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Early Pregnancy Diagnosis in Bulgarian Murrah Buffaloes by Measurement of Pregnancy-Associated Glycoproteins (PAGs) in Blood Serum and Milk

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ABSTRACT

***Background*:** The timely and accurate pregnancy diagnosis in productive animals allows improving reproductive performance by reduction of the calving interval. The tests for assay of maternal blood pregnancy-associated glycoproteins (PAGs) have been developed for pregnancy diagnosis in large ruminants. The present study aimed to evaluate the efficacy of commercial ELISA kits for detection of pregnancy-associated glycoproteins (PAGs) in blood serum and milk for early pregnancy diagnosis in Bulgarian Murrah buffaloes.

***Materials, Methods & Results*:** Blood samples were collected from 57 buffaloes and 27 heifers along with 55 milk samples on the 28th day following artificial insemination. The pregnancy was confirmed on the 35th day by transrectal ultrasonography. Blood serum and milk PAGs concentrations were assayed with commercial PAGs ELISA kit for early pregnancy diagnosis. The data were analyzed by a non-parametric Chi-square test for comparison of proportions with Statistica 7 (StatSoft, Microsoft Corp. 1984-2000 Inc.) statistical software. Serum PAGs values were statistically significantly higher than those in milk ($P < 0.05$). Based on the collected samples, S-N values for serum PAGs 28 days following artificial insemination of buffaloes, 17 animals were classified as pregnant and 40 – as non-pregnant. In heifers, 7 positive diagnosis of early pregnancy vs 20 negative diagnoses were obtained. The assay parameters - sensitivity, specificity and accuracy - were within acceptable ranges for both tests, with superior results for serum PAGs than for milk PAGs test. The tests had a high sensitivity and accuracy for early diagnosis of pregnancy in buffaloes and heifers on the 28th day after artificial insemination. The sensitivity of the test in the present study was acceptable, 94.4% for the serum and 87.5% for the milk PAGs ELISA in buffaloes, but low in heifers (55.6%). In terms of specificity, absolute values of PAGs tests in serum and milk were recorded in buffaloes and 88.9% for the serum ELISA in heifers. The accuracy of the serum and milk ELISA tests on the 28th day after artificial insemination was 98.2% and 96.4% respectively for buffaloes and 77.8% for the serum test in heifers. The rates of positively predicted diagnoses (PPV) for the serum and milk PAGs tests were 100% in buffaloes and 71.4% in heifers. The negative predicted values (NPV) for the milk and serum PAGs tests were 95.1% and 100% respectively in buffaloes and 80.0% in heifers.

***Discussion*:** The PAGs ELISA developed in the last years is a very useful tool in early pregnancy diagnosis, especially in large ruminants raised for milk. However, collection of blood causes stress to the animals. Thus, the use of milk as an alternative for performing pregnancy tests in buffaloes has attracted increasing interest from buffalo farmers. The present study highlights the advantage of using a milk sample rather than blood for PAGs assay as a non-invasive technique causing minimum stress to the potentially pregnant animals. More importantly, the presence of PAGs in milk provides the possibility to identify pregnant buffaloes within a relatively early period following insemination, allowing for timely inclusion of non-pregnant animals in breeding programs. The analysis of the sensitivity, specificity and accuracy of obtained results from PAGs in serum and milk, as well as the positive and negative diagnoses made showed that they were within acceptable limits. Based on the results obtained for PAGs in serum and milk it may be assumed that PAG ELISA tests are sufficiently specific, sensitive and accuracy for early pregnancy diagnosis on the 28th day after artificial insemination at buffalo farms.

Keywords: pregnancy-associated glycoproteins, serum and milk, ruminants, buffaloes, ELISA.

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INTRODUCTION

The timely and accurate diagnosis of pregnancy in productive animals allows improving reproductive performance by reduction of the calving interval [22,30]. In buffaloes, pregnancy diagnosis is mainly done by rectal palpation or transrectal ultrasonography but these methods give precise results after the 45th day following insemination [2].

The pregnancy-associated glycoprotein (PAGs) is a pregnancy-specific biomarker that is used as indicator of pregnancy in large ruminants. In maternal blood and milk, PAGs increase to detectable levels 24 to 28 days after insemination [5,12]. The isolation of PAGs from the placenta of buffaloes allowed developing the RIA 860 detection system and improving the accuracy of the early diagnosis of pregnancy in buffaloes to 99% on the 28th day following artificial insemination [4,6].

The tests for assay of maternal blood pregnancy-associated glycoproteins (PAGs) have been developed for pregnancy diagnosis in large ruminants [14,32]. During the years, the blood PAG ELISA became a useful practical tool for early pregnancy detection in dairy cattle and buffalo farms [13,28]. Later, a commercial test for assay of PAGs in milk has been developed, marketed and evaluated in field experiments [20] as alternative to blood PAGs test. A number of studies have determined milk PAG concentrations in dairy and beef cattle [10,16,19,24] and at a lesser extent, in buffaloes [3,6,27].

The objective of the study was to evaluate the efficacy of commercial ELISA kits for detection of pregnancy-associated glycoproteins (PAGs) in blood serum and milk for early pregnancy diagnosis in Bulgarian Murrah buffaloes.

MATERIALS AND METHODS

Animals

The study was performed on the buffalo farm Agricultural Institute - Shumen. It included 57 lactating buffaloes after 45 DIM and 27 buffalo heifers from the Bulgarian Murrah breed after application of estrus synchronisation protocols. The day of artificial insemination was accepted as day 0. All procedures were conducted in accordance with the guidelines for welfare and animals protection showed in Bulgarian legislation.

Blood and milk samples for pregnancy-associated glycoproteins assay

Blood samples were collected on the 28th day following artificial insemination from v. jugularis in 10 mL vacutainers[1], left to clot at room temperature before being transported at 4-6°C to the laboratory. Samples were centrifuged at 2000 × *g* for 10 min and separated sera were stored at -20°C.

Milk samples were collected from the buffalo cows on the 28th day following artificial insemination in 30 mL plastic containers during the morning milking. The samples were transported at 4-6°C and were assayed within an 1 h after their arrival at the lab. Serum and milk PAGs concentrations were assayed with commercial ELISA kit[2]. All tests were performed in the Laboratory of the Department of Obstetrics, Reproduction and Reproductive Disorders of the Faculty of Veterinary Medicine, Trakia University. The ELISA results (S-N values) were obtained by subtracting the reference wavelength OD of the sample (S) from the optical density (OD) of the sample, minus the negative control OD (N) at 450 nm after correction of both values by subtracting the reference wavelength OD of the negative control.

The pregnancy status was interpreted versus cut-off values specified by PAG ELISA's manufacturer[3]. For serum PAGs test, buffaloes with S-N values < 0.300 were classified "non-pregnant"; those with 0.300 < S-N values < 1.000 were classified "for recheck" and those with -N values >1.000: "pregnant". For milk PAGs, buffaloes with S-N values < 0.100 were classified "non-pregnant"; those with 0.100 < S-N values < 0.250 were classified "for recheck" and those with S-N values > 0.250: "pregnant".

On the basis of ELISA results on the 28th day after artificial insemination and transrectal ultrasonography results on the 35th day following artificial insemination, a 2 x 2 contingency table (Table 2) was made for calculation of ELISA sensitivity, specificity, accuracy, positive predictive value (PPV) and negative predictive value [24]. The results from PAGs tests were classified in the following categories: correct diagnosis "pregnant" (a); incorrect diagnosis "pregnant" (b); correct diagnosis "non-pregnant" (c) and incorrect diagnosis "non-pregnant" (d). These values served for calculation of test sensitivity [100 a/(a + d)], specificity [100 c/(c + b)], accuracy [100 (a + c) / (a + c + b + d)], PPV [100 a/(a + b)] и NPV [100 c/(c + d)].

Ultrasound pregnancy diagnosis

Pregnancy was diagnosed by ultrasound device[3] (Esaote MyLab One Vet) equipped with a linear multifrequency 7-12 MHz probe on the 35th day following artificial insemination. Criteria for pregnancy diagnosis included detection of enlarged uterine lumen, anechogenic alantoic fluid and embryo with visible cardiac activity, described by Fricke *et al*. [15].

Statistical analysis

The data were processed by the non-parametric Chi-square test for comparison of proportions with Statistica 7 (StatSoft, Microsoft Corp. 1984-2000) statistical software[4]. The results are expressed as mean, standard deviation (SD), relative proportion (%) and significance levels. Differences were assumed as statistically significant at $P < 0.05$.

RESULTS

Based on the obtained serum PAGs S-N values on the 28th day following the artificial insemination, 17 buffaloes were determined as pregnant and 40 as non-pregnant. The average serum PAGs S-N value in buffaloes with early pregnancy diagnosis was 1.342 ± 0.394 vs 0.063 ± 0.046 for non-pregnant animals. During the ultrasound examination performed on the 35th post-AI day, pregnancy was confirmed in all 17 buffaloes with positive result from serum PAGs ELISA. Negative diagnoses were confirmed in 39 animals, and pregnancy was recorded in 1 buffalo (S-N value 0.088) [Table 1].

In the buffalo heifers, 7 diagnoses of early pregnancy and 20 negative diagnoses were done on the basis of the registered PAGs values. The average serum S-N values in the heifers positive for pregnancy were 0.865 ± 0.375 vs 0.044 ± 0.026 for those that were tested negative. The ultrasound examination on the 35th day after the artificial insemination, confirmed pregnancy in 5 of the animals with high serum S-N values. Negative diagnoses were confirmed in 16 heifers, and pregnancy was registered in 4 animals (S-N values in the range 0.028-0.191). The serum concentrations of pregnant buffaloes were significantly higher than those of pregnant heifers ($P < 0.05$).

Serum PAGs S-N values in pregnant buffaloes exceeded significantly those in milk ($P < 0.05$). On the basis of the S-N values of milk PAGs on the 28th day after artificial insemination, 14 buffaloes were classified as pregnant, 34 as non-pregnant and 7 animals - for recheck. Two of the animals did not lactate. The mean milk PAGs in positive cases was 0.782 ± 0.325, for non-pregnant animals: 0.075 ± 0.014 and for those classified "recheck" - 0.145 ± 0.037 (Table 1). The transrectal ultrasound examination confirmed pregnancy in all 14 animals with high S-N values. In the group of non-pregnant animals, the absence of pregnancy based on the results of milk PAGs was confirmed in 34 animals. In the animals for recheck, 2 buffaloes were detected pregnant by ultrasound (S-N values 0.116 and 0.179, as well as low serum ELISA S-N values 0.088 and 0.212) while the other 5 were confirmed as not pregnant.

The sensitivity of the test in the present study was acceptable, 94.4% for the serum and 87.5% for the milk PAGs ELISA in buffaloes, but low in heifers (55.6%). In terms of specificity, absolute values of PAGs tests in serum and milk were recorded in buffaloes and 88.9% for the serum ELISA in heifers. The accuracy of the serum and milk ELISA tests on the 28th day after artificial insemination was 98.2% and 96.4% respectively for buffaloes and 77.8% for the serum test in heifers. The rates of positively predicted diagnoses (PPV) for the serum and milk PAGs tests were 100% in buffaloes and 71.4% in heifers. The negative predicted values (NPV) were 95.1% and 100% respectively in buffaloes and 80.0% in heifers (Table 2).

Table 1. S - N values of pregnancy-associated glycoproteins (PAGs) in serum and milk of buffalo cows and serum of buffalo heifers on the 28th day after the artificial insemination.

Parameters	Pregnant buffalo cows	Non-pregnant buffalo cows	Pre-check buffalo cows
PAGs in milk of buffalo cows	n=14	n=34	n=7
mean ± SD	0.782 ± 0.325	0.075 ± 0.014	0.145 ± 0.037
PAGs in serum of buffalo cows	n=17	n=40	
mean ± SD	1.342 ± 0.394*	0.063 ± 0.046	
PAGs in serum of buffalo heifers	n=7	n=20	
mean ± SD	0.865 ± 0.375	0.044 ± 0.026	

S-N: Sample minus negative control; *$P < 0.05$.

Table 2. Sensitivity, specificity, accurancy, positive predictive value (PPV), negative predictive value (NPV) of the PAGs tests for early diagnosis of pregnancy in serum and milk of buffalo cows and in serum of buffalo heifers of the Bulgarian Murrah on the 28th day after the artificial insemination.

Parameters	Sensitivity %	Specificity %	Accuracy %	Positive predictive value (PPV)	Negative predictive value (NPV)
PAGs in serum of buffalo cows	94.4(17/18)	100(40/40)	98.2(56/57)	100(17/17)	100(40/40)
PAGs in milk of buffalo cows	87.5(14/16)	100(39/39)	96.4(53/55)	100(14/14)	95.1(39/41)
PAGs in serum of buffalo heifers	55.6(5/9)	88.9(16/18)	77.8(21/27)	71.4(5/7)	80.0(16/20)

Sensitivity: the proportion of pregnant cows with a positive result of the applied method. Specificity: the proportion of non-pregnant cows with a negative result of the applied method. Accurancy: proportion of pregnancy status results, pregnant and non-pregnant, that were correctly classified by the method used. PPV: the proportion of cows diagnosed as pregnant using the applied method that were actually pregnant. NPV: fraction of cows diagnosed as non-pregnant using the applied method that were not really pregnant.

DISCUSSION

The average serum PAGs S-N values in early pregnancy-positive cases and for non-pregnant cases are in conformity with studies conducted in cows [24] and buffaloes [27]. This is explicable, since milk is a filtrate from the blood, and PAGs are relatively large glycoproteins (with molecular weight 40-80,000) found in the aqueous portion of milk [13]. Low PAGs levels in milk compared to serum have also been reported in other studies conducted in cows [21] and buffaloes depending on their breed and productivity [1,9]. The authors demonstrated a strong negative correlation between milk yield and PAGs concentrations, especially during the 1st trimester of pregnancy. Differences in milk PAGs may be due in part to other factors such as breed, placental mass, age of the animals or even the farm (genetic selection and feeding management) [29]. Furthermore, as previously described in cattle and sheep, the expression of PAGs during the gestation is complex. Some PAGs molecules are expressed earlier, others - later in pregnancy, while thirds are expressed throughout the entire gestation period [14].

The PAGs ELISA recently is a very useful tool in early pregnancy diagnosis, especially in large ruminants raised for milk [28]. However, collection of blood causes stress to the animals. Thus, the use of milk as an alternative for performing pregnancy tests in buffaloes has attracted increasing interest from buffalo farmers [27]. The present study highlights the advantage of using a milk sample rather than blood for PAGs assay as a non-invasive technique causing minimum stress to the potentially pregnant animals. More importantly, the presence of PAGs in milk provides the possibility to identify pregnant buffaloes within a relatively early period following insemination, allowing for timely inclusion of non-pregnant animals in breeding programs. Several studies have found significant individual variations in PAGs levels in both milk and blood samples from buffaloes and cows [21,25]. These different PAGs levels among pregnant cows may be due to differences in body weight, placental size and the number of binucleate cells in the trophectoderm, which affect the PAGs production [11,27]. Our study confirms the possibility of early pregnancy diagnosis by detecting PAGs in milk of lactating buffaloes. The test used was demonstrated to be highly accurate for diagnosing early pregnancy, similar to that of the blood test. However, the milk PAGs test has its limitations in heifers [27].

In the present study, serum PAGs ELISA in buffaloes gave no false positive results compared to ultrasound outcomes on the 35th day after artificial insemination. The absence of false positive diagnoses in serum PAGs assay in buffaloes on post-AI day 26, confirmed on day 40 by ultrasound was reported by Tadeo *et al*. [27]. In another study, 3 false positive diagnoses were established in Egyptian buffaloes using RIA-706 31-35 days following artificial insemination [17].

In the heifers, 2 false positive and 4 false negative diagnoses were recorded. The presence of detectable concentrations in samples from non-pregnant females could be explained by the existence of extraplacental sources of PAGs [32]. The existence of antigens immunologically related to PAGs was demonstrated in testes and in ovarian extracts, justifying the use of adjective “associated” rather than “specific” in the

name of this family of placental glycoproteins [31]. In cows, physiologically, pregnancy loss after the 28th day coincides with an active placentation period marked by endometrial remodeling, migration of binucleate cells and PAG production changes [23]. The false positive diagnoses identified in the study may be possibly due to embryonic mortality. This process is complex and is not always associated with a lower relative serum PAGs concentration. The relatively high PAGs concentrations in the absence of a detectable embryo are most likely the result of residual PAGs previously secreted by the already dead embryo. Elevated PAGs may be also due to cross-reactions with foreign proteins other than PAGs [13,18]. False negative diagnoses by the milk PAGs test may result from low levels of PAGs between the 25th and 32nd days following the artificial insemination [26,32].

The sensitivity and specificity of the method based on assay of serum progesterone concentrations by RIA were 92.0% and 82.6% to 91.9% whereas the sensitivity and specificity of the radioimmunoassay pregnancy test using PAG-1 in serum samples were 95.2% to 100.0% and 56.7% to 79.0%, 29-30 days after artificial insemination [26]. PAGs tests have been shown as reliable biomarkers for early pregnancy detection in buffaloes [7,17].

The analysis of the sensitivity, specificity and accuracy of obtained results from PAGs in serum and milk, as well as the positive and negative diagnoses made showed that they were within acceptable limits. The values obtained from the present study were higher than those in Nili-Ravi buffaloes, performed during between the 24th - 35th day of gestation, when the levels of PAGs in both serum and milk marked early peaks. Arshad *et al*. [3] reported sensitivity (77.9 vs. 89.7 vs. 93.3%), specificity (84.2 vs. 87.7 vs. 93.9%) and accuracy (82.1 vs. 88.4 vs. 93.7%) by ELISA test in serum on the 24th, 28th and 35th days after artificial insemination. The results for the milk PAGs test in terms of sensitivity (77.6 vs. 89.5 vs. 95.0%), specificity (89.1 vs. 91.9, vs. 93.9%) and accuracy (85.1 vs. 91.1 vs. 94.3%) were similar. Barbato *et al*. [6] and Barile *et al*. [8] reported comparable results for serum PAGs determined by RIA-860 on the 28th day following artificial insemination, with accuracy 99%, sensitivity 98% and specificity 100%. Thus, the PAGs test in milk is confirmed as a highly accurate method for the diagnosis of early pregnancy, similar to that of the blood PAGs test [27].

CONCLUSIONS

The present study highlights the advantage of using a milk sample rather than blood for PAGs assay as a non-invasive technique causing minimum stress to the potentially pregnant animals. More importantly, the presence of PAGs in milk provides the possibility to identify pregnant buffaloes within a relatively early period following insemination, allowing for timely inclusion of non-pregnant animals in breeding programs. Based on the results obtained for PAGs in serum and milk it may be assumed that PAG ELISA tests are sufficiently specific, sensitive and accurate for early pregnancy diagnosis on the 28th day after artificial insemination at buffalo farms, therefore we recommend their use as an alternative method for early pregnancy diagnosis under production conditions.

MANUFACTURERS

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***Ethical approval*.** The experiment was approved by the National Ethics Committee for animals in accordance with the Bulgarian legislation (Ordinance No 20/1.11.2012) on the minimum requirements for protection and welfare of experimental animals and requirements for use, rearing and/or their delivery.

***Declaration of interest*.** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of paper.

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