EARLY PREGNANCY DETECTION IN WATER BUFFALOES

Roseline D. Tadeo*, Eufrocina P. Atabay, Edwin C. Atabay,
Carlito F. Dela Cruz, and Jhon Paul R. Apolinario

ABSTRACT

The present study compared the accuracy of PAG-ELISA in blood and milk samples from water buffaloes. Fifty-three lactating buffaloes were subjected to fixed-time artificial insemination (FTAI). The PAG level in blood was determined on days 25 and 30, while in milk it was determined on days 26 and 30 post-FTAI. Transrectal ultrasonography (TRUS), the gold standard for pregnancy diagnosis, was done on day 40 post-FTAI. Out of 53 cows inseminated, 30 were confirmed pregnant by TRUS. In these pregnant animals, 93% and 100% sensitivity were achieved in blood at days 25 and 30, respectively, while 40% and 93% were achieved in milk at days 26 and 30, respectively. In terms of specificity, 100% of the assay was achieved in both blood and milk samples on all days of collection. On day 30, 100% overall accuracy for blood was achieved, while it was only 96% for milk. Blood PAG-ELISA is more accurate and can detect pregnancy earlier than its milk counterpart. Milk PAG-ELISA, however, can serve as an alternative for being a non-invasive and stress-free method. In conclusion, PAG-ELISA provides a highly efficient and reliable means to detect early pregnancy and facilitate rebreeding program in water buffaloes.

Keywords: blood, milk, Pregnancy-associated glycoprotein, pregnancy diagnosis, water buffalo

INTRODUCTION

Untimed and inaccurate pregnancy diagnosis are factors contributing to the long calving interval in cows, which is one of the major limiting factors for improving reproductive performance in buffalo and can lead to decreased milk yields and meat production in the dairy sector (Nanda *et al.*, 2003). Therefore, accurate and early detection of pregnancy in dairy cows is an essential component of today's reproductive management programs to enable faster rebreeding and shorten the calving interval, thereby maximizing milk production and farm revenue. There are several methods that have been developed and being used for pregnancy detection. Widely and commonly used pregnancy methods are rectal palpation, which is only accurate from day 45 (Arthur *et al.*, 1996), and transrectal ultrasonography (TRUS), which is accurate from days 30-39 (El-Shahat *et al.*, 2004; Youngquist, 2006). Both are simple and economic methods for pregnancy

Reproduction and Physiology Section, Philippine Carabao Center, National Headquarters and Gene Pool, Science City of Muñoz, Nueva Ecija, Philippines 3120 (*email: roselinetadeo@gmail.com).

diagnosis; however, these methods might increase the risk of iatrogenic embryonic mortality (Arthur *et al.*, 1996) and require expensive instruments with an experienced and skilled operator to perform the diagnosis (Lucy *et al.*, 2011). With the advancement of molecular techniques like proteomics and their applications in animal research, there is new hope to look for pregnancy biomarker molecules, especially in livestock species.

Most commonly used present day methods to detect pregnancy is the Enzyme-linked Immunosorbent Assay (ELISA) that has been used for the detection of proteins and hormones in different biological samples. ELISA provides a laboratory-based method for accurate detection of pregnancy and an important tool for the identification of open cows in dairy herd to shorten calving interval (Balhara *et al.*, 2013). Pregnancy-associated glycoprotein (PAG), on the other hand, is one of the most commonly known and used biomarkers for detecting early pregnancy in ruminant species. It is expressed in the outer epithelial layer of the placenta, which persists from the moment of conception throughout the calving period (Touzard *et al.*, 2013). Thus, PAG has become a useful tool for detecting and monitoring pregnancy in ruminant species (Reese *et al.*, 2018); however, maternal circulation profiles of PAG might differ in terms of the earliest day of pregnancy detection until gestation period and also their accuracy. Profiling of PAG level has been recently studied in water buffalo in the Philippines and showed early detection of pregnancy at day 26 in milk and day 25 in blood samples (Tadeo *et al.*, 2021). However, accuracy on these specified days of detection has not yet been examined. The aim of the present study was to evaluate the accuracy in terms of sensitivity and specificity of PAG-ELISA in milk (days 26 and 30) and blood samples (days 25 and 30) in water buffalo. Essentially, the present study constitutes the first attempt in the country to establish and determine the efficiency and use PAG as an early pregnancy diagnosis in water buffaloes and the incorporation of the technology into the current program of reproduction in this species.

MATERIALS AND METHODS

Induction of synchronous ovulation for Fixed Timed Artificial Insemination (FTAI)

A total of fifty-three open lactating water buffaloes at the Philippine Carabao Center, National Gene Pool with least 60 days' post-partum, free from clinical mastitis, with a body condition score of at least three, and with at least one ovary measuring 2 cm in size, were selected and subjected to ovulation synchronization and FTAI.

In brief, the animals received two (2) mL intramuscular (IM) injection of Gonadotropin-releasing hormone (GnRH) (100 µg; Cystorelin, Merial Ltd., GA, USA,) simultaneous with the insertion of Controlled Internal Drug Release (CIDR) (1.38 g progesterone; Eazi-Breed CIDR, DEC International, NZ. Ltd.) on day 0. CIDRs were removed, and buffaloes received five (5) mL injection of Prostaglandin F2α (PGF2α) (25 mg; Lutalyse, dinoprost tromethamine, Pharmacia & Upjohn Co., MI, USA) on Day 7. Two (2) mL of human Chorionic Gonadotropin (hCG) (1500 IU, Chorulon, Intervet Inc., Summit, NJ 07001, USA) was given on day 9, and timed Artificial Insemination was performed on day 10 of the FTAI program.

Blood and milk sample collection

All milk samples were collected prior to the scheduled milking of the animals in the Philippine Carabao Center Genepool Milking Parlor. Routine cleaning and disinfection

of functional quarters were followed. After the stripping of the foremilk, a total of five (5) mL of milk was collected from all healthy quarters of the udder of the water buffalo and placed into a 5-mL screw-cap tube. Milk samples were collected on days 26 and 30 post-FTAI. Samples were kept frozen at -20ºC prior to PAG-ELISA. All collected samples were triplicated during ELISA plating as a standard protocol.

On the other hand, ten (10) ml of blood samples from the jugular vein of the buffaloes were collected at days 25 and 30 post-FTAI. Plasma samples were collected and stored at -20ºC prior to the PAG ELISA. Similar to the milk samples, all blood samples were triplicated during ELISA plating as a standard protocol.

Pregnancy Associated Glycoprotein (PAG) ELISA analysis

The PAG ELISA analysis for both blood and milk samples were performed using commercial IDEXX kits (IDEXX Bovine Pregnancy Test and IDEXX Milk Pregnancy Test, IDEXX Laboratories, USA) following the manufacturer's instructions.

The 96-well plate format was coated with an anti-PAG monoclonal antibody raised against the PAG-55 protein fractions comprising PAG-4, PAG-6, PAG-9, PAG-16, PAG-18, and PAG-19 (Nagappan *et al.*, 2009). Plasma and milk samples in the 96-well plates were incubated at 37°C for 1 h for plasma and 2 h for milk with shaking. Following incubation, PAGs in the sample were determined by a detector solution and the secondary antibody (horseradish peroxidase conjugate). Unbound conjugates were washed using washing buffer solution, and 3,3′,5,5′- tetramethylbenzidine substrate was added for color development, which is relative to the amount of PAGs in the sample and was measured using a spectrophotometer. Results were calculated from the optical density (OD) of the sample [450 nm (for sample and control) and 620–650 nm (for reference)]. Corrected OD values for samples (S) and controls (N) was calculated as S-N= [Sample (450-ref)-Negative Control (NC)].

Pregnancy outcomes were determined based on cut-off values provided by the PAG ELISA manufacturer. As a standard reference for interpretation for milk PAG ELISA, when the S-N value is > 0.25, the cow is considered "pregnant", when the S-N value is > 0.1 to < 0.25, the result says "recheck" and if the S-N value is <0.1, the cow is considered "not pregnant" Meanwhile, for blood PAG ELISA, when the S-N value is ≥ 0.3, the cow is considered "pregnant" and if the S-N value is < 0.3, the cow is "not pregnant".

Transrectal Ultrasonography (TRUS)

All animals used in the study were subjected to pregnancy diagnosis by ultrasonography on day 40 post-FTAI. The ultrasound examination was performed using a transrectal ultrasound scanner (Honda, HS-1600V, Japan) equipped with a 7.5 MHz linear array transducer designed for intrarectal placement (Mehrajuddin *et al.*, 2013). The scanning of the uterine horns was performed on their dorsal and lateral surfaces. Pregnancy status was determined following the criteria described by Fricke *et al.* (2016) with some modifications. Briefly, the criteria include the presence or absence of corpus luteum (CL), uterine fluid, and embryo. Cows were considered pregnant when CL, uterine fluid, and an embryo with a heartbeat were present upon examination.

Data Analysis

The pregnancy diagnosis that was obtained by the PAG-ELISA test was categorized

based on (a) correct positive, (b) false positive, (c) correct negative, and (d) false negative. TRUS was set as the gold standard for pregnancy diagnosis on day 40 via conceptus heartbeat. The diagnostic values were calculated by using the number of animals used in each diagnostic category. Calculation formulas for the diagnostic values were obtained from Martin *et al.* (1987). The sensitivity (Se) indicates the probability that the ELISA correctly indicates pregnancy = number of correct positive pregnancy diagnoses/all pregnant cows based on TRUS (a/(a + d) x 100); the specificity (Sp) is the probability that the ELISA correctly indicates a cow as open = number of correct negative pregnancy diagnoses by the test/all nonpregnant cows based on TRUS (c/(c + b) x 100); the positive predictive (PPV) is the proportion of cows truly pregnant among cows identified as pregnant by ELISA = number of correct positive pregnancy diagnoses/number of diagnosed pregnant by blood test or TRUS (a/(a + b) × 100); the negative predictive value (NPV) is the proportion of cows truly open among cows identified as not pregnant by ELISA = number of correct negative pregnancy diagnoses/number of diagnosed non pregnant by blood test or TRUS (c/(c + d) × 100) and the overall accuracy (Ac) is the proportion of true results (both true positives and true negatives) as determined by the reference test method relative to the total number of ELISA = number of correct positive pregnancy diagnoses + number of correct negative pregnancy diagnoses/total number of cows tested by diagnostic tests ((a + c)/(a + b + c + d) × 100). Differences between pregnant and non-pregnant buffaloes in the level of PAG were statistically analyzed by using an independent t-test.

RESULTS

Based on TRUS pregnancy diagnosis at day 40 post-FTAI, 30 pregnant cows were confirmed out of 53 inseminated cows. TRUS served as the gold standard for the computation of sensitivity, specificity, predictive values, and overall accuracy of PAG-ELISA in milk and blood samples from water buffaloes. In these pregnant animals, 93% and 100% sensitivity were achieved in blood at days 25 and 30, respectively, while 40% and 93% were achieved in milk at days 26 and 30, respectively. In terms of specificity, 100% of the assay was achieved in both blood and milk samples on all days of collection. (Table 1). PAG-ELISA in blood showed a higher sensitivity as early as day 25 as compared with milk, which had a low sensitivity at day 26. Moreover, all the false negatives identified in both assays did not reach the threshold of PAG level that would consider the animals pregnant. In terms of predictive values, PPV in blood and milk obtained 100% in all periods of examination. On the other hand, NPV in blood at days 25 and 30 was 92% and 100%, respectively, while in milk at days 26 and 30, it was 56% and 92%, respectively. Overall accuracy of blood PAG-ELISA was 96% at day 25 and increased to 100% at day 30, while milk PAG-ELISA was 66% at day 26 and increased to 96% at day 30 post-FTAI.

The PAG level in pregnant cows was significantly higher ($P< 0.05$) than in non-pregnant cows in all periods of examination in both blood and milk PAG-ELISA tests. Moreover, pregnant cows PAG levels in both assays increased significantly at day 30, with blood levels observed being significantly higher than in milk (Table 2).

Table 1. Sensitivity (Se), specificity (Sp), positive predictive value (+PV), negative predictive value (-PV), and overall accuracy of the blood and Milk PAG-ELISA test in water buffaloes based on the actual gold standard (TRUS).

Evaluated Criteria	Blood PAG-ELISA (n=53)		Milk PAG-ELISA (n=53	
	Day 25	Day 30	Day 26	Day 30
Correct positive diagnoses (a)	28	30	12	28
False positive diagnoses (b)	0	0	0	0
Correct negative diagnoses (c)	23	23	23	23
False negative diagnoses (d)	2	0	18	2
Sensitivity (%)	93	100	40	93
Specificity (%)	100	100	100	100
Positive predictive value (%)	100	100	100	100
Negative predictive value (%)	92	100	56	92
Overall accuracy (%)	96	100	66	96

Table 2. Levels (mean ± S.D.) of blood and milk PAG (OD value) in pregnant and non-pregnant water buffaloes.

	Blood PAG-ELISA		Milk PAG-ELISA	
	25	30	26	30
Pregnant	$0.69 \pm 0.09^{a,1}$	$1.51 \pm 0.10^{a,2}$	$0.47 \pm 0.09^{a,1}$	$0.77 \pm 0.08^{a,2}$
Non-pregnant	$0.02 \pm 0.01^{b,1}$	$0.03 \pm 0.03^{b,1}$	$0.07 \pm 0.08^{b,1}$	$0.05 \pm 0.08^{b,2}$

[a,b] means with different superscript within the column differ significantly
[1,2] means with different superscript within the row differ significantly

DISCUSSION

Early and reliable pregnancy diagnosis is a valuable strategy to shorten calving interval, improve average milk production, and increase the overall reproductive efficiency and profitability from livestock and dairy production. Detection and measurement of PAG in blood and milk using ELISA tests offer an alternative, simple, early, and non-invasive technique for early pregnancy detection, especially in water buffalo. In our recent study in water buffalo, the earliest possible day PAG can be detected in milk and blood samples for pregnancy detection was determined, wherein pregnant cows were detected through PAG as early as day 25 post-FTAI in blood and 26 post-FTAI in milk (Tadeo *et al.*, 2021). However, the evaluation of accuracy of these test results at specified days in both samples has not been done yet; hence, the present study.

The present study compared the accuracy of blood and milk PAG ELISA as early pregnancy diagnosis tools in water buffalo. The sensitivity of blood PAG-ELISA at day 25 was relatively similar to the other finding in dairy cattle, with 92–98% between days 23–28 (Gajewski *et al.*, 2014; Durocher *et al.*, 2022), and reached 100% at day 30, similarly to the findings of Commun *et al.* (2016) in cattle. In buffalo cows, a study conducted using PAG-RIA showed a low sensitivity of 80% at days 25–30 (Karen *et al.*, 2007) as compared to the present study using an ELISA test. This indicates that PAG-ELISA is a more sensitive test as compared with PAG-RIA. On the other hand, sensitivity in milk PAG-ELISA is much lower at day 26 as compared to blood; however, it increased at day 30 but did not reach 100% sensitivity. The present result conforms with that in cattle with 93% at day 28 and 97% on day 35 (Gajewski *et al.*, 2014) and other findings with 98% at day 30 (Commun *et al.*, 2016). Since PAG levels in the blood were two-fold higher due to the abundance of PAG present in maternal blood circulation than in the mammary gland or in milk samples (Tadeo *et al.*, 2021), the chances of a false negative diagnosis using milk PAG-ELISA, especially at an early stage of gestation, were higher due to the delayed PAG increase in pregnant cows. Therefore, blood PAG-ELISA was a more sensitive test compared with milk PAG-ELISA for early detection of pregnancy.

The 100% specificity of blood and milk PAG-ELISA tests for diagnosing non-pregnant buffalo cows in all days of examinations in the present study was much higher than those reported in other studies conducted in cattle which ranged from 53%–60% after day 28 (Gajewski *et al.*, 2014) and increased from 88.9%–100% from days 30-35 (Gajewski *et al.*, 2014; Commun *et al.*, 2016; Durocher *et al.*, 2022). In buffalo, the PAG-RIA test in blood showed high (>90%) specificity from days 19–55 (Karen *et al.*, 2007). Lower specificity obtained from a false-positive diagnosis could be attributed to embryo death or residual PAG from the last calving. In the present study, there is no false positive diagnosis result since the collection of blood and milk samples was ensured to be more than 60 days post-partum to avoid the possible presence or overlap of PAG from previous pregnancy. Previous work supports the idea that PAG levels are high during the late gestation period and that it takes up to 60 days for PAG residuals to be cleared from maternal circulation after parturition (Haugejorden *et al.*, 2006; Giordano *et al.*, 2012).

The present study showed that blood PAG-ELISA has a higher accuracy when it comes to diagnosing pregnant and non-pregnant cows than milk PAG-ELISA, which just started to achieve high accuracy but not 100% at day 30, as compared to blood PAG-ELISA. Moreover, the present percent accuracy of PAG-ELISA in buffalo blood and milk are higher compared with those of cattle with 92% and 89% at day 32, respectively (Ricci *et al.*, 2014). Based on the results of the present study, the optimal time to conduct pregnancy diagnosis in blood, with 100% accuracy, is around day 30 post-FTAI, while in milk it is beyond 30 days after AI service. But PAG-ELISA levels at day 25 for blood and day 30 for milk were found high enough and provide reliable results. milk PAG-ELISA however offers advantage and alternative approach for adult lactating water buffalo, but not for heifer, as being convenient, stress-free sample collection for early pregnancy test.

Essentially, the present study demonstrated PAG-ELISA as an alternative, simple, early, and non-invasive technique for detecting and monitoring pregnancy in water buffaloes. Furthermore, early pregnancy detection through PAG-ELISA is a valuable reproductive tool to identify and rebreed non-pregnant or open cows which can shorten the calving interval and enhance reproduction in buffaloes. Lastly, PAG-ELISA in tandem with timed Artificial

insemination, as demonstrated in the present study, constitute technological innovations for intensified reproduction to maximize reproductive efficiency and profitability from buffalo and livestock production.

CONCLUSION

The present study demonstrated that blood and milk PAG-ELISA are effective, convenient, and reliable means of determining early pregnancy and can be used as an alternative method to the Transrectal Ultrasonography in water buffaloes. Blood PAG-ELISA revealed a higher accuracy compared to milk PAG-ELISA during the early stages of pregnancy. Milk PAG-ELISA though, has the limitation of not being suitable for use in heifers, but it can be an alternative approach for lactating adult buffaloes as being convenient and stress-free method for early pregnancy detection. PAG-ELISA, in general, is an effective and valuable addition to the reproductive toolbox for intensive and maximized reproduction in water buffaloes.

ACKNOWLEDGMENTS

This research work was supported by the Philippine Carabao Center, Department of Agriculture. Grateful acknowledgement is accorded to Joseph Oliver C. Lofranco, Joselito V. Del Rosario, Rodante V. De Vera and Mike V. Del Rosario for their valuable help in fixed time artificial insemination and sample collection activities.

REFERENCES

Arthur GH, Noakes DE, Pearson H and Parkinson TJ. 1996. Reproduction in the buffalo. In: Veterinary Reproduction and Obstetrics. London: WB Saunders Company Ltd.; p. 669.

Balhara, AK, Gupta M, Singh S, Mohanty AK and Singh I. 2013. Early pregnancy diagnosis in bovines: current status and future directions. *J Reprod Fertil* 13:1-5.

Commun L, Velek K, Barbry J, Pun S, Rice A, Mestek A, Egli C and Leterme S. 2016. Detection of pregnancy-associated glycoproteins in milk and blood as a test for early pregnancy in dairy cows. *J Vet Diagn Invest* 28(3):207–213.

Durocher J, Moore RK, Castonguay MH, Albaaj A, LeBlanc SJ and Dufour S. 2022. Bayesian estimation of sensitivity and specificity of a milk pregnancy associated glycoprotein ELISA test for pregnancy diagnosis between 23 and 27 days after insemination in Holstein dairy cows. *J Dairy Sci* 105(8):6985–6996.

El-Shahat KH, Hegab AO and El-Nattat WS. 2004. Ultrasonographic findings and progesterone profile during early pregnancy diagnosis in buffalo cows. *Egypt J Basic Appl Physiol* 3:57–63.

Fricke P, Ricci A, Giordano J and Carvalho P. 2016. Methods for and implementation of pregnancy diagnosis in dairy cows. *Vet Clin North Am Food Anim Pract 32*(1):165–180.

Gajewski Z, Petrajtis-Gołobów M, Melo de Sousa N, Beckers JF, Pawliński B and Wehrend A. 2014. Comparison of accuracy of pregnancy-associated glycoprotein (PAG) concentration in blood and milk for early pregnancy diagnosis in cows. *Schweiz Arch fur Tierheilkd* 156(12):585– 590.

Giordano JO, Guenther JN, Lopes G and Fricke PM. 2012. Changes in serum pregnancy-associated glycoprotein, pregnancy-specific protein B, and progesterone concentrations before and after induction of pregnancy loss in lactating dairy cows. *J Dairy Sci* 95(2):683-697.

Haugejorden, G, Waage S, Dahl E, Karlbert K, Beckers JF and Ropstad E. 2006. Pregnancy associated glycoproteins (PAG) in postpartum cows, ewes, goats and their offspring. *Theriogenology* 66(8):1976-1984.

Karen A, Darwish S, Ramoun A, Tawfeek K, Van Hanh N, de Sousa NM, Sulon J, Szenci O and Beckers JF. 2007. Accuracy of ultrasonography and pregnancy-associated glycoprotein test for pregnancy diagnosis in buffaloes. *Theriogenology* 68(8):1150-1155.

Lucy M, Green J and Poock S. 2011. Pregnancy determination in cattle: a review of available alternatives. In: *Proceedings, Applied Reproductive Strategies in Beef Cattle*. Joplin MO, pp. 367–375.

Martin SW, Meek AH and Willeberg P. 1987. *Veterinary Epidemiology, Principles and Methods*. Ames, IA: Iowa State University Press.

Mehrajuddin N, Patel DM and Derashri HJ. 2013. Early pregnancy diagnosis by transrectal ultrasonography in mehsana buffaloes (*Bubalus bubalis*). *Buffalo Bull* 32(2):120-125.

Nagappan M, McGrath M and Schenkel R. 2009. *Methods for early detection of pregnancy in cows*. Monsanto Technology LLC, assignee. United States. Patent No. 7,604,950 B2.

Nanda AS, Brar PS and Prabhakar S. 2003. Enhancing reproductive performance in dairy buffalo: major constraints and achievement. *Reprod Suppl* 61:27–36.

Reese ST, Pereira MHC, Edwards JL, Vasconcelos JLM and Pohler KG. 2018. Pregnancy diagnosis in cattle using pregnancy associated glycoprotein concentration in circulation at day 24 of gestation. *Theriogenology* 106:178–185.

Ricci A, Carvalho PD, Amundson MC, Fourdraine RH, Vincenti L and Fricke PM. 2014. Factors associated with pregnancy-associated glycoprotein (PAG) levels in plasma and milk of Holstein cows during early pregnancy and their effect on the accuracy of pregnancy diagnosis. *J Dairy Sci* 98(4):2502–2514.

Tadeo, RD, Atabay, EP, Atabay EC, Matias D, Fajardo ZP, Apolinario JPR, Dela Cruz C and Tilwani RC. 2021. Early pregnancy diagnosis in water buffaloes through detection of pregnancy-associated glycoprotein (PAG) in milk using Enzyme-link immunosorbent assay. *Thai H Vet Med 51*(1): 35–41.

Touzard E, Reinaud, P, Dubois O, Guyader-Joly C, Humblot, P, Ponsart C and Charpigny G. 2013. Specific expression patterns and cell distribution of ancient and modern PAG in bovine placenta during Pregnancy. *Reproduction* 146(4):347–362.

Youngquist RS. 2007. Pregnany diagnosis. In: Youngquist RS and Threlfall WR, eds. 2nd ed. *Current Therapy in Large Animal Theriogenology*, Missouri: Saunder Elsevier, pp. 294–303.