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Correlation of two radioimmunoassay systems for measuring plasma pregnancy-associated glycoproteins (PAG) concentrations during early pregnancy and postpartum periods in water buffalo

Running title: PAG radioimmunoassay in buffalo cows

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Contents

This is the first time that PAG determination using two different antisera raised against PAG-molecules purified from both caprine (RIA-706) and bubaline placentas (RIA-860) is reported in water buffalo. Ninety-eight buffalo cows, belonging to a buffalo herd subjected to a synchronization and artificial insemination (AI) program, were enrolled in this study. Blood samples were taken on days 0 (AI), 23, 25, 28, 30 and 45. Pregnancy was confirmed by ultrasonography on days 28 and 45. The blood of 20 buffaloes that had calved was tested every five days from the day of calving until day 50 post-calving. Differences in PAG concentrations were observed between pregnant and non pregnant buffaloes starting from day 23 post AI by using both RIA-706 and RIA-860 ($P < 0.001$). However, estimated mean concentrations of PAG measured by RIA-706 were higher than RIA-860 ($P<0.001$) and Bland-Altman analysis showed biases ranged from 0.0 ng/mL at day 23 to 0.79 ng/mL at day 28 post AI. Moreover, RIA-706 showed greater sensitivity and accuracy both at 23 and 25 days

of pregnancy. RIA-706 and RIA-860 decreased below 1 ng/mL from 40 and 30 days post-partum, respectively, suggesting that PAG are better recognized by the antisera raised against the caprine PAG in the post-partum period also. This is essential when using PAG as an appropriate marker of early pregnancy after post-partum for detecting new pregnancies. The results of this study show that the ability of RIA-systems to recognise early PAG could be improved by using antisera raised against PAG molecules isolated from caprine placenta.

Keywords: buffalo, pregnancy-associated glycoprotein, RIA-706, RIA-860, pregnancy.

INTRODUCTION

Pregnancy-associated glycoproteins (PAGs) are a multigene family related to aspartic proteinases that are expressed in the placenta in eutherian species (Zoli et al., 1991; Garbayo et al., 2008; Wallace et al., 2015). Phylogenetic analyses have shown that there are two distinct groups of PAGs known as “ancient” (PAG-II group: PAG-2, PAG-8, PAG-10 to PAG-13) and “modern” (PAG-I group: PAG-1, PAG-3 to -7, PAG-9, PAG-14 to -18, PAG-20 to -22) (Hughes et al., 2000).

Using various chromatographic procedures, some members of the PAG family have been isolated from the cotyledons of several species of the order Cetardiodactyla (Zoli et al., 1991; Szafranska et. al., 1995; Xie et al., 1997; Garbayo et al., 1998; El Amiri B. et al., 2004; Green et al., 2000; Sousa NM. et al., 2003; Majewska et al. 2006; Brandt et al., 2007; Beriot et al., 2014) including water buffalo (wbPAG; Barbato et al., 2008; 2013).

PAG concentrations in the maternal blood of ruminant ungulates have become useful tools for monitoring pregnancy. Purified and semipurified PAG preparations have been used to immunize rabbits; the antisera (AS) thus obtained have led to the development of homologous (Zoli et al., 1992a) and heterologous radioimmunoassays (RIA) (Zoli et al., 1992b; Perenyi et al., 2002).

Our research group (Barbato et al. 2013; Barbato et al. 2016) reported using antisera raised against buffalo PAG molecules, purified from buffalo placenta, in order to develop a specific RIA system (RIA-860) for pregnancy diagnosis in buffalo. Using this system, the plasma PAG profiles of water buffalo during pregnancy and postpartum periods have been recently described (Barbato et al., 2017).

In a previous study, (Barbato et al. 2009) using antisera raised against heterologous PAG-molecules such as RIA-497, RIA-706 and RIA-708 (antisera raised against $boPAG_{67kDa}$, $caPAG_{55kDa+62kDa}$ and $caPAG_{55kDa+59kDa}$ respectively) for detecting PAG in pregnant buffalo cows, we observed that from week 6 of gestation onward, analogous PAG antigens were better recognized using RIA-706 (antisera raised against $caPAG_{55kDa+62kDa}$). Other authors (Karen et al. 2007; El Battawy et al. 2009) also reported using RIA-706 system to determine PAG in buffalo species.

Various studies have demonstrated that heterologous RIA-706 is able to measure bovine PAG concentration (Perenyi et al., 2002; Lopez Gatius et al., 2007; Ayad et al., 2009).

For this reason, we wanted to determine the best RIA system (RIA-706 or RIA-860, developed by using antisera raised against PAG molecules purified from caprine or bubaline placentas) for early pregnancy diagnosis in this species. The correlation between concentrations measured by the aforementioned RIA systems, their ability to detect PAG molecules during pregnancy and the disappearance in the post-partum were investigated.

2. MATERIALS AND METHODS

2.1. Animals and experimental design

The experiment was carried out at the experimental farm of the CREA Research Centre for Animal Production and Aquaculture in Monterotondo (Rome, Italy, 42° N parallel).

Ninety-eight cows belonging to a Italian Mediterranean buffalo herd subjected to a synchronization and artificial insemination (AI) program were enrolled in the study and divided *ex post* into groups (pregnant and non-pregnant) as described below. The buffaloes were synchronized with a progesterone releasing intravaginal device (PRID; Sanofi, France) containing 1.55 g natural progesterone inserted *in situ* for 10 days. On day 7, an i.m. injection of 1000 IU of Pregnant Mare Serum Gonadotrophin (PMSG; Ciclogonina, Fort Dodge, Italy) and 0.15 mg of cloprostenol (PGF2α analogue; Dalmazin, Fatro, Italy) were administered. On day 10, the PRID was removed and the cows were artificially inseminated at 72h and 96h after device withdrawal. The day of the second AI was considered as day zero.

Blood samples were taken from the jugular vein and collected in 10 ml EDTA tubes at days 0 (0d), (23d), 25 (25d), 28 (28d), 30 (30d) and 45 (45d) after AI in order to detect the presence of PAG molecules in early pregnancy. The blood of 20 buffaloes that had calved was tested every five days from the day of calving until day 50 post-calving in order to determine PAG disappearance.

The plasma was immediately separated by centrifugation (1,200 x g for 15 minutes at 5 °C) and stored at -20 °C until assayed.

The animals involved in this experiment were treated in compliance with the animal testing regulations established under Italian law. The experimental design was carried out according to good veterinary practices under farm conditions. The CREA Research Centre for Animal Production and

Aquaculture is authorized to use farm animals for experimental design (as stated in DM 26/96-A of Italian Welfare Ministry).

2.2. Pregnancy diagnosis

The animals were classified *ex post* as pregnant (P group) and non-pregnant (NP group) based on ultrasound findings and PAG concentrations.

Pregnancy was diagnosed on days 28 and 45 from AI by transrectal ultrasonography (Aloka–SSD Prosound 2 scanner, Hitachi Medical System, Italy) using a 7.5 MHz linear-array transducer. The same operator performed all of the ultrasound scans. Positive pregnancy status on day 28 and day 45 was characterized by the presence of embryonic vesicles and embryo proper within the embryonic vesicle and heartbeat visualization (embryo viability). In the absence of embryonic vesicles or embryo proper in the uterine lumen on day 28 and day 45, the females were considered as non-pregnant. Pregnancy status was confirmed by transrectal palpation on day 60.

2.3. PAG radioimmunoassay

Two different radioimmunoassay systems (RIA-706 and RIA-860) obtained using the method previously described by Perenyi et al. (2002) and Barbato et al. (2009) were used to measure PAG concentrations. Pure boPAG$_{67kDa}$ preparation was used as standard and tracer for all PAG assays. Iodination (Na-I^{125}, Amersham Pharmacia Biotech, Uppsala, Sweden) was carried out according to the Chloramine T method previously described by Greenwood et al. (1963).

Polyclonal antisera were raised in rabbits using different PAG preparations according to the Vaitukaitis et al (1971). Anti-caPAG$_{55+62kDa}$ (AS#706, PAG accession numbers P80935; Garbayo et al., 1998) and anti-wbPAG$_{55+59kDa}$ (AS#860; PAG accession numbers P86369; Barbato et al., 2009) polyclonal antisera were used respectively in RIA-706 and RIA-860. The double antibody precipitation system was composed of a mixture of sheep anti-rabbit immunoglolobulin (0.83% v:v), normal rabbit serum (0.17% v:v), polyethylene glycol 6000 (20 mg/mL; Vel, Leuven, Belgium), cellulose microcrystalline (0.05 mg/mL; Merck, Darmstad, Germany) and BSA (2 mg/mL; Fraction V, ICN Biochemicals Inc.), diluted in Tris buffer (25 mM Tris, 10 mM $MgCl_2$ and 0.02% w/v NaN_3; pH 7.5).

Firstly, the plasma samples were assayed in a preincubated system. Briefly, standard (0.2-25 ng/mL) and plasma samples (100µL) were aliquoted into duplicate assay tubes and diluted respectively with 0.1 mL and 0.2 mL of Tris-BSA buffer (Tris-HCl containing 1 mg/mL of BSA; pH 7.5).

In order to minimize nonspecific interference of plasma proteins, 0.1 mL of bovine PAG-free plasma was added to all of the standard tubes. 0.1 mL of the diluted antiserum (AS#706: 1/100,000 and AS#860: 1/840,000) were then added and the tubes were incubated overnight at room temperature. The following day, 0.1 mL of radiolabelled ^{125}I-PAG (28,000 counts per min) was added to all of the tubes, which were further incubated at room temperature for 4 h before adding 1.0 mL of double antibody precipitation system. A further 30 min incubation at room temperature was carried out before adding 2.0 mL of assay buffer. Bound (B) and free PAGs were separated by centrifugation (1,500 x g for 20 min). The supernatants were discarded and the radioactivity of the pellet was determined using a Multigamma Counter (LKB Wallac 1261; Turku, Finland).

Samples with higher PAG concentrations than the estimated standard dose at which the percentage B/B_0 was 20% (ED_{20}) were re-assayed in non-preincubated systems in which the standard curves ranged from 0.8 to 100 ng/mL. Tracer was added to these systems simultaneously with one of the

aforementioned first antibodies (AS#706: 1/100,000 and AS#860: 1/840,000). The following day the double antibody precipitation system was added and further 30 min incubation was carried out before separating bound and free PAGs.

The minimum detection limit (MDL), calculated as the mean concentration minus twice standard deviation (mean – 2 SD) of 20 duplicates of the zero (B_0) standard (Skelley et al., 1973), were 0.1 ng/mL, and 0.4 ng/mL for RIA-706 and RIA-860 respectively.

2.4. Statistics analyses

The data were analysed using Linear Mixed models. Time (days post AI or post-partum) and Buffalo were included in the models as a repeated measure and as a random factor, respectively. We preliminarily analysed PAG-706 and PAG-860 concentrations during early pregnancy in order to investigate the differences between the two RIA systems in pregnant and non-pregnant animals. These models evaluated the effects of Time (6 levels: 0, 23, 25, 28, 30, and 45 days post AI), group (2 levels: P and NP), RIA systems (2 levels: PAG-706 and PAG-860) and their interactions. The model for the postpartum period evaluated the effects of Time (11 levels: every 5 days from calving to day 50post-calving), RIA system (2 levels: PAG-706 and PAG-860) and Time x RIA system interaction. Sidak corrections were used to obtain pairwise comparisons, while diagnostic graphics were used to check assumptions and outliers. Log(x+1) transformations were used for both PAG-706 and PAG-860 values. The results were expressed as estimated marginal means ± standard error (SE) while the raw data were presented in figures.

The *elimination rate constant* was calculated from the slope of the line during the post-partum period by linear regression analysis of the semilogarithmic plot of PAG concentrations versus time

while the *half-life* was obtained as follows: (ln 2 /elimination rate constant) (Barbato et al., 2017; Menchetti et al., 2018).

We used Bland-Altman plots to assess agreement between the two methods in early pregnancy (at 23, 25, and 28 days post AI in P group) and regression analysis for detecting proportional bias (Bland & Altman, 1999; Watson & Petrie, 2010). If model assumptions were not met, we adopted the nonparametric approach and presented median as bias, 2.5th and 97.5th percentiles of the differences as limits of agreements (LoA; Bland & Altman, 1999). Bland-Altman plots were performed using GraphPad Prism version 6.0 (San Diego, CA, USA).

After categorization of the PAG-706 and PAG-860 concentrations according to the threshold of 1.0 ng/mL (PAG < 1.0 = No Pregnant; PAG ≥ 1.0 ng/mL = Pregnant), we calculated accuracy, sensitivity and specificity at 23, 25, and 28 days post AI. Finally, we used Cohen's kappa (k; Watson & Petrie, 2010) to evaluate the degree of agreement in pregnancy diagnosis between the two radioimmunoassay systems at day 23, 25, 28, and 30 post AI. The extent of the agreement was judged poor if $k < 0.00$, slight if $0.00 \leq k \leq 0.20$, fair if $0.21 \leq k \leq 0.40$, moderate if $0.41 \leq k \leq 0.60$, substantial if $0.61 \leq k \leq 0.80$, almost perfect if $k > 0.80$, and perfect if $k = 1.00$ (Watson & Petrie, 2010).

Statistical analysis were performed with SPSS 23.0 (SPSS Inc. Chicago, USA) and considered as significant at a level of 0.05.

3. RESULTS

From 98 synchronized buffalo cows, 52 became pregnant (P group, n = 52) and 46 remained non-pregnant (NP group, n = 46) as revealed by ultrasonography and RIA analysis.

3.1. *PAG-706 and PAG-860 concentrations in pregnant and non-pregnant buffaloes until day 45 post AI*

Log- PAG concentrations were affected by Time ($P < 0.001$), Group ($P < 0.001$), radioimmunoassay systems ($P < 0.001$) and their interactions ($P < 0.001$).

Both PAG-706 and PAG-860 showed differences between P and NP starting from day 23 post AI (mean difference between P and NP at day 23 post AI: -0.4±0.1 ng/mL and -0.3±0.1 ng/mL for PAG-706 and PAG-860, respectively; $P < 0.001$) until the last day of observation (mean difference between P and NP at day 45 post AI: -19.1±0.1 ng/mL and -11.5±0.1 ng/mL for PAG-706 and PAG-860, respectively; $P < 0.001$). Mean concentrations of both PAG-706 and PAG-860 in NP animals constantly remained close to zero (0.2±0.5 ng/mL and 0.0±0.0 ng/mL for PAG-706 and PAG-860, respectively; $P < 0.001$).

Estimated marginal means were higher in PAG-706 (1.1±0.1 ng/mL) than PAG-860 (0.7±0.1 ng/mL; $P<0.001$). In particular, pairwise comparisons showed that in the P animals PAG-706 were higher than PAG-860 values from day 25 until day 45 post AI ($P < 0.001$; Figure 1).

3.2. *Agreement between PAG-706 and PAG-860 concentrations in early pregnancy (until day28 post AI): Bland-Altman analysis, accuracy and Cohen's kappa statistic*

Since differences were proportional to the mean, we used a nonparametric approach for the Bland-Altman analysis. The biases (median difference between 2 methods) at 23, 25, and 28 days post AI in pregnant cows were 0.00 ng/mL (LoA = -1.27, 2.53 ng/mL), 0.79 ng/mL (LoA = -1.07, 8.35 ng/mL), and 2.54 ng/mL (LoA = 0.00, 7.64 ng/mL), respectively (Figure 2A-C).

At day 23 post AI (Table 1), pregnancy was correctly diagnosed in 61/98 animals (accuracy = 62%, sensitivity 29%) using PAG-706 while it was correctly diagnosed in 58/98 animals using PAG-wb860

(accuracy = 59%, sensitivity 23%). At day 25 post AI, pregnancy was correctly diagnosed in 88/98 (accuracy = 90%, sensitivity = 81%) and 85/98 animals (accuracy = 87%, sensitivity = 77%) using PAG-706 and PAG-wb860, respectively. At day 28 post AI, accuracy and sensitivity were 99% and 98% for both RIA systems using both PAG-706 and PAG-860.

Agreement of diagnosis between the two radioimmunoassay systems (Table 2), as measured by Cohen's kappa, was deemed moderate at day 23 ($k = 0.443$, $P < 0.001$), substantial at day 25 ($k = 0.728$, $P < 0.001$) and perfect at days 28 ($k = 1.000$, $P < 0.001$) post insemination.

3.3. *Profile of PAG-706 and PAG-860 concentrations during post-partum*

During the post-partum period, PAG concentrations were affected by Time ($P < 0.001$), RIA system ($P < 0.001$) and RIA system x Time effect ($P < 0.001$). Both PAG-706 and PAG-860 concentrations progressively decreased after parturition (53.4±0.1 ng/mL and 42.1±0.1 ng/mL for PAG-706 and PAG-860 concentrations, respectively) to 25 days post-partum (2.4±0.1 ng/mL and 1.6±0.1 ng/mL for PAG-706 and PAG-860 concentrations, respectively; $P < 0.01$; Figure 3). PAG-706 and PAG-860 decreased below 1 ng/mL from 40 and 30 days post-partum, respectively, and were close to zero on the last day of observation (both 0.0±0.1 ng/mL at 50 d post-partum). However, PAG-706 were higher than PAG-860 from parturition until 40 days post-partum ($P < 0.01$).

We calculated the kinetic parameters on scatter plots of ln PAG-706 and ln PAG-860 concentrations *versus* post-partum days. The *elimination rate constants* were 0.11 d^{-1} and 0.12 d^{-1} while the half-lives were 6.3 d and 5.8 d for PAG-706 and PAG-860, respectively.

4. DISCUSSION

To the best of our knowledge this is the first time that PAG determination in water buffalo using two different antisera raised against PAG-molecules purified from both caprine (RIA-706) and bubaline placentas (RIA-860) is reported.

Measurement of PAG/PSPB concentrations in the peripheral circulation of pregnant and non-pregnant buffalo cows was carried out using different RIA methods (Barbato et al. 2009 and 2013; El Battawy et al. 2009; Karen et al., 2007). In our previous study (Barbato et al. 2009) that compared the effectiveness of RIA-497, RIA-706 and RIA-708 (antisera raised against PAG molecules isolated from bovine and caprine placenta) for detecting PAG molecules in pregnant buffalo cows, showed that analogous PAG antigens were better recognized using RIA-706 that use molecules isolated from caprine placenta. This is in agreement with this study in which the RIA-706 revealed much higher buffalo PAG concentrations from day 23 to day 45 after AI respect to RIA-860 that use antisera raised against PAG molecules isolated from bubaline placenta. Similarly, in bovine, Perényi et al. (2002) and Ayad et al. (2009) comparing anti-boPAG$_{67kDa}$ antiserum (AS#497) with different antisera raised against PAG molecules isolated from caprine placenta (PAG$_{55kDa+62kDa}$, PAG$_{55kDa+59kDa}$ and PAG$_{55kDa}$, AS#706, AS#708 and AS#809, respectively), showed the RIA-706 revealed much higher bovine PAG concentrations respect the other RIA systems. These differences may be due to the ability to detect different epitopes, as previously reported by Perenyi et al. (2002).

Bland-Altman analysis confirmed the presence of biases between the two methods. Although differences between the two values were small, they could have effects in the early diagnosis of pregnancy. In fact, RIA-706 showed greater sensitivity and accuracy both at 23 and 25 days of pregnancy. Instead at day 28 post AI, the measures of diagnostic accuracy were identical for both

RIA-706 and RIA-860 (accuracy= 99%, sensitivity=98%, specificity=100%). Moreover, Cohen's kappa confirmed a perfect agreement of diagnosis between the two radioimmunoassay systems after day 25 post AI. These findings indicate that the use of antisera raised against $caPAG_{55+62kDa}$ or against $wbPAG_{55+62kDa}$ provides both RIA systems able to detect pregnancy in buffalo species from 25d after conceiving.

The most surprising data observed in buffalo species concerns the very fast decrease in PAG concentrations described in the postpartum period.

In this species, PAG concentrations decrease rapidly reaching minimal levels (< 1ng/mL) at day 30 and day 40 when using both RIA-860 and RIA-706, respectively. A similar decline in PAG concentrations during the first month postpartum was reported by Ranilla et al. (1994, 1997) in Assaf, Churra and Merinos ewes and by Sousa et al. (1999) in native goats from north-east of Brazil. In these species, PAG concentration reach level lower than 1 ng/mL at day 30 postpartum. Differently, in the cow, PAG concentration decreased slowly after parturition and could be detected as late as 100 days postpartum (Zoli et al., 1992).

In the post-partum period, also, the buffalo pregnancy proteins are better recognized by the antisera raised against the caprine PAG. The rapid decrease in PAG concentration during post-partum period is essential when using PAG as an appropriate marker of early pregnancy after post-partum in buffalo. This rapid disappearance does not entail using a cut-off limit in post-partum animals as a means for detecting new pregnancies, since the time interval from calving to AI is at least 50 days, if AI program is scheduled to start 40 days post-partum.

As regards PAG half-life, it was found to be shorter in buffalo (6.3 d and 5.8 d for PAG-706 and PAG-860, respectively) than in cattle (from 7.0-8.8 days) (Sasser et al., 1986; Ali et al., 1997; Kirakofe et al., 1993; Haugejorden et al., 2006), goats (7.49 d) (Haugejorden et al., 2006) and Zebu (9.2-10.1 d) (Sousa et al., 2003), while, buffalo PAG half-life was similar to that of sheep (4.5 d) (Haugejorden et al., 2006). These differences may be due to the presence of N-linked carbohydrate and sialic acid chains on their structure (Rafferty et al., 1995)

In conclusion, the results of this study show that using antisera raised against $caPAG_{55+62kDa}$ or against $wbPAG_{55+62kDa}$ provides us RIA systems that are capable of detecting pregnancy in the buffalo species from 25d of gestation. The ability of RIA-systems to recognise early PAGs could be improved by using antisera raised against PAG molecules isolated from caprine placenta. Furthermore, the rapid disappearance of PAG concentrations in buffalo cows after calving does not entail using a cut- off day limit in post-partum animals for detecting new pregnancies.

CONFLICT OF INTEREST

The authors have declared no conflict of interests

AUTHOR CONTRIBUTIONS

Barbato O helped to design, conduct the experiment and process the samples analysed data and drafted manuscript. Menchetti L carried out the statistical analysis. Sousa NM and Beckers F helped to conduct and revised the manuscript. Brecchia G, Malfatti A, Canali C helped to conduct the experiment and process the samples. Barile VL helped to conduct the experiment and critically revised the manuscript.

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REFERENCES

Ayad, A., Sousa, N. M., Nornick, J. F., Iguer-Ovada, M., Beckers, J. F. (2009). Correlation of five radioimmunoassay systems for measurement of bovine plasma pregnancy-associated glycoprotein concentrations at early pregnancy period. *Research in Veterinary Science*, 86(3), 377-382.

Ali, M., Gebbie, F. E., Sinclair, K. D., Hutchinson, J. S. M., Beckers, J. F., Broadbent, P. J. (1997). Half-life of serum bPAG, the postpartum anoestrus and performance of beef cows. *Journal of Reproduction and Fertility*, 20, 64.

Barbato, O., Sousa, N. M., Klisch, K., Clerge,t E., Debenedetti, A., Barile, V. L., Malfatti, A., Beckers, J.F. (2008). Isolation of new pregnancy-associated glycoproteins from water buffalo (Bubalus bubalis) placenta by Vicia villosa affinity chromatography. *Research in Veterinary Sciences*, 85, 457-66.

Barbato, O., Sousa, N. M., Debenedetti, A., Canali, C., Todini, L., Beckers, J. F. (2009). Validation of a new pregnancy-associated glycoprotein radioimmunoassay method for the detection of early pregnancy in ewes. *Theriogenology*, 72, 993-1000.

Barbato, O., Melo de Sousa, N., Barile, V. L., Canali, C., Beckers, J. F. (2013). Purification of pregnancy-associated glycoproteins from late-pregnancy Bubalus bubalis placentas and development of a radioimmunoassay for pregnancy diagnosis in water buffalo females. *BioMedical Center Veterinary Research,* 9, 89.

Barbato, O., Chiaradia, E., Barile, V. L., Pierri, F., Melo de Sousa, N., Terracina, L., Canali, C., Avellini, L. (2016). Investigation into omocysteine, vitamin E and malondialdeyde as indicators of successful artificial insemination in synchronized buffalo cows (Bubalus bubalis). *Research in*

Veterinary Sciences, 104, 100-105.

Barbato, O., Menchetti, L., Sousa, N. M., Malfatti, A., Brecchia, G., Canali, C., Beckers, J. F., Barile, V. L. (2017). Pregnancy-associated glycoproteins (PAGs) concentrations in water buffaloes (Bubalus bubalis) during gestation and the postpartum period. *Theriogenology*, 97, 73-77.

Bland, J. M., Altman, D. G. (1998). Measuring agreement in method comparison studies. *Statistic Methods in Medical Research*. 8(2), 135-60.

Beriot, M., Tchimbou, A.F., Barbato, O., Beckers, J. F., Sousa, N. M. (2014). Identification of pregnancy-associated glycoproteins and alpha fetoprotein in fallow deer (Dama dama) placenta. *Acta Veterinaria Scandinava*, 56, 4.

Brandt, G. A., Parks, T. E., Killian, G., Ealy, A. D., Green, J. (2007). A cloning and expression of pregnancy-associated glycoproteins expressed in trophoblasts of the white-tail deer placenta. *Molecular Reproduction and Development*, 74, 1355-1362.

El-Battawy, K. A., Sousa, N. M., Szenci, O., Beckers, J. F. (2009). Pregnancy-associated glycoprotein profile during the First Trimester of Pregnancy in Egyptian Buffalo Cows. *Reproduction of Domestic Animals,* 44, 161-166.

El Amiri, B., Remy, B., Sousa, N. M., Joris, B., Ottiers, N. G., Perenyi, Z., Banga Mboko, H., Beckers, J. F. (2004). Isolation and characterization of eight pregnancy-associated glycoproteins present at high levels in the ovine placenta between day 60 and day 100 of gestation. *Reproduction, Nutrition and Development*, 44, 169–181.

Garbayo, J. M., Remy, B., Alabart, J. L., Folch, J., Wattiez, R., Falmagne, P., Beckers, J. F. (1998). Isolation and partial characterization of a pregnancy-associated glycoprotein family from the goat placenta. *Biology of Reproduction*, 58, 109-115.

Garbayo, J. M., Serrano, B., Lopez-Gatius, F. (2008). Identification of novel pregnancy-associated

glycoproteins (PAG) expressed by the perimplantion conceptus of domestic ruminants. *Animal of Reproduction Sciences*, 103,120-34.

Green, J. A., Xie, S., Quan, X., Bao, B., Gan, X., Mathialagan, N., Beckers, J. F., Roberts, R. M, (2000). Pregnancy-associated bovine and ovine glycoproteins exhibit spatially and temporally distinct expression patterns during pregnancy. *Biology of Reproduction*, 62, 1624-1631.

Greenwood, F. C., Hunter, W. M., Glover, J. S. (1963). The preparation of 131-I labelled human growth hormone of high specific radioactivity. *Biochemical Journal,* 89,114-123.

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

Hughes, A.L., Green, J.A., Garbayo, J.M., Roberts, R. M. (2000). Adaptive diversification within a large family of recently duplicated, placentally expressed genes. *Proceedings of the National Academy of Sciences USA*, 97, 3319-23.

Karen, A., Darwish, S., Ramoun, A., Tawfeek, K., Van Han, N., Sousa, N. M., Sulon, J., Szenci, O., Beckers, J. F. (2007). Accuracy of ultrasonography and pregnancy-associated glycoprotein test for pregnancy diagnosis in buffaloes. *Theriogenology*, 68, 1150-55.

Kiracofe, G. H., Wright, J. M., Schalles, R. R., Rude,r C. A., Parish, S., Sasser, R. G. (1993). Pregnancy specific protein B in serum of postpartum in beef cows. *Journal of Animal Sciences*, 71, 2199-2205.

Lopez-Gatius, F., Garbayo, J. M., Santolaria, P., Yaniz, J., Serrano, B., Ayad, A., Sousa, N. M., Beckers, J. F. (2002). Plasma pregnancy-associated glycoprotein-1 (PAG-1) concentrations during gestation in Neospora caninum- infected dairy cows. *Theriogenology*, 67(3), 592-508.

Majewska, M., Panasiewicz, G., Majewski, M., Szafranska, B. (2006). Localization of chorionic

Pregnancy-Associated Glycoprotein family in the pig. *Biology of Reproduction*, 6(3), 205-230.

Menchetti, L., Canali, C., Castellini, C., Boiti, C., Brecchia, G. (2018). The different effects of linseed and fish oil supplemented diets on insulin sensitivity of rabbit does during pregnancy. Research in Veterinary Science, 118, 126-133.

Perenyi, Z., Szenc,i O., Drion, P.V., Banga-Mboko, H., Sousa, N. M., El Amiri, B., Beckers, J. F. (2002). Aspartic proteinase members secreted by the ruminant placenta: specificity of three radioimmunoassay systems for the measurement of pregnancy-associated glycoproteins. *Reproduction in Domestic Animals*, 37, 324-329.

Rafferty, B., Mower, J. A., Ward, H. L., Rose, M. (1995). Defferences in carbohydrate composition of FSH preparations detected with lecithin-ELISA systems. *Journal of Endocrinology*, 145, 527-533.

Ranilla, M. J., Sulon, J., Carro, M. D., Mantecón, A. R., Beckers, J. F. (1994). Plasmatic profiles of pregnancy associated glycoprotein and progesterone levels during gestation in churra and merino sheep. *Theriogenology*, 42, 537-545.

Ranilla, M. J., Sulon, J., Mantecón, A. R., Beckers, J. F., Carro, M. D. (1997). Plasma pregnancy associated glycoprotein and progesterone concentrations in pregnant Assaf ewes carrying single and twin lambs. *Small Ruminant Research,* 24, 125-131.

Sousa, N. M., Garbayo, J. M., Figueiredo, J. R., Sulon, J., Goncalves, P. B. D., Beckers, J. F. (1999). Pregnancy-associated glycoprotein and progesterone profiles during pregnancy and postpartum in native goats from the north-east of Brazil. *Small Ruminants Research*, 32, 137-147.

Sousa, N. M., Zongo, M., Pitala, W, Boly, H., Sawadogo, L., Sanon, M., Figueiredo,

S.R., Gonçalves, P. B. D, El Amiri, B., Perènyi, Z., Beckers, J. F. (2003). Pregnancy-associated

glycoprotein concentrations during pregnancy and post-partum period in Azawak Zebu Cattle. *Theriogenology*, 59, 1131-1142.

Sasser, R. G., Ruder, C. A., Ivani, K. A., Butler, J. E., Hamilton, W.C. (1986). Detection of pregnancy by radioimmunoassay of a novel pregnancy-specific protein in serum of cows and a profile of serum concentrations during gestation. *Biology of Reproduction*, 35, 936-942.

Skelley, D. S., Brown, L. P., Besch P. K. Radioimmunoassay. (1973). Clinical Chemistry, 19, 146-186.Szafranska, B., Xie, S., Green, J., Roberts, R.M. (1995). Porcine Pregnancy-Associated Glycoproteins: new members of the aspartic Proteinase gene family expressed in the trophectoderm. *Biology of Reproduction*, 53, 21–28.

Szafranska, B., Xie, S., Green, J., Roberts, R. M. (1995). Prorcine pregnancy-associated glycoproteins: new members of the aspartic Proteinase gene family expressed in the trophectoderm. *Biology of Reproduction*, 53, 21-28.

Vaitukaitis, J., Robbins, J. B., Nieschlag, E., Ross, G. T. (1971). A method for producing specific antisera with small doses of immunogen. *Journal of Clinical Endocrinology and Metabolism,* 33,988–991.

Wallace, R. M., Pohler K. G., Smith, M. F., Green, J. A. (2015). Placental Pags: gene origins, expression patterns and use as marker of pregnancy. *Reproduction,* 149(3), R115-26.

Watson, P. F., Petrie, A. (2010). Method agreement analysis: a review of correct methodology. *Theriogenology,* 73(9), 1167-79.

Xie, S., Green, J. A., Bao, B., Beckers, J. F., Valdez, K. E., Hakami, L., Roberts, R. M. (1997). Multiple pregnancy-associated glycoproteins are secreted by day 100 ovine placental tissue. *Biology of Reproduction*, 57, 1384-1393

Zoli, A. P., Beckers, J. F., Wouters-Ballman, P., Closset, J., Falmagne, P., Ectors, F. (1991). Purification

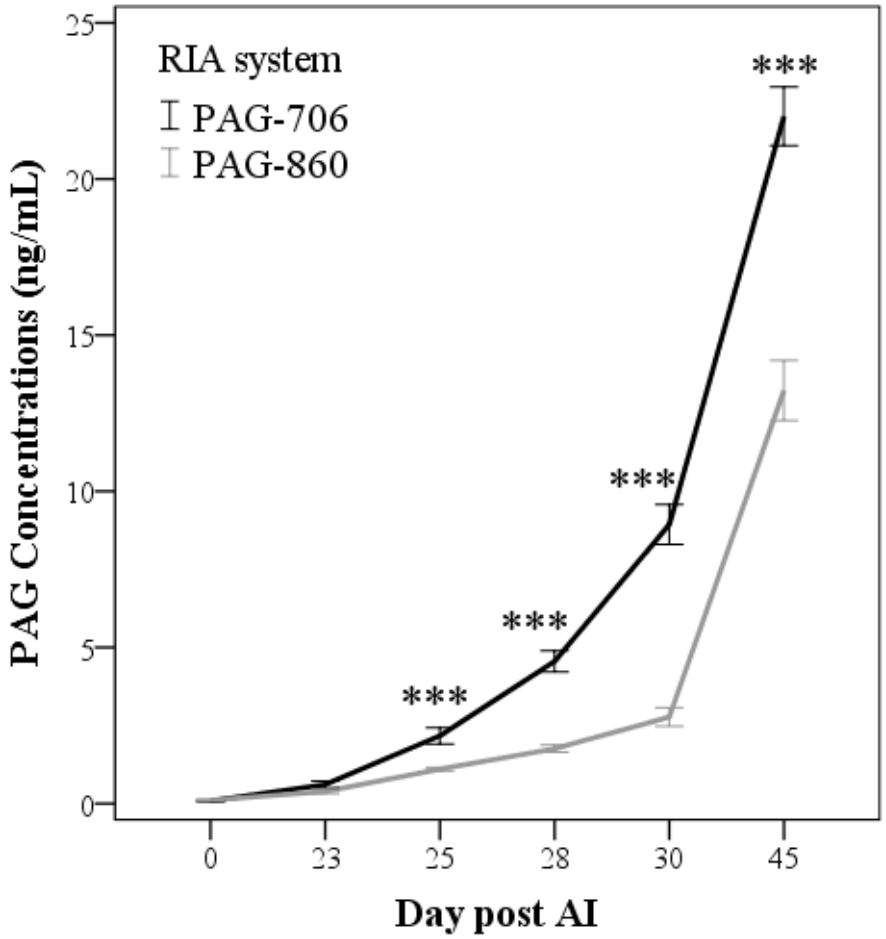
and characterization of a bovine pregnancy-associated glycoprotein. Biology of Reproduction, 45, 1-10.

Zoli, A., P, Guilbault, L. A., Delahaut, P., Ortiz, W. B., Beckers, J. F., (1992). Radioimmunoassay of a bovine pregnancy-associated glycoprotein in serum: its application for pregnancy diagnosis. *Biology of Reproduction*, 46, 83-92.

Day	Radioimmunoassay systems	Diagnosis	Outcome*		Sensitivity	Specificity	Accuracy	PPV	NPV
			Non pregnant	Pregnant					
23 d	PAG-706	Non pregnant	46 (100.0%)	37 (71.2%)	29	100	62	100	55
		Pregnant	0 (0.0%)	15 (28.8%)					
	PAG-860	Non pregnant	46 (100.0%)	40 (76.9%)	23	100	59	100	53
		Pregnant	0 (0.0%)	12 (23.1%)					
25 d	PAG-706	Non pregnant	46 (100.0%)	10 (19.2%)	81	100	90	100	82
		Pregnant	0 (0.0%)	42 (80.8%)					
	PAG-860	Non pregnant	45 (97.8%)	12 (23.1%)	77	98	87	98	79
		Pregnant	1 (2.2%)	40 (76.9%)					
28 d	PAG-706	Non pregnant	46 (100.0%)	1 (1.9%)	98	100	99	100	98
		Pregnant	0 (0.0%)	51 (98.1%)					
	PAG-860	Non pregnant	46 (100.0%)	1 (1.9%)	98	100	99	100	98
		Pregnant	0 (0.0%)	51 (98.1%)					

*determined *ex post* on the basis of pregnancy diagnosis at days 28, 45 and 60

Day	Radioimmunoassay systems	Diagnosis	PAG-wb860		Cohen's kappa	P value for H_0: kappa = 0
			No pregnant	Pregnant		
23 d	**PAG-706**	**Non pregnant**	78 (79.6%)	5 (5.1%)	0.443	<0.001
		Pregnant	8 (8.2%)	7 (7.1%)		
25 d	**PAG-706**	**Non pregnant**	50 (51.0%)	6 (6.1%)	0.728	<0.001
		Pregnant	7 (7.1%)	35 (35.7%)		
28 d	**PAG-706**	**Non pregnant**	47 (48.0%)	0 (0.0%)	1.000	<0.001
		Pregnant	0 (0.0%)	51 (52.0%)		
Total	**PAG-706**	**Non pregnant**	175 (59.5%)	11 (3.7%)	0.808	<0.001
		Pregnant	15 (5.1%)	93 (31.6%)		



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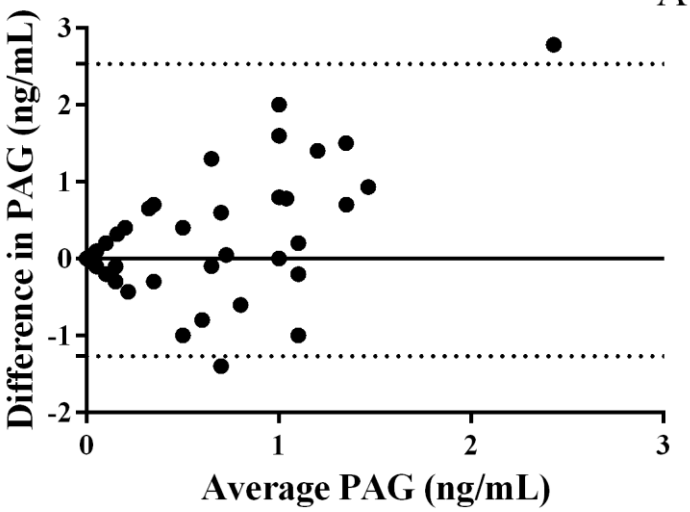

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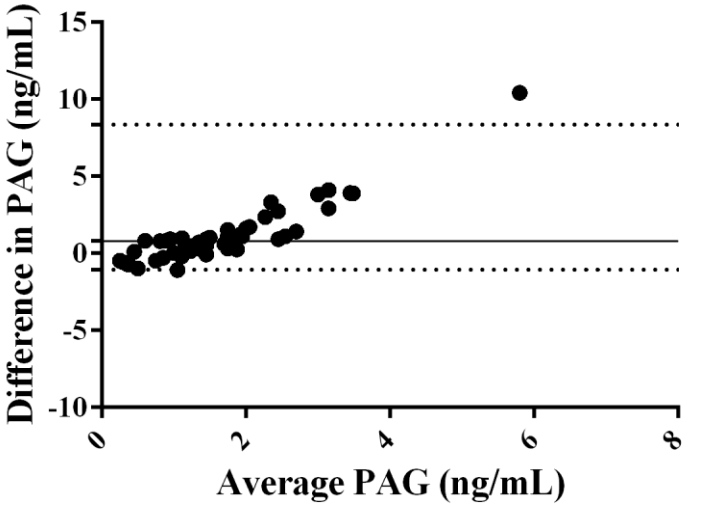

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