

# Early pregnancy diagnosis in water buffaloes through detection of Pregnancy-associated glycoprotein (PAG) in milk using Enzyme-link immunosorbent assay

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## *Abstract*

Early pregnancy diagnosis following insemination is an important component of the reproductive management program to shorten the calving interval in water buffaloes. A recent method to detect pregnancy in dairy animals is through pregnancy-associated glycoproteins (PAGs) that are present during implantation and throughout the gestation period. In this study, milk PAG Enzyme-Link Immunosorbent Assay (ELISA) technique was used and run in parallel with blood PAG ELISA for its efficiency in detecting early pregnancy in lactating buffaloes. Fixed Time Artificial Insemination (FTAI) was conducted in lactating buffaloes followed by the collection of milk and blood samples post-FTAI. Samples were then assayed using commercial PAG ELISA test kits (IDEXX, USA). Trans-rectal ultrasonography was conducted to confirm pregnancy. Of the 22 inseminated lactating buffaloes, 12 (54 %) were considered pregnant based on PAG concentration in milk on day 26 and similarly in blood on day 25 post-FTAI. These twelve animals were all confirmed pregnant by ultrasonography on day 40 post-FTAI. In addition, variation in PAG expressions in milk samples was observed among animals until at least day 60 of gestation. Essentially, milk PAG ELISA is an accurate and non-invasive approach to detect early pregnancy in lactating buffaloes which can help reduce the calving interval and improve reproduction in water buffaloes.

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**Keywords:** buffaloes, blood, milk, pregnancy-associated glycoproteins, pregnancy diagnosis

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## Introduction

In dairy herds, economic profitability is directly related to the efficiency of reproductive performance of cows and milk yield (Leblanc, 2010). One strategy for improving reproductive performance is to shorten the calving interval through rapid, accurate and timely diagnosis of pregnant cows (Whitlock and Maxwell, 2008; Ott *et al.*, 2014). Traditionally, pregnancy diagnosis is conducted by rectal palpation or trans-rectal ultrasonography; however, this method is only accurate from day 45 of pregnancy in water buffaloes and might increase the risk of iatrogenic embryonic mortality (Arthur *et al.*, 1996). Advancement in pregnancy diagnosis has been developed over time and utilizing reproductive hormones such as progesterone has been employed as a tool for early pregnancy diagnosis as early as day 20-24 post-breeding. The test however is effective in detecting non pregnancy (91-100%) but low in identifying pregnant buffaloes (57-75%) due to the variation of the animals' estrous cycles (Perera *et al.*, 1980; Singh *et al.*, 1980; Gupta *et al.*, 1990; Ghoneim *et al.*, 1994). Some animals persist with high progesterone concentration that undergoes an extended cycle as well as the incidence of early or late embryonic death (Gowan *et al.*, 1982; Pennington *et al.*, 1985; Parkinson *et al.*, 1994).

Pregnancy Associated Glycoprotein (PAG) is one of the recent pregnancy biomarkers that has been used as a pregnancy indicator in dairy animals. PAGs start their expression during implantation time and persist throughout pregnancy; thus, several studies are now focused on the detection of PAG level to rapidly evaluate pregnancy status (Silva *et al.*, 2007; Peter, 2013). Pregnancy-detection assay based on the measurement of PAGs in serum or plasma samples became available to dairy farmers in 2002 and PAGs were then reported synthesized and secreted directly by placental tissue and serve as direct indicators of pregnancy (Telugu *et al.*, 2009). However, in spite of successful application of this technology, one main set back is the stress it may cause to the presumed pregnant animals during blood sample collection. In the past few years, a commercial test detecting PAG levels in milk has been developed, marketed and being assessed in the field trials for the dairy industry (Leblanc, 2013) as alternative to blood for PAG test. There are several studies conducted on PAG assay in milk on dairy animals especially in cattle, but to our knowledge, there is no reported study on milk PAG in water buffaloes to date.

The present study was conducted specifically to determine the earliest day possible that inseminated water buffaloes can be detected pregnant through PAGs in milk. Furthermore, it aims to investigate PAGs potential to facilitate rebreeding by identifying non-pregnant lactating cows less than 30days post-AI, as against ultrasonography which can detect pregnancy at more than 30 days post-AI. Water buffaloes have inherent characteristics as silent heat animals wherein signs of estrus are not manifested and it is very difficult to determine the best time to bring the animals for artificial insemination or for the services of bulls for re-breeding. Early detection of pregnancy through PAG assay will enable

management decision to initiate effective rebreeding strategies to shorten the calving interval and to improve reproductive efficiency. Essentially, the present study constitutes the first attempt in the country to determine the efficiency and possibility of using PAG ELISA in pregnancy diagnosis in water buffaloes and for the potential incorporation of the technology into the current programmed reproduction in this species.

## Materials and Methods

All procedures involving the use of animals for scientific research purposes were approved by the Ethics Committee of the Philippine Carabao Center, National Headquarters and Gene Pool.

**Induction of Synchronous Ovulation for Fixed Time Artificial Insemination:** A total of twenty-two multiparous lactating Bulgarian water buffaloes at the Philippine Carabao Center, National Gene Pool at Science City of Muñoz, Nueva Ecija, Philippines, were subjected to ovulation synchronization and FTAI. In brief, the animals received two mL intramuscular (IM) injection of Gonadotropin-releasing hormone (GnRH) (100 µg; Cystorelin (Gonadorelin diacetate tetrahydrate), 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 on Day 7 and the water buffaloes received an intramuscular (IM) injection of five mL Prostaglandin F<sub>2α</sub> (PGF<sub>2α</sub>), (25 mg; Lutalyse, dinoprost tromethamine, Pharmacia & Upjohn Co., MI, USA) . Two mL of human Chorionic Gonadotropin (hCG) (1500 IU, Chorulon, Intervet Inc. Summit, NJ 07001, USA) was likewise injected (IM) on day 9. Finally, FTAI was performed twice (am/pm) on day 10, 14-16h post hCG injection (Fig. 1).

**Blood and Milk sample collection:** Lactating water buffaloes under the FTAI program were ensured to be at more than 60 days post-partum before collection of milk and blood samples to avoid the possible presence or overlap of PAGs from previous pregnancy.

Milk samples (5ml) were collected on days 18, 21-30, 40 and 60 post-FTAI during the morning milking schedule and samples were stored at -20°C until analysis.

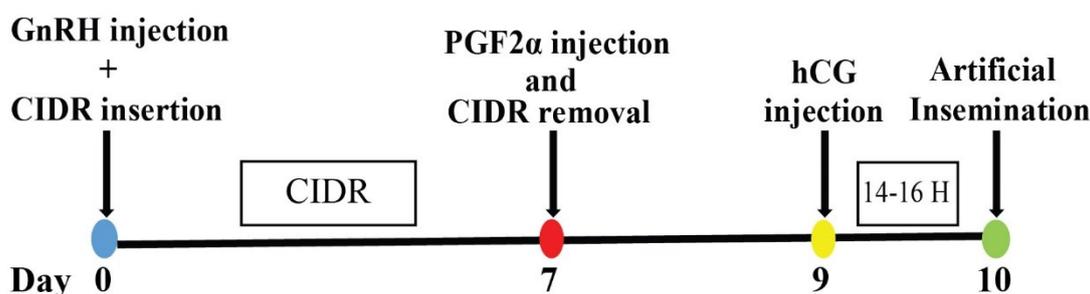
Meanwhile, the parallel collection of blood samples (10ml) was done on days 18, 25, 30, 40, and 60 post-FTAI. Blood samples were collected through the jugular vein of water buffaloes and placed in a tube containing Heparin as an anticoagulant. Samples were then centrifuged for 20 mins at 200 x g under room temperature. After which, plasma samples were collected and stored in microcentrifuge tubes at -20°C until analysis.

**PAG ELISA (Enzyme-Link Immunosorbent Assay) 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). PAG ELISA were conducted according to the

manufacturer's instructions by trained technicians who were blinded as to the pregnancy status of the animals.

The 96 well plate format was coated with 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 microplate were incubated at 37°C (1 h for plasma, 2 h for milk with shaking). Following incubation, PAGs in the sample were determined by a detector solution and the secondary antibody (horseradish peroxidase

conjugated). Unbound conjugate was washed away and 3,3',5,5'- tetramethylbenzidine substrate was added for color development which was relative to the amount of PAGs in the sample and were measured using a spectrophotometer (Multiskan GO, version 1.00.40, Thermo Fischer Scientific, Vantaa, Finland). Results were calculated from the optical density (OD) of the samples (450nm, for sample and control) and 650nm (for reference). Corrected OD values for samples (S) and controls (N) were calculated as  $S-N = (\text{Sample (450-ref)} - \text{Negative Control (NC)})$ .



**Figure 1** Fixed Time AI using CIDR-Synch Protocol. GnRH injected on day-0 induces ovulation of a dominant follicle. Insertion of CIDR on day 0 and removal on day 7 prevents the expression of estrus before induced luteolysis. Injection of PGF2α on day 7 induces luteolysis of the CL formed after 1<sup>st</sup> GnRH injection and initiates new follicular growth and estrus occurs. hCG injection on day 9 induces ovulation of the new follicles formed after luteolysis. Artificial insemination is done on day 10 (AM/PM) 14-16 h after hCG injection.

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

**Trans-rectal Ultrasonography:** All animals used in the study were subjected to pregnancy diagnosis by ultrasonography on days 30, 40 and 60 post-FTAI, respectively. Pregnancy diagnosis by rectal palpation was done on day 60 post-FTAI for confirmation and to serve as control.

The ultrasound examinations were performed using a trans-rectal ultrasound scanner (Honda, HS-1600V, Japan) equipped with 7.5 MHz linear array transducer designed for intra-rectal placement (Mehrajuddin *et al.*, 2013). The scanning of 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 modification. Briefly, the criteria include the presence or absence of corpus luteum (CL), uterine fluid, and embryo. Cows are considered pregnant when CL, uterine fluid, and embryo with a heartbeat are present upon examination by ultrasonography which is considered as the gold standard for pregnancy tests in livestock.

## Results

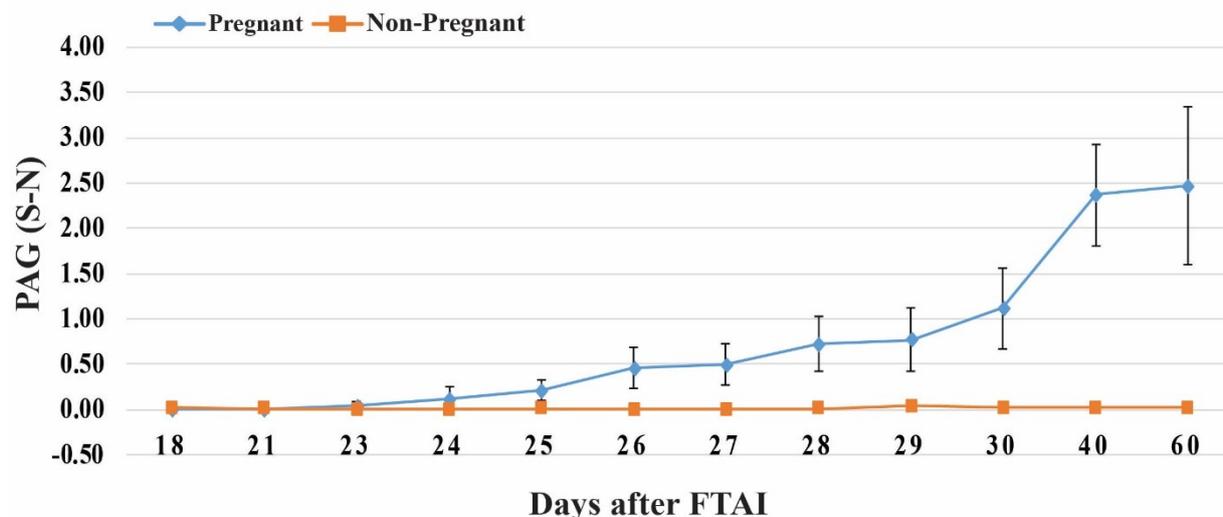
**PAG ELISA:** The result of milk PAG ELISA performed in 22 inseminated lactating water buffaloes revealed that the expression of PAGs had already been observed in the majority of milk samples as early as day 23 post-FTAI, although values were considerably low to be substantial. Further increase of expression and more elevated PAG level, (concentrations of > 0.25) were noted on days 24-25 in three animals which were then categorized as pregnant. On day 26, these PAG concentrations (> 0.25) were observed in milk samples in a total of twelve water buffaloes which were then confirmed as pregnant. It is interesting to note the variations of PAG levels and expression in milk samples among pregnant water buffaloes at least until day 60 of gestation in the present study.

Generally, however; a linear increase in average PAG concentration was observed in pregnant animals until day 60 post-FTAI. This is in contrast with animals identified as non-pregnant which consistently showed no detection of PAGs or with an insignificant amount of PAGs in their milk samples (Fig. 2).

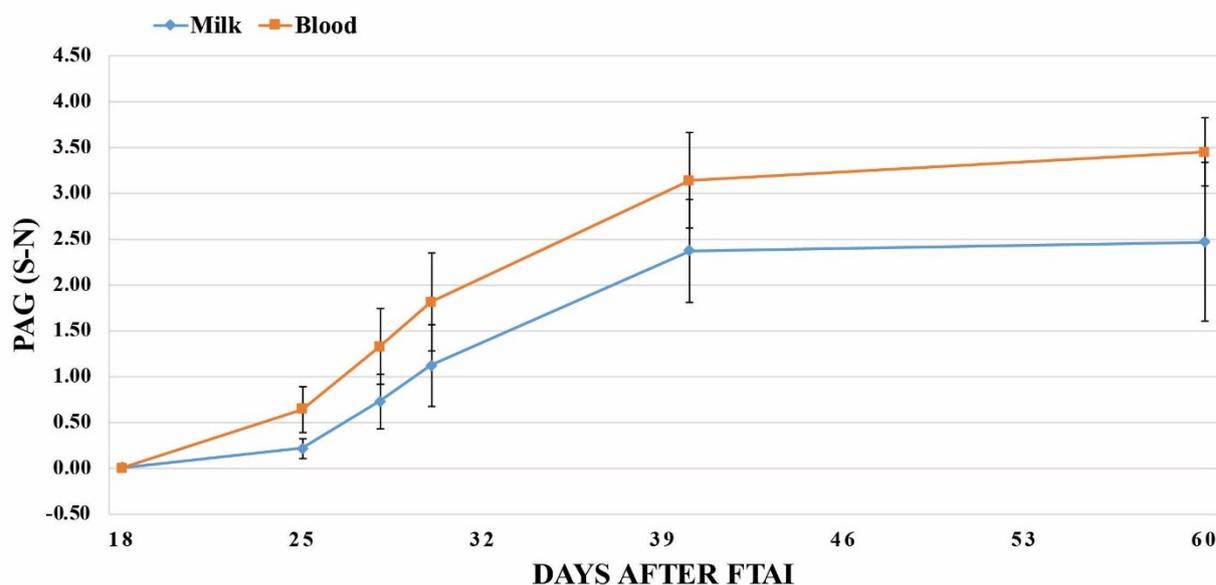
Meanwhile, blood PAG ELISA, which was performed simultaneously with milk PAG ELISA on certain days, revealed that the 22 blood samples were negative or with insignificant levels of PAGs on day 18 post-FTAI (Fig.3). On day 25 however, the twelve animals reached the level of PAGs (>0.3), thus were categorized as pregnant. Similarly, a linear increase of PAG levels in the blood was observed until day 60 of gestation (Fig. 3). It is worth noting that the PAG levels in the blood were consistently higher compared with milk PAG levels.

Lastly, no false-positive results were observed in either blood or milk PAG ELISA tests, following confirmation by ultrasound at days 30, 40 and 60 post-FTAI. With trans-rectal ultrasound though, only 83% (10/12) was achieved at 30 days post-FTAI, and the highest efficiency of 100% (12/12) was only realized on

day 40 and 60 post-FTAI. In contrast, 100% (12/12) pregnancies were achieved as early as day 25 and 26 post-FTAI following pregnancy detection using blood and milk PAG ELISA, respectively, in water buffaloes in the present study.



**Figure 2** Average PAG levels in milk days after FTAI in water buffaloes (pregnant and non-pregnant). PAG levels in milk were calculated from Optical Density, corrected OD values for samples (S) and controls (N) were calculated as S-N= (Sample (450-ref)- Negative Control (NC)). Variations of PAG levels in milk samples among pregnant water buffaloes were observed, wherein, PAG levels started to increase from day 24 until day 60.



**Figure 3** Average PAG level of milk and blood in pregnant water buffaloes. PAG levels in both milk and blood were calculated from Optical Density, corrected OD values for samples (S) and controls (N) were calculated as S-N= (Sample (450-ref)- Negative Control (NC)).

### Discussion

The systematic determination of the PAG levels enable the earliest detection of pregnant and non-pregnant cows to shorten the calving interval and increase the reproductive efficiency and profitability from dairy farming activities. Establishment of blood PAG ELISA in the past several years is now a remarkably useful tool in the pregnancy diagnosis

especially in the dairy industry (Telugu et al., 2009); however, blood collection causes stress in animals. Thus, the use of milk as an alternative sample to blood to perform pregnancy tests in water buffaloes has recently generated interest from many farm owners and livestock raisers. The present study underscores the advantage of using milk over blood sample for PAG analysis in view of its being a non-invasive technique, and therefore, with minimum disturbance

to the possibly pregnant cows. More importantly, the presence of PAG in milk enabled the earliest day the buffalo can be identified as pregnant after insemination, allowing not pregnant animals to return to breeding pool. In the present study, twelve water buffaloes detected pregnant reached detectable level of PAGs in milk ( $>0.25$ ) at day 26 post-FTAI, while PAG in blood of same animals were detected earlier on day 25 post-FTAI at relatively higher level ( $\geq 0.3$ ). The present results conform with the previous report in cattle wherein the milk PAG level started to show up and increase between day 24 to day 25 post-FTAI (Lawson *et al.*, 2012; Ricci *et al.*, 2015; Gatea *et al.*, 2018). In this study, PAG levels in the milk though was observed as early as day 23 post-FTAI in some animals and became significantly detectable on day 26 in all animals as mentioned earlier. Earlier work has provided evidence for the idea that prior to these days, adhesion of mononuclear cells of the trophoctoderm to the uterine luminal epithelium and appearance of giant binuclear cells (BNC) occurred (Wooding, 1983). Accordingly, these cells arise in trophoctoderm and migrate across microvillar junction and fuse with nearby uterine epithelial cells (Wooding, 1982 (A); Wooding, 1982 (B)). The fusion results in the exocytosis of BNC secretory granules with many PAGs being packed in these granules and thereafter, PAGs begin to appear in the maternal circulation. Thus from day 24 to 25 of the gestation period, an extensive tissue remodeling associated with bovine placentation is implied to have occurred (Assis *et al.*, 2009).

Meanwhile, it is important to note that PAG transcripts are expressed at different times throughout the gestation period. It was reported earlier that PAG-1 is not expressed during early gestation while PAG -4, -5 and -9 are expressed and detectable as early as Day 25 post-FTAI (Green *et al.*, 2000; Patel *et al.*, 2004). This may explain the observed variation in PAG expression through gestation period.

Moreover, significant variations of PAG levels in each pregnant water buffaloes observed in both milk and blood samples in the present study were also demonstrated among pregnant cows (Melo *et al.*, 2003; Lopez-Gatius *et al.*, 2007). These varying levels of PAGs among pregnant cows can be attributed to their differences in body weight, placental size, and number of binucleated cells in trophoctoderm that influences the production of PAG (Gajewski *et al.*, 2009; Mialon *et al.*, 1993). Furthermore, the doubled concentration of PAG levels in water buffalo blood compared with milk PAG in the present study had also been shown in cattle (Ricci *et al.*, 2015). The earlier pregnancy detection with blood PAG ELISA (day 25) compared with milk PAG ELISA (day 26) can be attributed to the abundance of PAGs present in blood systemic circulation compared with those PAGs localized in the mammary gland or found in milk samples.

The earlier positive pregnancy results obtained with PAG ELISA using both milk or blood samples were confirmed by ultrasound on day 40 post-FTAI, thus PAG ELISA yielded 100% detection accuracy in the present study. Cautioned on the possibility of the presence of PAGs until day 60 post-parturition and so as not to have false-positive pregnancy result, the present work ensured selection of inseminated

lactating water buffaloes to be more than 60 days after parturition. Mounting evidence supports the idea that PAG levels in milk and blood are high during the late gestation period and it takes up to 60 days for PAG residual to be cleared from maternal circulation after parturition (Haugejorden *et al.*, 2006; Giordano *et al.*, 2012). In the present study, pregnant lactating water buffaloes were already beyond 80 days post-partum when they were tested for PAGs in their milk and blood samples, indicating complete clearance of PAGs from previous gestation.

Lastly, trans-rectal ultrasonography has become a gold standard in farms with advanced reproductive management programs and the recent incorporation of the PAG test for early pregnancy detection represents an innovative strategy for identifying non-pregnant early after AI. This will shorten the calving interval by rebreeding open animals at the earliest opportunity thus may play a key role in management strategies to improve reproductive efficiency and profitability from dairy farming.

The present study successfully demonstrated the possibility of detecting early pregnancy through milk PAG ELISA in lactating water buffaloes, wherein the majority of inseminated cows were diagnosed pregnant by day 26 post-FTAI. It is found a highly accurate and an effective method of early pregnancy diagnosis similar to blood PAG ELISA. Pregnancy detection using a milk sample is the desired alternative to avoid causing stress to presumed pregnant lactating animals during sample collection, as this is crucially important to ensure milk productivity. Milk PAG ELISA however has its limitation of being not suitable for use in heifers; thus in this case, blood PAG ELISA is used instead. In general, the present study underscores the importance of early pregnancy detection in the reproduction program to enable rebreeding of not pregnant animals to increase reproductive efficiency and economic profitability from water buffalo raising. Lastly, the PAG test can be used as well to determine embryonic loss and fetal mortality during early gestation period. In essence, PAG ELISA is an important reproductive tool not only to manage early pregnancy detection but also pregnancy loss to enhance pregnancy outcome in water buffaloes and other livestock species.

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