

# Comparison of accuracy of pregnancy-associated glycoprotein (PAG) concentration in blood and milk for early pregnancy diagnosis in cows

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## Summary

The aim of this study was to compare two methods of early pregnancy diagnosis by determining pregnancy-associated glycoprotein (PAG) concentration in blood and PAG concentration in milk. Blood and milk samples were obtained on days 0 (AI day), 14, 21, 28, 35, 49, 63, 77, 91 and 105 of gestation from 60 lactating Holstein Frisian cows from one herd, carrying live fetuses. To determine PAG concentration a specific RIA system (RIA-706) was used. PAG concentration in blood and milk increased after 28 days of pregnancy, with blood concentrations being significantly higher than in milk. However, the accuracy of both tests at this time point was similar (sensitivity: 92 % in blood, 93 % in milk; specificity 53 % and 60 % respectively). None of the tests were able to detect open cows properly at this stage. On day 35 of gestation sensitivity (100 % for blood, 97 % for milk) and specificity (100 % for blood, 100 % for milk) were high enough to be used for reliable pregnancy diagnosis. The accuracy (sensitivity and specificity) for PAG concentrations in blood and milk for the rest of the study was 100 %. Our investigation shows that PAG determination in milk is a stress-free and non-invasive method for early pregnancy diagnosis in cattle.

Keywords: pregnancy-associated glycoproteins, PAG, pregnancy diagnosis, cow

## Vergleich der Genauigkeit trächtigkeitsassoziierter Glykoproteine (PAG) in Blut und Milch zur frühen Trächtigkeitsdiagnose beim Rind

Das Ziel dieser Studie war es, die Konzentrationen trächtigkeitsassoziierter Glykoproteine (PAG) in Blut und Milch zur frühen Trächtigkeitsdiagnose bei der Kuh zu vergleichen. Blut- und Milchproben wurden an den Tagen 0 (KB), 14, 21, 28, 35, 49, 63, 77, 91 und 105 der Trächtigkeit von 60 laktierenden Holstein Frisian Kühen mit lebenden Feten gewonnen. Zur Bestimmung der PAG-Konzentration wurde ein spezifisches RIA-System (RIA-706) verwendet. PAG-Konzentrationen in Blut und Milch erhöhten sich ab Tag 28 der Trächtigkeit, wobei die Blutkonzentrationen deutlich höher waren, als diejenigen in der Milch. Die Genauigkeit der beiden Tests war zu diesem Zeitpunkt ähnlich (Sensitivität: 92 % im Blut, 93 % Milch, Spezifität: 53 % bzw. 60 %). Keine der beiden Methoden war in der Lage, in diesem Stadium nicht trächtige Kühe richtig zu erkennen. Am Tag 35 der Trächtigkeit waren Empfindlichkeit (100 % für Blut, 97 % für Milch) und Spezifität (100 % für Blut, 100 % für Milch) beider Methoden hoch genug, eine Trächtigkeit zuverlässig zu diagnostizieren. Die Genauigkeit (Empfindlichkeit und Spezifität) zur Messung der PAG-Konzentrationen in Blut und Milch betrug im weiteren Verlauf der Trächtigkeit 100 %. Diese Untersuchungen zeigen, dass die PAG-Bestimmung in der Milch eine stressfreie und nicht-invasive Methode zur frühen Trächtigkeitsdiagnose beim Rind darstellt.

Schlüsselwörter: trächtigkeitsassoziierte Glykoproteine, PAG, Trächtigkeitsdiagnose, Rind

## Introduction

Early pregnancy diagnosis is a fundamental tool for dairy cattle reproduction management. Efficient identification of open cows allows for reduction of economic losses through faster implementation of treatment, or elimination of infertile individuals from the herd. In addition to the well-developed rectal palpation and transrectal ultrasonography for pregnancy diagnosis in cows in parallel, the development of new methods of laboratory diagnosis is observed. One such method is the determination of pregnancy-associated glycoproteins concentration in blood or milk samples (Gajewski et al., 2008; Gajewski et al., 2009; Green et al., 2011; Zoli et al., 1992).

Pregnancy-associated glycoproteins (PAGs) constitute a large family of proteins isolated from many species of farm (cow, sheep, goat, buffalo) and wild animals (bison, fallow deer) during gestation (de Sousa et al., 2006). In cows, 22 molecules have been identified (Xie et al., 1991; Garbayo et al., Green et al., 2000, 2008; Gajewski et al., 2014). Proteins from bPAG (bovine PAG) family differ from each other regarding their amino acid content, the duration of their expression at placental tissue (Green et al., 2000), and probably regarding their secretion in the blood of pregnant cows. The entire family of glycoproteins is classified to the group of proteolytic enzymes as aspartic proteinases (AP), but enzyme activity was so far not confirmed for native proteins (Xie et al., 1991; Green et al., 2000).

For the bovine species, there are many publications relating to the PAG concentration in blood (Zoli et al., 1992; Perenyi et al., 2002; Gajewski et al., 2008; Gajewski et al., 2014). However, only a few publications report PAG analysis in milk by using RIA (Tainturier et al., 2006; Gajewski et al., 2008; Gajewski et al., 2014) or ELISA techniques (Metelo et al., 2002; Friedrich and Holtz, 2010). Different studies have indicated that PAG concentration in milk can be easily used for pregnancy diagnosis in small ruminants (González et al., 2001; El Amiri et al., 2003). The sensitivity and specificity for radioimmunoassay (RIA) used for PAG detection are very high and enable gestation diagnosis and embryo status control in small ruminants (González et al., 2001; El Amiri et al., 2003).

In cattle, Metelo et al. (2002) reported uselessness of a homologous RIA (hm PAG-RIA) for bPAG-1 detection in bovine milk (bovine PAG as standard and tracer, and for production of antisera). The same authors (Metelo et al. 2002) reported that the detection of PAG through the use of antibodies raised against glycoproteins from caprine and ovine origins (heterologous RIA systems; ht PAG-RIA) proved to be accurate in sheep and goats, but not in cows. In their work, PAG concentration in cows varied from 0.1 to 0.3 ng/ml until 70 days of gestation, which excludes this method to distinguish pregnant from non-pregnant cows. However, another study (Gajewski et al. 2014) indicates legitimacy of ht PAG-RIA (antisera raised against caprine PAG) application for pregnancy

diagnosis in cow's milk. The research of Gajewski et al. (2008) also demonstrated statistically significant positive correlation between progress of pregnancy and PAG concentration in cow's milk. Regarding the use of an ELISA test, Friedrich and Holtz (2010) described that PAG could not be detected in milk before day 60 after insemination. By using this technique, PAG concentrations amounted to only 3.2% of that measured in serum (Friedrich and Holtz, 2010). The goal of this study was to evaluate PAG concentration in blood and milk by using an ht PAG-RIA and to compare these methods with each other.

## Animals, Material and Methods

In this study 70 cows were examined. All animals were Holstein-Friesian breed, 3–8 years old and BCS 3 to 3.5. All cows were divided into 2 groups: an experimental group (pregnant cows,  $n = 60$ ) and a control group (non-pregnant cows,  $n = 10$ ). All cows underwent ultrasound pregnancy diagnosis (Mindray M5, LA, with linear-array probe 5–10 MHz) on days 35, 50 and 105 of gestation. Plasma and milk samples collection started from the insemination day and on weeks 2, 3, 4, 5, 7, 9, 11, 13 and 15 after insemination. Blood samples were taken from tail vein (*v. cava caudalis*) into EDTA tubes. After centrifugation (6'000 rpm for 10 minutes), plasma and defatted milk were stored at  $-20^{\circ}\text{C}$  until analysis. At the time of sampling the milk yield for each cow was also identified.

## PAG analysis

To determine PAG concentration in plasma and milk, a heterologous (ht) PAG RIA was used according to the method of Zoli et al. (1992), with modifications described by Perenyi et al. (2002). The difference between heterologous and homologous RIA systems concern the use of antisera raised in rabbits against antigens derived from caprine placenta (caPAG<sub>55+62kDa</sub>; AS#706) and not from bovine placenta (boPAG<sub>67kDa</sub>; AS#497). Pure boPAG<sub>67kDa</sub> preparation was used as standard and tracer (Zoli et al., 1992). Radiolabelling was carried out using lactoperoxidase method. To minimize non specific interference of proteins in plasma and milk, PAG-free sera and PAG-free milk were added to each tube of the standard curve (Gajewski et al., 2008).

## Results

The present work demonstrated the presence of PAG in blood and milk samples from all cows in the experimental group from the 4<sup>th</sup> week of gestation until the end of the study. In the control group the presence of PAG was observed in blood and milk of some cows ( $n = 5$ ) at the

3<sup>rd</sup> week of gestation. Average PAG concentration obtained from cows of the control group at days 0 to 21 of pregnancy was 0.22 ng/mL. All other samples of the control group (from 28 to 115 days of the study) were negative for PAG. Concentrations obtained in the blood were about 10 times higher than those measured in the milk.

In the experimental group, average PAG concentration in blood of pregnant cows (days 0 to 21) was 0.41 ng/mL. From days 28 to 63, the average concentration was 6.31 ng/mL, and from days 70 to 115 the mean value was 15.1 ng/mL plasma (Fig. 1). Average PAG concentration in milk varied between 0.24 ng/mL (days 0 to 21 of gestation), 0.68 ng/mL (days 28 to 63) and 1.02 ng/mL (days 70 to 115) (Fig. 2). The sensitivity and specificity results of both tests during the first 15 weeks of pregnancy are shown in Table 1.

Milk yield in the experimental group except 2 cows was stable and increased from 27 to 35 liters of average daily milking. In 2 cows mastitis was diagnosed and milk yield varied between 17 and 24 liters per day. No correlations were seen between milk yield and PAG concentration in the blood nor between milk yield and PAG concentration in the milk.

## Discussion

This study demonstrates the high efficiency of blood and milk testing for PAG as a method for early pregnancy di-

agnosis in cows starting at 5 weeks of gestation. During the first 4 weeks of gestation, the results were unacceptable for pregnancy diagnosis, primarily because of the low specificity of the test (Tab. 1). This is also the period in which PAG molecules from a previous pregnancy (especially when interpregnancy intervals are less than 70 days) are still detectable and representing also the greatest risk for embryo mortality (Prvanović et al., 2009). It is noteworthy that PAG is still detectable even 80–100 days after calving (Zoli et al., 1992). This aspect does not matter when examining heifers, but is important in multiparous animals when inseminated early (60 days) after parturition (Sousa et al., 2006).

The origin of detectable levels of PAG in blood (0.22 to 0.41 ng/mL) and milk (0.24 ng/mL) in the first 3 weeks after insemination was probably due to the remaining level of PAG from a previous pregnancy. The relatively long time needed for PAG to be cleared from maternal circulation can be explained by the very high concentrations present in maternal blood at parturition and by a long half-life of this glycoprotein (Kiracofe et al., 1993). PAG concentrations decrease steadily in the postpartum period, reaching undetectable levels by day 100 postpartum (Tainturier et al., 1996). PAG molecules were detected in a few samples at 97 days after previous parturition. All cows with longer than 97 interpregnancy intervals were negative for PAG in day “0” (day of AI). This is a crucial parameter in selecting the method of early pregnancy diagnosis. For these reasons, determi-

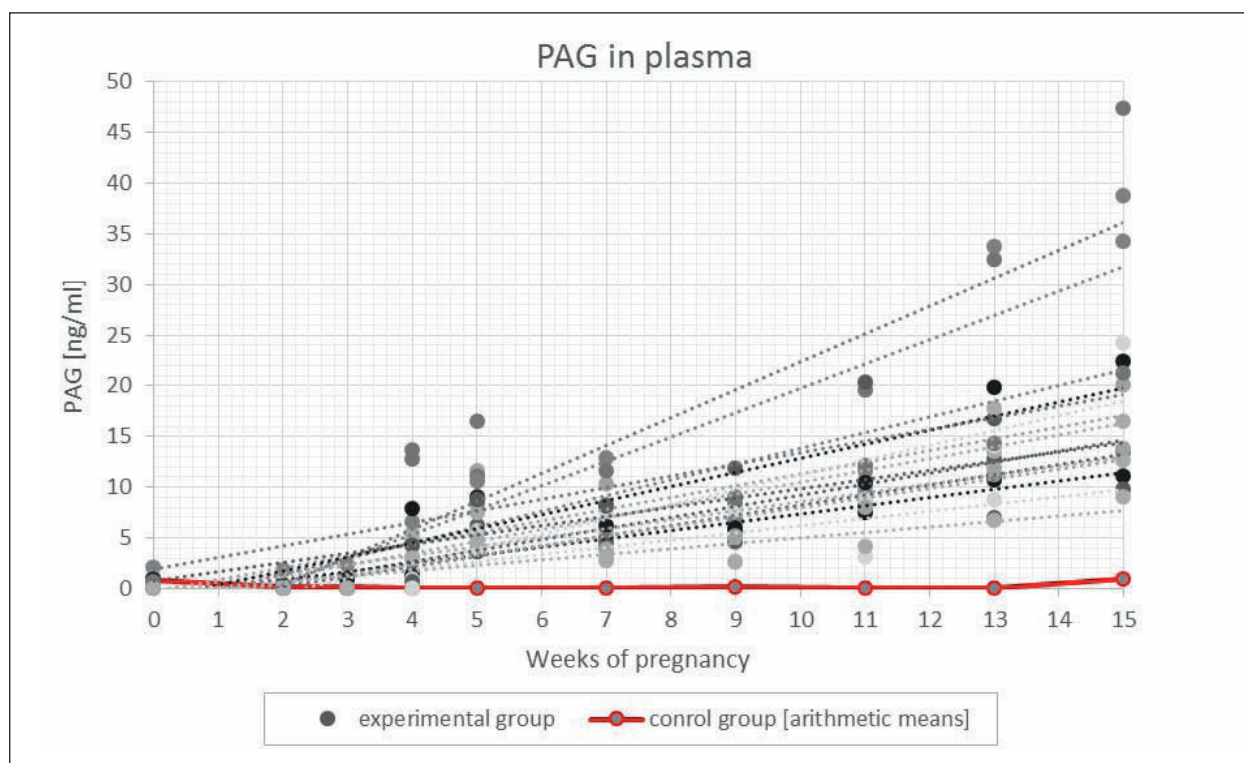


Figure 1: PAG concentrations in blood plasma of the experimental (pregnant cows) and control group (non-pregnant cows) during 15 weeks of pregnancy.

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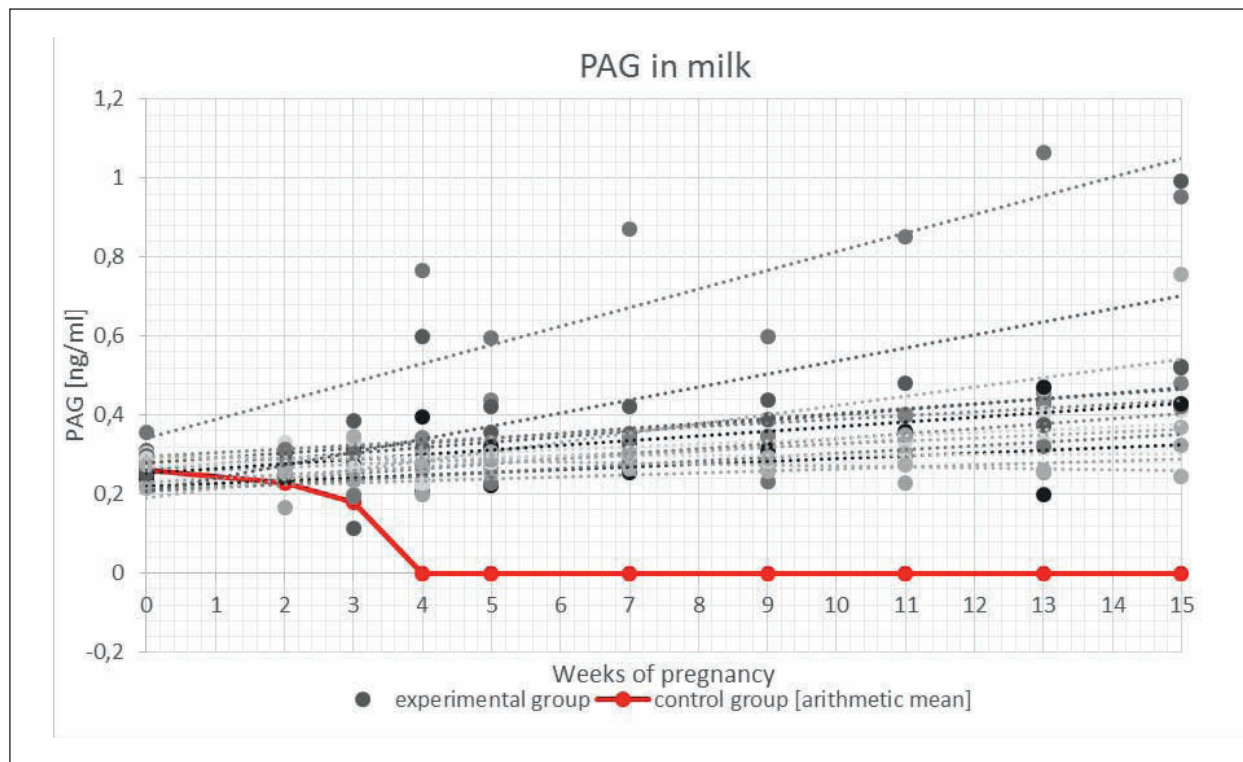


Figure 2: PAG concentrations in milk of the experimental (pregnant cows) and control group (non-pregnant cows) during 15 weeks of pregnancy.

Table 1: Sensitivity and specificity results of PAG analysis in blood and milk from 3 to 15 weeks of pregnancy.

| PAG in bovine plasma |        |        |      |      |      |      |      |      |
|----------------------|--------|--------|------|------|------|------|------|------|
| Week after AI        | 3      | 4      | 5    | 7    | 9    | 11   | 13   | 15   |
| Sensitivity          | 87,10% | 92,20% | 100% | 100% | 100% | 100% | 100% | 100% |
| Specificity          | 22,60% | 52,90% | 100% | 100% | 100% | 100% | 100% | 100% |

| PAG in bovine milk |        |        |      |      |      |      |      |      |
|--------------------|--------|--------|------|------|------|------|------|------|
| Week after AI      | 3      | 4      | 5    | 7    | 9    | 11   | 13   | 15   |
| Sensitivity        | 100%   | 93,30% | 100% | 100% | 100% | 100% | 100% | 100% |
| Specificity        | 90,40% | 60,00% | 100% | 100% | 100% | 100% | 100% | 100% |

nation of PAG concentrations during the first 4 weeks of pregnancy is not recommended. As expected, PAG concentrations were much lower in milk than in blood plasma: 9.2 times lower between days 28–63 and 14.8 times lower between days 70 and 115. However, this ratio of PAG concentrations in milk versus blood was smaller than by using ELISA technique (30 times lower in milk) (Friedrich and Holtz, 2010). In all examined cows, pregnancy was confirmed by rectal palpation and ultrasound examination. It is worth noting that the efficiency of all methods used was similar. This was also observed by LeBlanc (2013) reporting high specificity and sensitivity of pregnancy diagnosis when these methods were performed in parallel with rectal palpation.

In summary, low concentrations of PAG in milk samples is still a problem for pregnancy diagnosis. However over the last 6 years, an increasing sensitivity of the PAG method is noted which can be successfully implemented in co-management programs of assisted reproduction in cattle and for research procedures as well.

### Acknowledgements

The Belgian part of the present work was supported by grants from the SPW-DGARNE (D31-1284) to J. F. Beckers.

### Comparaison de l'exactitude entre les glycoprotéines associées à la gestation (PAG) dans le sang et le lait pour le diagnostic précoce de gestation chez la vache

Le but de cette étude était de comparer les concentrations de glycoprotéines associées à la gestation (PAG) dans le sang et le lait en vue d'un diagnostic de gestation précoce chez la vache. Des échantillons de sang et de lait ont été prélevés aux jours 0 (IA), 14, 21, 28, 35, 49, 63, 77, 91 et 105 de la gestation sur 60 vaches Holstein Frisonnes en lactation avec un fœtus vivant. On a utilisé pour mesurer la concentration de PAG une méthode RIA spécifique (RIA-706). Les concentrations de PAG dans le sang et le lait s'élevaient à partir du 28<sup>ème</sup> jour de gestation, les concentrations mesurées dans le sang étant nettement plus élevée que celles mesurées dans le lait. L'exactitude des deux tests était à ce moment-là similaires (sensitivité: 92 % dans le sang, 93 % dans le lait, spécificité: 53 % respectivement 60 %). Aucune des deux méthodes n'était, à ce stade, à même de distinguer une vache non portante avec justesse. Au 35<sup>ème</sup> jour de gestation, la sensibilité des deux méthodes (100 % dans le sang, 97 % dans le lait) et leur spécificité (100 % dans le sang, 100 % dans le lait) était assez élevées pour permettre un diagnostic de gestation sûr. L'exactitude (sensitivité et spécificité) des mesures de concentration de PAG dans le sang et le lait durant la suite de la gestation était de 100 %. Ces études montrent donc que la mesure de PAG dans le lait représente une méthode non-invasive et exempte de stress pour le diagnostic précoce de gestation chez la vache.

### Confronto sulla precisione delle glicoproteine (PAG) associate alla gravidanza nel sangue e nel latte per la diagnosi precoce della gravidanza nei bovini

Scopo di questo studio era di confrontare la precisione delle glicoproteine (PAG) associate alla gravidanza nel sangue e nel latte per la diagnosi precoce della gravidanza nei bovini. Campioni di sangue e di latte sono stati raccolti nei giorni 0 (KB) 14, 21, 28, 35, 49, 63, 77, 91 e 105 durante la gestazione di 60 mucche Holstein Frisian con fœti vivi. Per determinare la concentrazione delle PAG è stato utilizzato uno specifico sistema RIA (RIA-706). Le concentrazioni delle PAG nel sangue e nel latte sono aumentate al giorno 28 di gestazione e le concentrazioni nel sangue erano significativamente superiori a quelle nel latte. La precisione di entrambi i test era al momento simile (sensibilità: nel sangue 92 %, nel latte 93 %, specificità: 53 % risp. 60 %). In questa fase nessuno dei due metodi è stato in grado di riconoscere correttamente le mucche non gravide. Al 35<sup>o</sup> giorno di gestazione i valori della sensibilità (100 % per il sangue, 97 % per il latte) e della specificità (100 % per il sangue, 100 % per il latte) di entrambi i metodi erano sufficientemente elevati per diagnosticare una gravidanza. La precisione (sensibilità e specificità) per misurare i livelli di concentrazione delle PAG nel latte e nel sangue era del 100 % nel corso della gravidanza. Questi esami dimostrano che la determinazione delle PAG nel latte è un metodo libero da stress e non invasivo per la diagnosi precoce della gravidanza nei bovini.

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*Received: 2 June 2014*

*Accepted: 26 August 2014*