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CONCENTRATION OF BOVINE PREGNANCY ASSOCIATED GLYCOPROTEIN IN PLASMA AND MILK: ITS APPLICATION FOR PREGNANCY DIAGNOSIS IN COWS

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Pregnancy diagnosis is an important part in reproduction management of ruminants. The aim of the study was to use a new method for evaluating the bPAG and cPAG in milk and blood bPAG and compare this results with the other method for pregnancy diagnosis in the cows. The study was carried out in 220 Holstein Frisian cows. Heparinised blood samples were taken from the jugular vein and stored at -20°C until PAG assay by RIA. For bPAG and cPAG, RIA test, milk samples were homogenized. Pure bPAG was used as a standard tracer described by Zoli et al. (1992). The cows were diagnosed as pregnant by means of USG (Aloka SSD 210) and by rectal palpation. bPAG and cPAG concentration in milk increased after 28 day of pregnancy and showed the rapid increase near the parturition. The same results of bPAG concentration we obtained in the blood samples. The decline of bPAG concentration was faster in the milk than in the blood. The data showed that the RIA method is precise enough to measure PAG concentrations in the maternal blood and milk of cows. The data indicate that milk samples can be used for pregnancy diagnosis in cows. The sensitivity and specificity of RIA measurement of PAG are very high.

Key words: pregnancy-associated glycoproteins, pregnancy diagnosis, cows, markers

INTRODUCTION

Accurate early detection of pregnant and nonpregnant cows is an essential factor for optimizing reproductive performance in dairy cattle. In dairy cattle, pregnancy diagnosis is an important tool to measure the success of a reproductive management
Different studies clearly indicate that milk can be used for pregnancy diagnosis in small ruminants. Published results by Tainturier et al. (6) on concentration of bPAG in milk, confirm the detection of traces of bPAG in milk. The sensitivity and specificity of this method are very high (6). The results showed the possibility of the used PAG in milk and in blood as pregnancy test (6-8). It is especially helpful in the gestation diagnosis and in detection of embryonic mortality as a non stressed method in the pregnancy management in the ruminants (9-19). In the preliminary test concerning pregnancy-associated protein measurements in cow milk, Metelo et al. (20) reported concentration from 0.1 to 0.3 ng/ml PAG until 70 days after insemination. These concentrations cannot be used as reference for confirming pregnancies (20, 21) It is a completely different matter with PAG measurements in goat milk (7). Gonzalez et al. (7), reported that pregnancy-associated protein concentrations in goat milk is 10 times higher than for cows. The reported concentrations are min. 2.12 +/- 1.11 ng/ml on the 21st day of pregnancy and 4.65 +/- 1.42 on the 42nd day. Gonzalez et al. (7), proposes 1.6 ng/mL as a threshold value confirming pregnancy. The test reaches diagnostic significance after 32 days from insemination. The history of pregnancy-associated protein research goes back to 1982 when Butler et al. (22) isolated the first two pregnancy associated proteins. The first was alpha fetoprotein (pregnancy specific protein - A, PSPA), with a molecular mass of 65-70 kDa and pI 4.6 – 4.8. The other discovered protein turned out to be a completely new antigen. It was named Pregnancy Specific Protein B (PSPB). Its molecular mass is 47 to 52 kDa and pI 4.0 – 4.4. In 1988 Beckers et al. (23) reported partial isolation of cattle chorionic gonadotrophin (bCG) which was later identified as a protein from the group of aspartyl proteins. In 1991 Zoli et al. (24), using the method developed by Prof. Becker’s team (23), isolated another protein with a molecular mass of 67kDa and four pls : 4.4; 4.6; 5.2; 5.4; it was named pregnancy associated glycoprotein (PAG). The aim of the study was to use a new developed method for evaluating the PAG in milk and blood and compare this with other pregnancy diagnosis methods in the cows.

MATERIALS AND METHODS

This study was approved by Institutional Ethic Committee of Warsaw University of Life Sciences.

Total 220 Holstein-Friesian dairy cows, 3 to 8 years old were examined 30-55 days after AI for the first time using a real-time, B-mode diagnostic ultrasound Scanner (Alloka SSD 500, Japan) equipped with a 5.0 MHz linear-array rectal transducer and and rectal control after 55th day after AI. All ultrasonographic examinations and rectal palpation were performed by the same operator. Blood and milk were used for PAG tests. 10 ml of blood samples were taken from jugular vein to heparinized tubes. After centrifugation (1000 r/min) plasma was stored at -20°C and used for further tests. 50 ml samples of milk were taken into special containers and stored at -20°C until analysis.

bPAG and cPAG assays in blood plasma and milk

The concentration of two glycoproteins bPAG and cPAG were measured in milk and bPAG was determined in plasma. RIA I method was used in plasma tests. The milk was tested with the use of...
two methods: RIA II and RIA III. In RIA I method and RIA II researchers used antibodies produced after immunization of rabbits with purified pregnancy associated protein extract from cow placenta (bPAG 67kDa). In RIA III antibodies were produced after immunization (25).

Reagents

Tris BSA buffer (25 mM Tris, 10 mM MgCl₂, 0.02% w/v NaNO₃, pH 7.5 containing 1 mg/mL BSA; INC Biochemicals, Aurora, OH) was used in all PAG assays. Bovine PAG-1 preparation (boPAG 67kDa, accession number A61232) was used as standard and tracer (25). The standard curve ranged from 0.2 ng/mL to 25 ng/mL. The iodination (Na-I¹²⁵, Amersham Pharmacia Biotech, Uppsala, Sweden) was carried out according to the Chloramine T method (26). In order to minimize non-specific interference of plasma or milk proteins, PAG-free sera or PAG-free milk were added to each tube of the standard curve. Anti-bPAG-1₆₇kDa (27) and anti-cPAG₅₅+₆₂kDa (accession numbers P80935 and P80933) (28) polyclonal antisera were used respectively in bPAG-RIA and cPAG-RIA. The first antibody titers were determined to obtain a tracer binding ratio in the zero standard of approximately 20-30% (final dilution bPAG-RIA: 1/200,000 and cPAG-RIA: 1/80,000 for assay in plasma samples; final dilution bPAG-RIA: 1/100,000 and cPAG-RIA: 1/60,000 for assay in milk samples). Second antibody PEG solution was constituted by sheep anti-rabbit immunoglobulin (0.83 % v/v), normal rabbit serum (0.17 % v/v), polyethylene glycol 6000 (20 mg/mL; Vel, Leuven, Belgium), microcrystalline cellulose (0.05 mg/mL; Merck, Darmstadt, Germany) and 0.4 % (w/v) BSA (INC Biochemicals, Aurora, OH) diluted in Tris buffer.

Plasma samples RIA

The PAG measurement in plasma samples was performed according to the method of Perenyi et al. (2002) (29-31) with some modifications. Briefly, standard and plasma samples (0.1 mL) were diluted into crystal polystyrene tubes (75 x 12 mm, in duplicate) containing 0.1 mL and 0.2 mL of Tris BSA buffer, respectively. Total count (Tc) tubes, zero standards tubes (B0; 0.3 mL Tris-BSA) and tubes for assessing non-specific binding (NSB; 0.4 mL Tris-BSA) were also prepared. PAG-free sera (0.1 mL) was added to tubes of the standard curve, to NSB and B0. Appropriate dilution of antisera was added to samples, standards and B0. All tubes were incubated overnight at room temperature. The following day, 0.1 mL of I¹²⁵-PAG (25,000 cpm) was added and the tubes were incubated for 4 hours at room temperature. The total assay volume was 0.5 mL.

After the tubes had been incubated for 30 min with 1.0 mL the second antibody PEG solution, 2.0 mL of Tris-BSA buffer was added and the tubes were centrifuged (20 min at 1,500 x g). After centrifugation, the tubes were aspirated and the pellet containing the ¹²⁵I-PAG was counted using a gamma counter (LKB Wallac 126 Multigamma counter, Turku, Finland) with a counting efficiency of 75 %.

Milk samples RIA

Measurement of PAG in milk samples was based on the method previously described for plasma, excepting the use of a 0.5 mL of milk sample. Briefly, a volume of 0.5 mL of each milk sample was dispersed into polystyrene tubes (in duplicate). A similar volume of PAG-free milk was added to each point of the standard curve. Thereafter, an appropriate dilution of each antisera was added to all tubes excepting Tc and NSB, then the samples were incubated overnight at room temperature. In the following day, 0.1 mL of I¹²⁵-PAG (25,000 cpm) was added and the tubes were incubated during 4 additional hours at room temperature. The total assay volume was 0.7 mL.

The separation between free and bound fractions following the addition of the second antibody solution was identical to that previously described for plasma samples (31).
RESULTS

Average bPAG concentration in blood plasma for cows 18-42 days after AI varied from 1.25 ng/ml on the 19th day to 3.47 ng/mL on the 41st day. Exceptionally, for same cows a very high bPAG concentration of 15.4 ng/mL was noticed (Fig. 1, 2). For cows in the period from 44 to 90 days from AI the average bPAG concentration reached 9.4 ng/mL, showing a tendency to increase. Also in that group significant variations between single cases were seen. For cows in the

![Fig. 1 bPAG concentration in blood plasma of cows until 273 days of pregnancy.](image1)

![Fig. 2. cPAG concentration in milk of cows after AI until 220 days of pregnancy.](image2)
period from 91 to 200 days after insemination, bPAG concentrations increased rapidly, exceeding 100 ng/mL on day 196 of pregnancy (Figs 1-5).

In the last group of animals in their 7-9 months of pregnancy the increasing tendency was sustained, for cows in their last 10 days of pregnancy sudden release of bPAG occurred reaching a maximum > 2900 ng/mL on day 273 of pregnancy (Fig. 1). Variation analysis with the reliability level of 99% indicates a statistically significant correlation between progress of pregnancy and bPAG concentrations in blood (p < 0.1). The correlation rate is 0.414201, which indicates a comparatively weak correlation between the variables. In the group of cows tested, significant differences in plasma bPAG concentrations in the same period of pregnancy were recorded.

In 152 tested milk samples, bPAG presence was detected in 144 cases. bPAG was not detected in milk of all the cows during the first 3 weeks of pregnancy and some of the cows on days 41.50 and 62 of pregnancy. If 0.2 ng/mL of milk is taken as the threshold value, in 28 cases a negative test value was found for cows between 5 – 22 weeks (35 -151 days) of pregnancy (Fig. 2).

Average bPAG obtained varied from 0.06 ng/mL in the 6th week of pregnancy, through 0.2 ng/ml on average on the 119th day of pregnancy, 1.28 ng/mL on the 168th day, to 4.84 ng/mL on the 201st day. Variation analysis with the reliability level of 99% indicates statistically significant correlation between progress of pregnancy and bPAG concentration in blood (p < 0.1). The correlation rate is 0.56 which

![Fig. 3. bPAG concentration in milk of cows after AI until 220 days of pregnancy.](image)

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<th>Table 1. Correlation between bPAG concentration in blood plasma, bPAG in milk and cPAG in milk.</th>
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indicates a strong correlation between the variables (Fig. 4). The standard deviation of the test was 1.14 and the variation value: 1.31. Average cPAG concentrations obtained for cows 18 to 39 days after insemination were 0.236 ng/mL, between 40 and 90 days after insemination 0.68 ng/mL, between 90 and 200 days 1.02 ng/mL and between 200 and 220, 2.07 ng/mL. Variation analysis with the reliability level of 99% indicates statistically significant correlation between progress of pregnancy

![Relationship between serum bPAG concentrations (Y) and pregnancy progress (X)](image1)

*Fig. 4. The relation between bPAG concentration in blood and stage of pregnancy.*

![Relationship between milk cPAG concentration and pregnancy progress](image2)

*Fig. 5. Relationship between bPAG in milk and stage of pregnancy.*
and cPAG concentration in milk ($p < 0.1$) (Fig. 4). The correlation rate of 0.50 is higher than bPAG concentration in plasma and indicates quite a strong correlation between the variables. Standard deviation for cPAG was 1.09, and the variation value for this test was 1.20. The table above indicates a strong correlation between cPAG and bPAG in milk, and quite strong correlation between bPAG concentration in milk and bPAG in plasma, cPAG in milk and bPAG in plasma.

The relation between bPAG concentration in blood and stage of pregnancy is illustrated by the following regression equation, $Y = -164.651 + 13.4564 \times X$, ($r=0.17$, $p<0.1$), (red lines indicate reliability bracket, pink line – prediction limit) (Fig. 4).

Variation analysis with the reliability level of 99% indicates a statistically significant correlation between progress of pregnancy and bPAG concentrations in blood ($p<0.1$). The correlation rate is 0.414201, which indicates a comparatively weak correlation between the variables. In the group of cows tested, significant differences in serum bPAG concentrations in the same period of pregnancy were recorded.

Relationship between bPAG concentration in milk and stage of pregnancy is illustrated by the following regression equation $Y = -0.05985 + 0.00877 \times X$ ($r=0.25$, $p < 0.1$), (red lines indicate reliability bracket, pink line – prediction limit) Fig. 5.

Variation analysis with the reliability level of 99% indicates statistically significant correlation between progress of pregnancy and cPAG concentration in milk ($p < 0.1$). The correlation rate of 0.50097 is higher than bPAG concentration in serum and indicates quite a strong correlation between the variables. Standard deviation for cPAG was 1.0967, and the variation value for this test was 1.20275.

**DISCUSSION**

Systematic determinations of PAG in the blood of pregnant cow allow us to follow pregnancy development and also to study early embryo or foetal death (32-36). In our study, all the cows were examined for pregnancy using USG method in the period from 30 – 55 days after AI. After 55 days, all the cows were examined rectally to confirm pregnancy. All the examined cows were recognized as pregnant. Four cows aborted later. RIA I test for measuring bPAG concentration in the blood displayed 100% effectiveness in detecting pregnant cows, as well as non-pregnant cows. The sensitivity of the test and its positive prognostic value were 100% ($n=196$). This result is better than results obtained in Zoli tests (27), who obtained 94.6%. On account of the fact that only four cows were not pregnant, despite a positive RIA result, no conclusion regarding its prognostic value can be drawn. It is worth noticing that, with the use of RIA, the pregnancies of 9 tested animals were diagnosed 18 and 19 days after AI.

Important to know that the amount of PAG showing up in the milk in the early stages of pregnancy was only 4.5 to 16.7% of the amount of PAG present in blood. In the later stages of pregnancy these concentrations decreased significantly to 0.6% and
raised again to 2% during lactation. The sensitivity of the RIA II test to measure bPAG in milk was 64%, (20% up to day 60 of pregnancy) and for cPAG concentration 84.2% and 94.6%, on average 92%. These results indicate low usefulness of bPAG determination in milk but high for cPAG. Very low concentrations and high variables of obtained bPAG concentrations in milk in the early stages of pregnancy disqualify RIAII method in pregnancy diagnosis. cPAG concentrations obtained in RIA III tests are higher than in RIA II, however they rarely exceeded 1 ng/mL, which makes them unreliable in diagnostic tests, when the threshold value of 0.2 ng/ml is taken as the least detectable amount with a measurement error of 0.1 ng/mL. This method prognosticates some hopes for its further use, if the detectability of cPAG in milk can be raised. This issue requires further laboratory research. So far, research on PAG presence indicates low concentrations of these proteins in milk and it will be difficult to use them as pregnancy diagnosis.

In all cows tested, a significant variation of PAG concentrations in the same stages of pregnancy was obvious. Similar variations were seen in other study (32,37), where some cows were diagnosed pregnant 40 days after insemination by ultrasonography but had only minimal amounts of PAG in the blood (3, 4, 8). The explanation for differences in PAG concentration may be differences in body weight of individual cows influencing placental size and indirectly the number of binucleated cells in trophectoderm, which influences the production of pregnancy-associated glycoproteins. The work of Mialon et al. (12) confirms this and indicates that PAG concentrations in blood were much lower for cows which gave birth to calves with lower body mass (Holstein) compared to breeds (Charolais) that usually give birth to calves with large body mass.

To conclude, our data showed that the RIA methods were very precise for measuring PAG concentrations in the maternal blood and milk of the investigated cows. The results showed the possibility of the use cPAG in milk and bPAG and cPAG in blood as pregnancy test to replies the traditional rectal investigation or USG method.

Conflicts of interest statement: None declared.

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Received: October 30, 2008
Accepted: December 15, 2008

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