Early pregnancy diagnosis by measurement of pregnancy-associated glycoproteins (PAGs) in blood serum and milk of dairy cows

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Abstract

The present study aimed to determine the efficiency of two commercial ELISA tests for measuring pregnancy-associated glycoproteins (PAGs) in blood serum and milk in relation to early pregnancy diagnosis in lactating Holstein-Friesian cows. A total of 144 blood and milk samples form lactating cows were collected 30 days after artificial insemination and subjected to PAGs assay. Pregnancy-specific glycoproteins were determined by ELISA method using of Ruminant Pregnancy PAG test for milk and blood. A transrectal ultrasonography for pregnancy detection was performed 45 days after insemination. The obtained data of PAGs and ultrasound diagnoses were compared. The results for PAGs concentrations in serum and milk and indicators for efficiency of both tests were processed by computer statistical program (Statistica 7, Microsoft Corp. 1984-2000 Inc.) and compared using non-parametric method of comparison of means and percentages. In conclusion, the PAGs concentrations in blood serum were significantly ($P < 0.05$) higher than those in milk. Sensitivity, specificity and accuracy of both tests were close with a tendency to better result after measuring PAGs in blood serum. The evaluated tests have high efficiency and can be used as an alternative of other methods for early pregnancy diagnosis in dairy cows.

Key words: Pregnancy-associated glycoproteins, serum and milk, lactating cows, ELISA

Ранна диагностика на бременността при млечни крави чрез измерване на свързани с бременността гликопротеини (PAGs) в кръвен серум и мляко

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Резюме

Целта на настоящото изследване беше да се определи ефективността на два търговски ELISA теста за измерване на специфични за бременността гликопротеини (pregnancy-associated

glycoproteins – PAGs) в кръвен серум и мляко във връзка с ранна диагностика на бременност при лактиращи крави от породата Холщайн-фризийско говедо.

Изследвани бяха общо 144 проби от кръв и мляко, получени 30 дни след изкуствено осеменяване на кравите. Бременността беше потвърдена 15 дни след вземането на кръвни и млечни проби чрез трансректална ехография.

Специфичните за бременността гликопротеини се определяха чрез ELISA метод и използване на Alertys Bovine Pregnancy kit за кръв и мляко. Трансректално ултразвуково изследване за бременност се извършваше 45 дни след осеменяването. Получените резултати от анализа на PAGs бяха сравнени с тези от ултразвуковото изследване. Данните за концентрациите на PAGs в серум и мляко и праметрите за измерване на ефективността на двата теста се обработваха чрез компютърна статистическа програма (Statistica 7, Microsoft Corp. 1984-2000 Inc.) и сравняваха чрез непараметричен метод за сравнение на средни стойности и проценти. В заключение, нивата на PAGs в кръвен серум са значително ($P < 0,05$) по-високи от тези в мляко. Стойностите за чувствителност, специфичност и точност за двата теста са близки с тенденция към по-добър резултат при измерване на PAGs в кръвен серум. Изследваните тестове имат висока ефективност и могат да се използват като алтернатива на други методи за диагностика на ранна бременност при млечни крави.

Key words: свързани с бременността гликопротеини, серум и мляко, лактиращи крави, ELISA

Introduction

Pregnancy diagnosis in dairy cows is extremely important for successful practices in reproductive management and identification of the open cows and also can decrease the intervals between insemination and calving which will allow for early resynchronization and following artificial insemination. The methods for pregnancy detection (rectal palpation, ultrasound examination or chemical tests) vary greatly.

In the last few years many assays for pregnancy associated glycoproteins (PAGs) were created. PAGs are secreted from the binucleate trophoblast cell in the placenta (Wallace et al., 2015). Entering the maternal blood circulation (Wooding and Wathes, 1980; Wooding and Burton, 2008; Pohler et al., 2013) glycoproteins can be found in blood samples of pregnant cows as early as 21–24th day of insemination using RIA or ELISA. Early pregnancy diagnosis shows relative success by measuring the PAGs serum concentration earlier than the 28th of insemination (Perényi et al., 2002; Silva et al., 2007; Sousa et al., 2006).

Early pregnancy detection is of extreme importance for the management and economics of a dairy farm. Each potential method should be compared with the gold standard which is transrectal ultrasonography. It has been proven that the success of early pregnancy detection based on PAGs concentration on the 31th day is similar to ultrasound examination on the same gestational day (Karen et al., 2015; Reese et al., 2016; Ricci et al., 2015).

Commercial test for PAG detection can detect pregnancy in cows with great accuracy around 95–98% using blood or milk samples after the 28th day after insemination (Breukelman et al., 2012; Le Blanc and Short, 2013; Romano and Larson, 2010; Silva et al., 2007).

The present study aimed to determine the efficiency of two commercial ELISA tests for measuring pregnancy-associated glycoproteins (PAGs) in blood serum and milk in relation to early pregnancy diagnosis in lactating Holstein-Friesian cows.

Materials and Methods

Experimental animals

The study was conducted in 2020 at dairy farm Elit, Popovitsa, Plovdiv. In the experiment

were included 144 Holstein-Friesian dairy cows, 60 days after calving, divided of heifers (n = 57) and cows (n = 87) and subjected to estrus synchronization and artificial insemination. The day of artificial insemination was considered as Day 0.

Blood and milk samples used for pregnancy-associated glycoproteins' detection

Blood samples were collected from all the cows on the 30th day of the artificial insemination from *v. jugularis* in 10 ml tubes. Then the samples were placed in room temperature for 1 h so that coagulation may occur, afterwords they were stored at 4–6 °C and transported to the laboratory. The samples were then centrifuged at 2000 × g for 10 minutes, the serum was collected and stored at -20 °C.

Milk samples were collected from all the cows on the 30th day after artificial insemination in 30-ml plastic containers. The samples were then transported at 4–6 °C and examined 1 h their arrival in the laboratory.

Blood and milk samples were collected before the midday milking.

The PAG concentrations in blood and milk samples were measured using commercial tests Ruminant PAG ELISA (IDEXX Laboratories, Inc., Westbrook, ME, USA). All tests were performed in the laboratory of the Department of "Obstetrics, reproduction, reproductive disorders", Faculty of Veterinary Medicine. The results of the assays were measured as optic density (OD) of sample, corrected by subtracting OD of the sample (S) and the OD of the negative control (N) measured with 450 nm wavelength the final result is S-N.

Pregnancy results were recorded on the base of reference values for pregnancy defined by manufacturer. For Bovine Pregnancy serum, when S-N is < 0.300, the cow is considered open, or „non pregnant ", when S-N is > 0.300 and < 1.000, the result is "pregnant-check again" and when S-N is ≥ 1.000, the cow is labeled „pregnant ". For Bovine Pregnancy milk, when S-N is < 0.100, the cow is classified as „non pregnant"; when S-N is > 0.100 and < 0.250, the result is "pregnant-check again" and when S-N is ≥ 0.250, the cow is considered „pregnant ".

Pregnancy diagnosis

Pregnancy examination was performed using ultrasound scanner SonoScape A5Vet (SonoScape, Co. LTD, Shenzhen, China), multifrequency (7–12 MHz) linear transducer and transrectal approach on the 45th day after artificial insemination. Pregnancy diagnosis was based on a few criteria – uterine lumen fluid, visualization of embryo and embryo heart rate.

The experiment was conducted according to the recommendations of the Local Animal Ethics Committee and regulation for human attitude and animal protection.

Statistical analysis

The results were processed using computer program StatSoft (Statistica 7, Microsoft Corp. 1984-2000 Inc.). We used the ANOVA method, LSD test for multifactor comparison and non-parametric analysis for comparison of proportions. Results are presented as (Mean), standard deviation (SD), relative share (%) and degree of credibility. Values of $P < 0.05$ were considered statically significant.

Results

Depending on the S-N values of PAG in milk samples on the 30th day after insemination, the cows were classified as 73 pregnant, 67 non pregnant and 4 cows were classified as pregnant-check again. Mean values of PAGs in milk for positive cases were 1.555 ± 0.420, for non pregnant 0.023 ± 0.021 and for the pregnant-check again – 0.132 ± 0.042 (Table 1). During the ultrasound transrectal examination 55 of the 73 cows with high S-N values were confirmed as pregnant. From the non-pregnant group based on PAGs in milk samples 66 cows were confirmed as open and only one cow with low S-N value (0.024) was found pregnant. From the pregnant-check again group one cow was confirmed pregnant and the remaining 3 were found to be open.

The readings for the PAGs measured in serum on the 30th day after artificial insemination confirmed 69 cows as pregnant, 70 were non pregnant and 5 cows were assigned for the pregnant-check again group. The positive mean values for serum PAGs were 3.840 ± 0.031, negative were 0.018 ± 0.045 and for the re-check were 0.509 ± 0.031 (Table 1). During the ultrasound transrectal examination pregnancy was confirmed for 55 cows with positive results in PAGs. Negative diagnosis was found in 69 cows but there was one exception in one of the cows with confirmed pregnancy (the same cow which showed low S-N values in milk PAGs).

To grade the results from PAG ELISA tests in serum and milk for the cows with unknown pregnancy status, a table was created. The table consists of a few indices – sensitivity, specificity,

Table 1. S-N values of pregnancy-associated glycoproteins (PAGs) in serum and milk on the 30th day after the artificial insemination

Таблица 1. S-N стойности на гликопротеините свързани с бременността (PAGs) в серум и мляко на 30-ия ден след изкуствено осеменяване

Pregnant cows / Бременни крави		Non-pregnant cows / Небременни крави		Pre-check cows / Повторна проверка	
PAGs in milk / мляко n = 73	PAGs in serum / серум n = 69 /	PAGs in milk / мляко N = 67	PAGs in serum / серум N = 70	PAGs in milk / мляко N = 4	PAGs in serum / серум N = 5
Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
1.555 ± 0.420	3.840 ± 0.031*	0.023 ± 0.021	0.018 ± 0.045	0.132 ± 0.042	0.509 ± 0.031

*S-N – „sample minus negative control"; *P < 0.0001*

*SN – „стойност на пробата минус отрицателната контрола"; *P < 0,0001*

Table 2. Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPVand accuracy of PAG ELISA tests in serum and milk for pregnancy diagnosis on the 30th day after artificial insemination. *Adapted from Ricci et al., 2005.*

Таблица 2. Чувствителност, специфичност, положителна прогнозна стойност (PPV), отрицателна прогнозна стойност (NPV) и точност на PAG ELISA тестове в серум и мляко за диагностика на бременност на 30-ия ден след след изкуствено осеменяване. *Адаптирана от Ricci et al., 2005.*

PAG	Sensitivity / Чувствителност	Specificity / Специфичност	Accuracy / Точност	PPV / ППС	NPV / ОПС
ELISA	% (n/n)	% (n/n)	% (n/n)	% (n/n)	% (n/n)
Milk / Мляко	98,2 (55/56)	78,6 (66/84)	86,4 (121/140)	75.3 (55/73)	98.5 (66/67)
Serum / Серум	98.2 (55/56)	82,9 (68/82)	88,5 (123/139)	79.7 (55/69)	98,6 (69/70)

Sensitivity – ratio of pregnant cows with positive results based on PAG ELISA

Specificity – ratio of non pregnant cows with negative results based on PAG ELISA

Accurancy – proportion of the results for pregnant, non pregnant and their correct classification based on PAG ELISA

PPV – ratio of pregnant cows based on PAG ELISA, which were confirmed pregnant

NPV – ratio of non-pregnant cows based on PAG ELISA, which were confirmed non pregnant

Чувствителност – делът на бременни крави с положителен резултат от PAG ELISA

Специфичност – делът на небременните крави с отрицателен резултат от PAG ELISA

Точност – пропорция на резултатите от състоянието на бременността, бременни и не бременни, които са били правилно класифицирани от PAG ELISA

ППС – делът на кравите, диагностицирани бременни с помощта на PAG ELISA, които наистина са били бременни

ОПС – делът от кравите, диагностицирани като небременни с помощта на PAG ELISA, които наистина не са били бременни

positive prognosis value (PPV), negative prognosis value (NPVand accuracy on the 30th day and is based on the transrectal ultrasound examination on the 30th and 45th day after artificial insemination (Table 2).

The results of the current experiment show that the sensitivity of the PAG ELISA tests in serum and milk is high (98.2% for both type of testing), in comparison the specificity is (82.9% and 78.6%). NPV for serum and milk samples of PAG ELISA test is found to be very high (98.5% for both tests) compared to PPV values (79.7% and 75.3%). The accuracy of both type of PAG ELISA tests on the 30th day after artificial insemination is 88.5% and 86.4%, for serum and milk samples. These calculations exclude the samples classified as Pregnant-check again.

Discussion

The mean S-N values in serum samples in the positive cases were significantly higher ($P < 0.001$) than those in milk samples. Relative levels of PAGs measured in milk samples were approximately half of the those measured in plasma. This was expected because milk is a filtrate from blood and PAGs are relatively large glycoproteins (molecular weight = 40–80,000) associated with the liquid composition of milk (Green et al., 2005).

The sensitivity analysis of the obtained results supports the opinion that the accuracy of using PAGs levels in serum and milk pregnancy detection in dairy cows on day 30 after artificial insemination is high. Our results are in agreement with other studies conducted which investigated the period 27–39 days of gestation, at this period PAGs levels in both serum and milk are at a peak (Lawson et al., 2014; LeBlanc, 2013; Silva et al., 2007; Sinedino et al., 2014). The differences found between individual studies are most likely due to the commercial tests used, experience of operator and individual variability in serum and milk PAGs concentrations. These differences were more pronounced when PAG ELISA tests for pregnancy diagnosis were administered between days 26 and 30 after artificial insemination (Humblot, 2001; Kaya et al., 2016; Zoli et al., 1992).

To realize a positive economic effect of using tests for early pregnancy detection, the sensitivity should be greater than 96% when the test is applied at day 30 and greater than 94% when used at 24-day after artificial insemination (Giordano et al., 2013). The sensitivities of both the serum and milk PAG ELISA assays evaluated in the present study (Table 2) and those reported by other authors (Silva et al., 2007; Romano and Larson, 2010) exceed these values. Recorded results show that commercial PAG ELISA tests for pregnancy diagnosis at day 30 after artificial insemination are applicable and economically viable for dairy farms.

In our study, two cases of pregnancy were registered by the ultrasound examination with low S-N values of serum and milk PAGs or negative predictive value of 98.6% and 98.5%, respectively. False negative findings may result from low PAGs levels between days 25 and 32 after artificial insemination (Szenci et al., 1998; Zoli et al., 1992).

The positive predictive value for milk PAGs of 75% and serum PAG of 79.7% are low compared to those reported by other authors (Kaya et al., 2016). Silva et al. (2007) reported that incorrect (false) positive results were observed in individual cows up to 100 days after calving from their first pregnancy. Lactating cows in this study were on average 137 days in lactation. In the present study, cows with incorrect (false) positive PAGs results in serum and milk were on average 133 days in lactation.

Commercial tests provide 98–99% true positive (pregnant) readings and false positives (reported as pregnant but open) within 1–5% (Reese et al., 2016). Variations between outcomes and consequences of misclassifications of reproductive status (false positives and false negatives) were reported by Szenci et al. (1998), Silva et al. (2007), Green et al. (2009) and Friedrich and Holtz (2010). In our study, 18 (12.5%) false-positive results were found in the PAG ELISA test in milk and 14 (9.7%) in the PAG ELISA in serum. Incorrect (false) positive results may be due to embryonic death before maternal blood

PAGs levels fall below the detection threshold using the ELISA test. The half-life of PAG in maternal blood after induced embryonic death has been reported to be 2.7 to 7 days (Szenci et al., 1998). Therefore, false positive PAG ELISA test results can sometimes be due to embryonic death or fetal loss (Kaya et al., 2015). Thompson et al. (2010) noted that cattle suffered late embryonic losses between days 28 and 45 after artificial insemination, with day 30 plasma PAGs concentrations being lower compared to cows maintained the pregnancy. This is also confirmed by our results regarding mean S-N values (3.840 ± 0.031 vs. 3.644 ± 0.617; $P < 0.01$) in serum and milk (1.555 ± 0.420 vs. 1.288 ± 0.628; $P < 0.0212$) which are lower than those of the animals confirmed in the ultrasound examination. The relatively high concentrations of PAGs in the absence of a detectable embryo are most likely the result of residual PAGs previously secreted by the already dead embryo. Embryonic lethality is a complex process that is not always associated with lower PAGs concentration (Lawson et al., 2014).

Green et al. (2005) found the presence of PAGs in blood in five of 42 cows on day 15 after artificial insemination. However, PAGs detected at this stage are unlikely to be due to pregnancy as the placenta is not yet developed. Therefore, false positive results may be due to cross-reactions with foreign proteins.

Conclusion

In conclusion, the PAGs concentrations in blood serum were significantly ($P < 0.05$) higher than those in milk. Sensitivity, specificity and accuracy of both tests were close with a tendency to better result after measuring PAGs in blood serum. The evaluated tests have high efficiency and can be used as an alternative of other methods for early pregnancy diagnosis in dairy cows.

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