Kafkas Univ Vet Fak Derg
24 (4): 497-502, 2018
DOI: 10.9775/kvfd.2018.19437

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://submit.vetdergikafkas.org

Research Article

Comparison of Bovine Pregnancy Associated Glycoproteins (bPAG-Serum and Milk), Bovine Pregnancy Specific Protein B (bPSP-B) Tests with Each Other and with Transrectal Ultrasonographic Findings for Early Pregnancy Diagnosis [1] [2]

Osman ERGENE [1,3,a] Isfendiyar DARBAZ [1,3,b] Serkan SAYINER [2,3,c] Selim ASLAN [1,3,d]

[1] *A part of this study was presented as poster presentation in the 21st Annual Conference of the European-Society-for-Domestic-Animal-Reproduction (ESDAR) on 24-26 August 2017, at Bern, Switzerland*

[2] *This study was supported by the Scientific Research Unit, Center of Excellence of Near East University (Grant number: CE064-2015)*

[1] Near East University, Faculty of Veterinary Medicine, Department of Obstetrics and Gynaecology, 99138 Nicosia, TURKISH REPUBLIC of NORTHERN CYPRUS

[2] Near East University, Faculty of Veterinary Medicine, Department of Biochemistry, 99138 Nicosia, TURKISH REPUBLIC of NORTHERN CYPRUS

[3] Near East University, Faculty of Veterinary Medicine, Animal Hospital, 99138 Nicosia, TURKISH REPUBLIC of NORTHERN CYPRUS

[a] ORCID: 0000-0002-7607-4044; [b] ORCID: 0000-0001-5141-8165; [c] ORCID: 0000-0002-3297-5369; [d] ORCID: 0000-0001-6411-5489

Article Code: KVFD-2018-19437 Received: 12.01.2018 Accepted: 31.05.2018 Published Online: 01.06.2018

How to Cite This Article

Ergene O, Darbaz I, Sayiner S, Aslan S: Comparison of bovine pregnancy associated glycoproteins (bPAG-serum and milk), bovine pregnancy specific protein B (bPSP-B) tests with each other and with transrectal ultrasonographic findings for early pregnancy diagnosis. *Kafkas Univ Vet Fak Derg,* 24 (4): 497-502, 2018. DOI: 10.9775/kvfd.2018.19437

Abstract

The aim of this study was to compare the accuracy of bovine pregnancy-associated glycoproteins (bPAG) and bovine pregnancy-specific protein B (bPSP-B) tests with transrectal ultrasonography (TRUS) for early pregnancy diagnosis in dairy cows. The study was carried out on 58 Holstein cows. Blood and milk samples were collected on days 20, 25, 28, 30, 32 and 40 following artificial insemination (AI). TRUS was performed on all cows on days 30 and 40 following AI. The positive predictive values for bPAG-serum and bPAG-milk tests were 100% on days 28 and 30. For bPSP-B tests, positive predictive values were 93.7% and 100% on days 28 and 30, respectively. The Kappa test revealed a high agreement between TRUS and the three tests on days 28 and 30 with the values of 0.954 and 0.946 (P<0.001), respectively. Specificity was found to be 100% for bPAG-serum and bPAG-milk, and 96.7% for bPSP-B on day 28. Sensitivity values were calculated as 94.1% and 93.8% for bPAG-serum and bPSP-B, respectively. The area under ROC curve was between 0.967 and 0.991 for on day 28 following AI. In conclusion, all three tests were found to be reliable methods for pregnancy diagnosis in cows on days 28 and 30 following AI. However, test results may give false positive values in case of embryonic deaths.

Keywords: *Pregnancy diagnosis, Bovine, Pregnancy-associated glycoprotein, Pregnancy-specific protein-B, ELISA*

Erken Gebelik Teşhisi Amacıyla Sığır Gebelik İlişkili Glikoprotein (bPAG-Serum ve Süt) ve Sığır Gebelik Spesifik Protein B (bPSP-B) Testlerinin Birbirileriyle ve Transrektal Ultrasonografik Bulgularla Karşılaştırılması

Öz

Bu çalışmanın amacı, süt ineklerinin erken gebelik tanısında sığır gebelik ilişkili glikoproteinlerin (bPAG) ve sığır gebelik spesifik protein B (bPSP-B) testlerinin transrektal ultrasonografiyle (TRUS) doğruluğunun karşılaştırılmasıdır. Araştırma 58 adet Holstein ineğinde gerçekleştirildi. Suni tohumlamayı (AI) takiben 20, 25, 28, 30, 32 ve 40. günlerde kan ve süt örnekleri toplandı. TRUS, AI'yi takiben 30. ve 40. günlerde tüm ineklerde yapıldı. bPAG-serum ve bPAG-süt testleri için pozitif prediktif değerler, 28 ve 30. Günlerde %100 idi. bPSP-B testleri için, pozitif prediktif değerler sırasıyla 28 ve 30. Günlerde %93.7 ve %100 idi. Kappa testi TRUS ile 28 ve 30. günlerde yapılan üç test arasında sırasıyla 0.954 ve 0.946 (P<0.001) değerlerinde yüksek bir mutabakat olduğunu ortaya koymuştur. Spesifiklik, bPAG-serum ve bPAG-sütü için %100 ve bpsp-B için 28. Günde %96.7 olarak bulundu. Duyarlılık değerleri, bPAG-serum ve bPSP-B için sırasıyla %94.1 ve %93.8 olarak hesaplandı. ROC eğrisi altındaki alan AI'yi takip eden 28. günde 0.967 ile 0.991 arasında idi. Sonuç olarak, üç testinde AI sonrası 28 ve 30. günlerde ineklerde gebelik tanısında güvenilir yöntemler olarak kullanılabileceği tespit edildi. Bununla birlikte, test sonuçları embriyonik ölüm durumunda yanlış pozitif değerler verebilir.

Anahtar sözcükler: *Gebelik tanısı, Sığır, Gebelik ilişkili glikoprotein, Gebelik spesifik protein-B, ELISA*

İletişim (Correspondence)
+90 392 6751000/3146
osman.ergene@neu.edu.tr; ergene67@yahoo.com

INTRODUCTION

In cows, it is important to diagnose early pregnancy, especially on farms where oestrus is not well-monitored [1,2]. In general, even though transrectal palpation and ultrasonographical methods are applied for the detection of pregnancy, ultrasonographical methods have been shown to be more successful [3] the main diagnostic methods used for reproductive control in cattle included rectal palpation, inspection of vaginal discharge and vaginoscopy. Since the 1990 s, the use of ultrasound (US. In one study, it was determined that when ultrasonographic controls were compared with progesterone (P_4), bovine pregnancy-specific protein B (bPSP-B) and bovine pregnancy-associated glycoprotein 1 (bPAG-1), there was indeed no difference between those three methods. When heart rate was used as a parameter during ultrasonography, it was determined that significantly fewer pregnancies were verified by ultrasonography than with the two tests [4].

Trophoblast binuclear cells synthesize proteins during early pregnancy. bPAG or bPAG-1 and bPSP-B participate in the circulatory system. bPSP-B is identified as a foetal protein while PAG is identified as placenta specific protein. This molecule is present in the maternal circulation and can reliably be determined on days 29 and 30 following insemination. It was reported that both factors (bPAG 1; bPSP-B) could be determined in the serum of some pregnant cows on days 15 to 22 following insemination, but more reliable results were determined on days 28 to 30 following conception [4].

It was determined based on the results obtained from the comparison of transrectal ultrasonography (TRUS) with both RIA [4] and ELISA [5] tests, that the bPSP-B test can be used for the diagnosis of pregnancy. There is also evidence that the bPSP-B can be measured until 70-100 days after parturition due to the persistence of postpartum high peripheral concentrations [4].

The aim of this study was to determine, during the here observed period, on which day highest levels of the bPAG-Serum, bPAG-Milk and bPSP-B serum tests can be measured in pregnant and non-pregnant cows. Furthermore, which of these three tests is the most accurate and suitable for determination of early pregnancy. We also aimed to determine the statistical reliability of the cut-off points (ideal test values) determined in the tests according to Youden J index in the early stages of pregnancy.

MATERIAL and METHODS

Animals

In the study, 58 clinically healthy Holstein cows, from five different commercial dairy farms were used. The study was performed with blood and milk samples sent from these farms. The average age of the animals studied was between 3 and 7 years and the average milk yield ranged from 6000 to 8000 L in one lactation. On all farms where the study was conducted, the animals were housed in a free system and all were fed with the same food (Corn silage, hay, green grass, TMR, admixture of minerals and vitamins). Cows were housed in free stall barns and had ad libitum access to feed and water.

Transrectal Ultrasonography (TRUS) and Test Groups

Transrectal ultrasonography was carried out in order to diagnose pregnancy on days 30 and 40 after insemination by using a portable scanner (B-Mode, 5.0 to 7.5 MHz, linear probe, ECM IMAGO®Veterinary; Angouleme/France). Animals were furthermore determined to be pregnant or non-pregnant on day 40 by using the ELISA test kits for the bPAG-milk tests and for measurement of bPAG, bPSP-B samples. The samples collected until the 28th day were compared with ultrasonography results on day 30. Comparisons on day 40 were made to determine whether the pregnancy was continued and to reaffirm the diagnosis in cows previously determined to be non-pregnant.

When pregnancies where assessed by ELISA on day 30 but not determined by ultrasonography on day 40, animals were allocated to the group of animals diagnosed as pregnant on day 30.

When cows were examined as not being pregnant on day 40, but pregnancy had been determined by using TRUS on day 30, this was diagnosed as embryonic death. These animals (n=9) were not taken into the statistical evaluation.

The following groups were formed after evaluation of the obtained blood and milk samples (N=49): A=PAG-Serum (PAG-S; N=49); B=PAG-Milk (PAG-M; N=49); C=PSPB-Serum (PSPB-S; N=49)

Collecting Blood and Milk Samples

Blood and milk samples were collected (*v. jugularis*) from each animal on days 20, 25, 28, 30, 32 and 40 following insemination (day 0).

Blood and milk samples were then brought to laboratory within 2 h under cold chain Serum was obtained by centrifugation of blood samples at 1500 X g for 10 min. Milk samples were centrifuged at 4800 X g for 20 min to obtain skimmed milk and were stored at -20ºC until analyses. The laboratory staff was blinded to the results of the TRUS exams.

Bovine Serum PAG, Milk PAG and Serum PSP-B ELISA Analysis

Sandwich ELISA test (IDEXX Bovine Pregnancy Test Ref 99-41169 Lot. E171, One IDEXX Drive, Westbrook, ME, USA; IDEXX Milk Pregnancy Test Ref 99-41209 Lot. E881, One IDEXX Drive, Westbrook, ME, USA; BioPRYN test Lot No: 5P106, Biotracking LLC, Moscow, ID, USA) was performed

for the measurement of serum bPAG; milk bPAG; and serum bPSP-B. The tests were carried out in accordance with the directives of the manufacturers. The washing steps were performed using an automated microtiter washer (MW-12A Microplate washer, Mindray, Shenzhen, China); analysis results (as optical density) of the samples were obtained using a microtiter plate reader (MR-96A Microplate reader, Mindray, Shenzhen, China). Test results were calculated according to the manufacturer's instructions and expressed as sample-negative (S-N) (all optical densities of the animals were subtracted from the optical density of the negative control). For bPAG serum samples ≥0.300 optical density was assessed as pregnancy positive (pregnant), and <0.300 optical density as pregnancy negative (not pregnant). The intra- and inter-assay coefficients of variation (CV) were 4.2% and 8.8%, respectively. For bPAG milk samples ≥0.150 OD was evaluated as pregnancy positive (pregnant), and <0.100 OD as pregnancy negative (not pregnant). The results, which ranged between 0.100 and 0.150, were considered uncertain. The intra- and inter-assay coefficients of variation (CV) were 3.2% and 9.6%, respectively. And for PSP-B, the test results were evaluated as >0.210 OD pregnancy positive (pregnant); OD between 0.135 and 0.210, uncertain; and <0.135 OD pregnancy negative (not pregnant). The intra- and inter-assay coefficients of variation (CV) were 2.6% and 5.4%, respectively.

Statistical Analyses

Statistical evaluations were performed using SPSS (version 17 for Windows, SPSS Ltd, Hong Kong). Crosstabs were used for specificity, sensitivity, false negative and false positive calculations. The Chi-Square test was used to compare percentages. The significance of the intersection value, which was obtained by defining Roc-Curve and the Area under Roc-Curve, was demonstrated. Normality and homogeneity of groups were determined by the Shapiro-Wilk test. The Kruskal-Wallis test was used to identify potential differences. Due to the relatively small sample numbers, a non-parametric Mann-Whitney U test was performed for comparisons between groups (i.e., treatment vs. control). Mean values and standard deviation were determined by descriptive calculation. Estimates of the agreement between TRUS and PSP-B results were determined by Kappa values and 95% confidence intervals (95% CI) were also determined. Differences were considered statistically significant when P<0.05.

RESULTS

Transrectal ultrasonography examinations performed on the days 30th and 40th; cows (n=9) who were found to be pregnant on the 30th day but not on the 40th day were evaluated as embryonic death was occurred.

In bPAG-Serum test, positive values were obtained in 8 cows (88.9%) whereas negative values were obtained in 1 cow (11.1%) on day 28. In bPAG-Milk positive values in 7 cows, and negative values in two animals were obtained whereas in bPSP-B test, positive values in 7 cows, uncertain values in 1 cow and negative values in 1 cow were obtained. TRUS findings obtained on day 30 were mostly found to be in agreement with bPAG-serum findings (positive in 8 cows, negative in one cow). After ultrasonographical detection of embryonic deaths in 9 cows, both positive and negative results could be obtained from all three tests on day 40; in 44.4% of cases the bPSP-B test results were false positive, in 77.7% the bPAG-Serum test and in 44.4% the bPAG-Milk test.

On day 25 of pregnancy, positive results obtained by TRUS could be confirmed by the bPSP-B-serum test in 42.4% of cases, by the bPAG-Serum test in 90.6% of cases and by using the PAG-Milk test in 78.1% of cases. On days 28 and 30, bPAG-serum and bPAG-milk tests showed a 93.7% and 100% accordance with TRUS, respectively. From day 40 on, more than 93% of results were in accordance with the TRUS results, in all tests evaluated.

Negative test results obtained by measurement of bPAG in serum and milk on day 20, could be confirmed by TRUS in 100%. On day 25 and 28, the accordance with TRUS was 94.1%, and increased to 100% later on with the bPAG-serum test. In bPAG-milk test, the accordance remained the same with 94.1% from day 25 to 40. With the bPSP-B-serum test, the accordance was 94.1% on day 20, 88.2% on day 25 and 30, 94.1% on day 32 and 100% on day 40 *(Table 1)*.

Similarly, the measure of agreement calculations made with the Kappa statistical test showed that the accordance with TRUS was highest with the bPAG-Serum and -Milk test on day 28 (both 0.954; P<0.001). With the bPSP-B Test, the highest accordance was found on day 30 (0.946; P<0.001) *(Table 2)*.

With bPAG-serum and -milk tests, a specificity of 94.1% and 100 % could already be determined on day 25 and 28, respectively; whereas with bPSP-B test a specificity of >90% was only detectable from day 28 *(Table 3)*.

As to the bPAG-serum test, the ideal cut-off points according to Youden J index of the statistical test results calculated with ROC-Curve was determined to be 0.865 on day 28, with a 100% of positive results. Sensitivity of the test was 1.0 and the specificity value was 0.059. The area under the curve value of 0.974 indicated that the test was reliable (P<0.001). Likewise, the ideal cut-off point for bPAG-milk was 0.2695 and the sensitivity of the test was 1.0; and the specificity value was 0.059 (P<0.001). The area under the curve value was 0.991, indicating that the test was reliable (P<0.001). The bPSP-B test was also found reliable on day 28 (cut-off point: 0.21; sensitivity: 0.938; specificity value: 0.059; Area under the curve: 0,967; P<0.001) *(Table 4)*.

Table 1. *Positive and negative pregnancy detection rates with the ELISA test (bPAG-Milk, bPAG-Serum, bPSPB-Serum)*

Day	Positive (bPAG/M) (n/x)	Positive (bPAG/S) (n/x)	Positive (bPSPB/S) (n/x)	Negative (bPAG/M) (n/x)	Negative (bPAG/S) (n/x)	Negative (bPSPB/S) (n/x)	Uncertain (bPAG/M) (n/x)	Uncertain (bPAG/S) (n/x)	Uncertain (bPSPB/S) (n/x)
D20	-	3.1%a (32/1)	6.3%a (32/2)	100% (17/17)	100% (17/17)	94.1% (17/16)	-	-	-
D25	78.1%a (32/25)	90.6%b (32/29)	42.4%b (32/14)	94.1% (17/16)	5.9% (17/1)	88.2% (17/15)	9.4% (32/3)	-	24.2% (33/8)
D28	100%b (32/32)	100%b (32/32)	93.7%c (32/30)	94.1% (17/16)	94.1% (17/16)	88.2% (17/15)	-	-	3.1% (32/1)
D30	100%b (32/32)	100%b (32/32)	100%c (32/32)	94.1% (17/16)	76.5% (17/13)	88.2% (17/15)	-	-	-
D32	93.6% (32/30)	93.7% (32/30)	93.9% (32/31)	94.1% (17/16)	94.1% (17/16)	94.1% (17/16)	-	-	-
D40	100% (32/32)	96.9% (32/31)	93.5% (32/29)	94.1% (17/16)	100% (15/15)	100% (15/15)	-	-	3.2% (31/1)

Table 2. *Measure of Agreement (Test/TRUS. Kappa) Test*

Test	Days	Value	SE	P
bPAG - Serum (N=49)	25	0.825	0.084	0.001
	28	0.954	0.045	0.001
	30	0.809	0.090	0.001
	32	0.867	0.074	0.001
	40	0.958	0.042	0.001
bPAG - Milk (N=49)	25	0.811	0.081	0.001
	28	0.954	0.045	0.001
	30	0.910	0.062	0.001
	32	0.867	0.074	0.001
	40	0.954	0.045	0.001
bPSP-B - Serum (N=49)	25	0.630	0.089	0.001
	28	0.920	0.044	0.001
	30	0.946	0.037	0.001
	32	0.921	0.044	0.001
	40	0.895	0.049	0.001

Kappa value as a measure of agreement between TRUS and ELISA

DISCUSSION

It has great importance to diagnose early pregnancy in order to increase the production volume of livestock sector. This will also lead to increased profitability and rural development [6]. bPAG produced in the ruminant trophoblast cells can be detected in the blood of pregnant cows [7]. Similarly, serum concentration of bPSP-B can be measured to determine early bovine pregnancy by using an ELISA test [5] and a bPAG-milk test has been developed to assess early pregnancy by using milk, which can be collected more easily than serum [8].

In this study, three tests (bPAG-Serum, bPAG-Milk and bPSP-B-Serum) were performed in order to determine early pregnancy in cows. The aim was to assess the rate of positive and negative test results between days 20 and 40 of pregnancy, and to determine the earliest day, on which each test can be used with sufficient reliability.

Embryonic deaths were detected in 9 cows during TRUS examinations performed on day 40; although pregnancy was detected in these cows by TRUS on day 30, this could not be confirmed on day 40. When all early results of the three ELISA tests were compared with the laboratory test results obtained on day 28 and TRUS findings obtained on day 30, the bPAG-Serum test was 100% compatible with

Table 3. *The specificity and sensitivity values of all tests examined (by days)*

Test	Specificity (%)					Sensitivity (%)				
	Days					Days				
	25	28	30	32	40	25	28	30	32	40
bPAG-Serum	94.1 (n=32)	100 (n=32)	100 (n=32)	93.7 (n=30)	96.9 (n=31)	90.6 (n=17)	94.1 (n=16)	76.5 (n=13)	94.1 (n=16)	100 (n=15)
bPAG- Milk	94.1 (n=32)	100 (n=32)	100 (n=32)	93.7 (n=30)	100 (n=32)	86.2 (n=17)	94.1 (n=16)	94.1 (n=16)	94.1 (n=16)	94.1 (n=16)
bPSPB- Serum	55.1 (n=33)	96.7 (n=30)	100 (n=32)	93.7 (n=30)	96.7 (n=30)	52 (n=17)	93.8 (n=15)	88.2 (n=15)	94.1 (n=16)	100 (n=15)

Table 4. The Cut-off Values calculated by ROC curve results, sensitivity, specificity and significance (AUC) of obtained values

Test	Cut-off Point (J index)	Sensitivity	Specificity	AUC*
bPAG-Milk	0.2695	1.0	0.059	0.991
bPAG-Serum	0.865	1.0	0.059	0.974
bPSPB-Serum	0.21	0.938	0.059	0.967

** Area under the Curve*

the TRUS findings. The other tests were found to be 88.9% compatible with TRUS on day 28. In cows, embryonic deaths is frequently seen until day 45 of pregnancy [4]. It is known that uncertain or negative test results can be caused by embryonic death. It has also been reported that false positive findings can be obtained due to embryonic mortality [9]. In the present study, in case of embryonic death, reliability of TRUS was greater. Even though no embryo was detected in TRUS controls performed on day 40, false positive results were obtained in bPSP-B test, bPAG-serum test and bPAG-milk in 44.4%, 77.7% and 44.4% of cases, respectively.

In this study, it was determined that in case of normal pregnancies, positive results obtained with bPSP-B serum ELISA on day 25 correlated with positive TRUS findings in 42.4% of cases; this percentage was 90.6 with the bPAG-serum test and 78.1 with the bPAG-milk test, which increased on days 28 and 30 to 100%; whereas with the bPSP-B test a 100% was not reached before day 30.

In case of negative pregnancies on day 20, compliance between bPAG serum and milk ELISA results, and TRUS findings was 100%, and 94.1% on the other days. With bPSP-B serum ELISA, this percentage only ranged between 94.1% and 88.2% in the early pregnancy period. Uncertain negative/positive test results were detected on some days in the other tests, however, no uncertain test results were obtained with the bPAG-serum ELISA.

Therefore, based on these findings, all the tests in this study were able to detect positive and negative pregnancies at high rates on day 28 and 30; however, with the bPAG-serum and milk tests only, compatibility with TRUS was 100% on days 28 and 30 of the pregnancy. In another study, the bPAG serum test was used on day 27 of pregnancy, and the compliance with TRUS findings was 93.7% and 97.8% [10]. With the bPAG-Milk test, results were found to be more variable than with the serum test, which was supposed to be mainly due to milk storage [11]. In other studies, it was suggested that milk amount [12], milk composition, contamination of milk samples [13] or lactation period [14]. In this study, no difference was found between bPAG-Serum and bPAG-Milk in the detection of pregnancy on day 28 and 30 following insemination.

Compliance between ultrasonography and these tests can be calculated using the Kappa test [5]. Considering all positive and negative comparisons, the results obtained with the Kappa test were found to be significant at the level of 0.954 ($P<0.001$) on day 28 with the bPAG-serum and milk ELISA, and 0.9446 ($P<0.001$) on day 30 with the bPSP-B test. In one study, comparison of results of transrectal palpation with the bPSP-B test by using the kappa test, resulted in 0.83 [15]. In another study, the kappa data of the agreement between bPAG ELISA test and determination of pregnancy increased from 0.87 (first insemination) up to 0.94 (third insemination) depending on the number of inseminations [10].

In bovine plasma, bPSP-B was detected between days 15 and 22 of pregnancy after insemination. It has been determined that because this glycoprotein is synthesized in the binuclear trophoblastic cells of placenta, good results can be obtained when the test is applied after day 30 post-conception [4]. In this study, with the bPSP-B test, compliance with TRUS on day 28 was 93.7% whereas 100% compliance was found on day 30. Others reported a 90% positive and a 99.5% negative compatibility with PSPB test on the same day [4,16].

In most studies, bPAG and bPSP-B tests were found to be similarly useful for the detection of pregnancy [4]. On the other hand, on days 28, 30 and 35 of pregnancy, uncertain bPSP-B sample rates were found to have changed through the rates of 8.5%, 4.8% and 3.3%, respectively [5]. In our study, with bPSP-B-Serum ELISA, uncertain results were found on days 25 (24.2%), 28 (3.1%) and 40 (3.2%) of pregnancy, with bPAG-Milk test, on day 25 (9.4%) only, whereas no uncertain results were found in bPAG serum test.

In bPAG serum and milk, the negative pregnancy rate, which was determined to be compatible with TRUS, was 100% on day 20 following insemination, and was determined as 94.1% on the consecutive days, including day 32. This ratio was determined to be 94.1% in bPSP-B test on day 20, and between 88.2% and 100% (on day 40) on the consecutive days. When the test results were examined, in bPAG-serum test, the specificity was 100% on days 28 and 30; in bPAG Milk, 100% on day 28 only; and in bPSP-B test, 100% on day 30 (96.7% on day 28). On the contrary, sensitivity in bPAG Serum was 94.1% on day 28; and in bPAG milk, 94.1% on both days 28 and 30. Sensitivity was determined to be 93.8% on day 28 in bPSP-B Serum test. Silva et al.[10] tested cows on day 27 for bPAG by ELISA and compared those results with TRUS 32 days after AI. They reported sensitivity ranging between 93.5% and 96.3% and specificity between

91.7% and 96.8%. In another study, bPAG analysis correctly diagnosed pregnancy on day 28 post-AI [17]; however only samples from females that delivered live calves were analysed. Finally, when bPAG-1 was analysed by RIA on day 35 and compared with transrectal palpation on day 45, the sensitivity and negative predictive values were 98.8% and 97.9%, respectively [16]. In this study, the fact that sensitivity was achieved at 94.1% rate on day 28 in bPAG Serum and milk test shows that if samples are examined at this stage of pregnancy, reliable pregnancy results can be obtained.

Szenci et al.[4], testing for bPSP-B by RIA, reported a sensitivity of 92% from samples collected on both days 29 and 30, and 98.1% on days 33 and 34. Howard et al.[18], using the same bPSP-B ELISA test, reported a sensitivity of 100% and a specificity of 87.8% for cows between days 30 and 36 compared with US performed between days 37 and 43. Interestingly, they found no uncertain results in 336 samples. In bPSP-B test, both sensitivity and specificity were high, again, on day 28. It is necessary to bear in mind that the specificity was calculated as 100% (specificity as 88.2%) on day 30.

The numerical Cut-off values obtained by the Area under the ROC curve on day 28 ranged from 0.967 to 0.991 and even approached 1, which means high specificity and sensitivity as well as reliability of the test.

In conclusion, the data obtained show that all of these tests are reliable and suitable for determination of pregnancy, with highest accuracy on days 28 and 30, when both specificity and sensitivity are considered. In some animals, when pregnancy was detectable by ELISA on days 28 and 30, positive results were obtained later, on the day when embryonic deaths was detected by TRUS.

Acknowledgements

The authors appreciate the dedication of all veterinarians, technicians and farmers who contributed to this study.

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