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Short communication: Blood samples before and after embryonic attachment accurately determine non-pregnant lactating dairy cows at 24 d post-artificial insemination using a commercially available assay for pregnancy-specific protein B

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ABSTRACT

Early pregnancy diagnosis is critical to reproductive success on dairy farms. Reproductive success depends on cows becoming pregnant before 130 d in milk and then maintaining that pregnancy. The earlier non-pregnant cows are identified, the sooner they can be reinseminated, thus reducing days to pregnancy. Assays for pregnancy-specific protein B (PSPB) and pregnancy-associated glycoproteins can be used to diagnose pregnancy >28 d post-artificial insemination (AI) in lactating cows. The objective of this study was to determine whether percentage change in serum levels of PSPB within cow from d 17 to 24 can be used to identify non-pregnant cows using a commercially available assay. This study was performed on a large commercial dairy. Blood samples were taken at d 17 and 24 post-AI. The d 17 sample served as a baseline based on previous data. Cows with a 10% increase in serum PSPB levels from d 17 to 24 were considered pregnant. Lactating dairy cows (n = 206; 39% primiparous and 61% multiparous) were synchronized using G6G-Ovsynch. The PSPB diagnosis was compared with the herd veterinarian's diagnosis via ultrasound on d 34. The sensitivity for a 10% cutoff as a non-pregnant diagnosis was 100%, and the specificity was 93.58%. The positive predictive value was 93.27%, and the negative predictive value was 100%. Low PSPB levels at d 24 were predictive of early pregnancy loss by 60 d post-AI. To our knowledge no other method can diagnose non-pregnancy with 100% accuracy and predict pregnancy loss earlier than 24 d post-AI. Using comparative PSPB samples at d 17 and 24 post-AI provides an accurate non-pregnancy diagnosis earlier than any other pregnancy diagnosing method.

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Short Communication

Reproductive success on a dairy farm depends on a cow becoming pregnant and maintaining the pregnancy until parturition. Data indicate that dairy cows need to become pregnant before 130 DIM to be profitable (Giordano et al., 2011). To maximize chances for pregnancy by 130 DIM, reproductive programs need to increase pregnancies per AI at first AI and decrease reinsemination intervals. Fertility programs such as Presynch-11, G6G, or Double-Ovsynch improved pregnancies per AI at first AI (Moreira et al., 2001; Bello et al., 2006; Souza et al., 2008), but generally these programs are too long to use in a resynchronization program. Aggressive resynchronization strategies that reduce the reinsemination interval depend on early non-pregnancy diagnoses (Fricke, 2002; Giordano et al., 2013). Blood and milk samples that assay for pregnancy-associated glycoproteins can be used to diagnose pregnancy. Pregnancy-specific protein B (**PSPB**) can be measured in the maternal serum with a single sample taken between 28 and 35 d post-AI with 98% accuracy for diagnosing pregnancy (Sasser et al., 1986; Piechotta et al., 2011).

Pregnancy-specific protein B is produced in the binucleate cells of the trophectoderm of the embryo. As the trophectoderm begins to attach to the uterine epithelium, PSPB is released via exocytosis and enters the maternal circulation (Wooding, 1992). Bovine placental attachment is believed to begin near d 17 of gestation (Roberts et al., 1996). Data from our laboratory indicated that serum levels of PSPB start to increase at d 22 post-AI in pregnant cows (Arnold et al., 2012), but this differs between cows and heifers. Nulliparous heifers appear to initiate an increase in PSPB before lactating cows.

Martins et al. (2018) used blood sampling before and after increases in PSPB to determine pregnancy

23 d post-AI. Blood sampling at d 16 and 20, before an increase in PSPB, was quite homogeneous within cow. This allowed for an accurate assessment of within-cow increases in PSPB using 2 blood samples at d 20 and 23 post-AI. Pregnancy was determined using an increase of 28% or greater in serum levels of PSPB from d 20 to 23 post-AI. Accuracy of pregnancy diagnosis had a sensitivity of 98% and specificity of 97% compared with a single PSPB determination at 28 d post-AI.

The objective of this study was to determine whether percentage change in serum levels of PSPB within cow from d 17 to 24 could more accurately identify non-pregnant cows. We hypothesized that within-cow PSPB samples before and after the time of attachment could diagnose cows that are not pregnant with very high accuracy.

This study was conducted in November and December 2017 on a commercial Holstein dairy farm (Nobis Dairy Farm, St. Johns, MI). The farm milked approximately 1,000 dairy cows 3 times a day with daily average milk production of 42 kg/cow per day. Cows were fed a TMR once a day with free access to feed and water and were housed in a 4-row freestall barn with sidewall curtains and fans. The TMR consisted of corn, wheat, and alfalfa silages and corn–soybean meal-based concentrates formulated to meet nutrient recommendations for lactating dairy cows (NRC, 2001). The Institutional Animal Care and Use Committee at Michigan State University approved all animal handling and procedures.

Weekly cohorts of lactating dairy cows (n = 206; 39% primiparous and 61% multiparous) were synchronized with G6G-Ovsynch and received timed AI on d 0. Cows ranged from first to fourth AI and were between 75 and 250 DIM. Blood samples for measurement of PSPB were collected from the coccygeal vein or artery by trained laboratory personnel on d 17 and 24 post-AI using Vacutainer tubes without anticoagulant (BD, Franklin Lakes, NJ). Following collection, samples were refrigerated at 4°C. The d 17 and d 24 samples for each cohort were mailed to a BioPRYN laboratory (West Michigan Veterinary Service, Coopersville, MI) on d 24 post-AI. This laboratory used a commercially available quantitative sandwich ELISA assay kit (BioTracking LLC, Moscow, ID) to measure serum concentrations of PSPB. Within-cow samples on d 17 and 24 were assayed together on the same plate. The BioPRYN assay is a sandwich ELISA in which rabbit anti-PSPB serum is coated on 96-well microtiter plates to capture PSPB. Detector solution containing a primary antibody against PSPB is used as the detection antibody. Enhancer solution containing an enzyme (horseradish peroxidase)-linked secondary antibody is used to detect the primary antibody from the detector solution. The development of color occurs with the addition of 3,3′,5,5′,-tetramethylbenzidine, the substrate for horseradish peroxidase. A fluoride stop solution was added to the reaction, and optical density (**OD**) for each well was obtained from a plate reader with a filter wavelength of 650 nm. The assay provided a semiquantitative analysis of samples using 4 standards on each plate (0.5, 1, 2, and 4 ng/mL). A curve was fitted to the standard wells on each plate using a linear least squares regression. This assay was validated with this standard curve with samples taken on d 28 post-AI or greater. The commercial laboratory that performed these analyses did not calculate sensitivity of the assay. The PSPB results were reported as OD and received by email 2 d later.

The difference in serum PSPB levels was obtained by subtracting the basal d 17 serum level from the d 24 serum level. Percentage change in serum PSPB levels for each cow was calculated by dividing the difference in serum levels by the basal d 17 serum level and then multiplying by 100. All cows received pregnancy diagnoses on d 34 and 62 post-AI by the farm veterinarian using ultrasound (**US**).

All information was recorded in an Excel (Microsoft Corp., Redmond, WA) spreadsheet for organization before statistical analysis. Milk production data and pregnancy confirmation information were used for each cow as recorded in PCDART (Dairy Records Management Systems, Raleigh, NC). The 305-d mature equivalent milk (**M305M**) was chosen for analyses. The d 17 PSPB OD, the d 24 PSPB OD, the difference in serum PSPB levels, and percentage change in serum PSPB from basal level to d 24 post-AI were recorded as continuous variables. Pregnancy diagnoses and pregnancy loss diagnoses were recorded as binomial variables. Sensitivity, specificity, positive predictive value, negative predictive value, and quartiles of PSPB OD values were analyzed using the FREQ procedure of SAS (version 9.4, SAS Institute Inc., Cary, NC). The veterinarian's pregnancy diagnosis on d 34 post-AI was the reference test. Sensitivity determined accuracy and measured the proportion of cows that were diagnosed non-pregnant by percentage change in serum PSPB from basal level to d 24 post-AI and diagnosed non-pregnant by US. Specificity measured the proportion of cows that were diagnosed pregnant by percentage change in serum PSPB from basal level to d 24 post-AI and diagnosed pregnant by US. The positive predictive value was the probability that the pregnancy diagnosis based on percentage change in serum PSPB from basal level to d 24 post-AI accurately identified cows that were pregnant. The negative predictive value was the probability that the non-pregnancy diagnosis based on

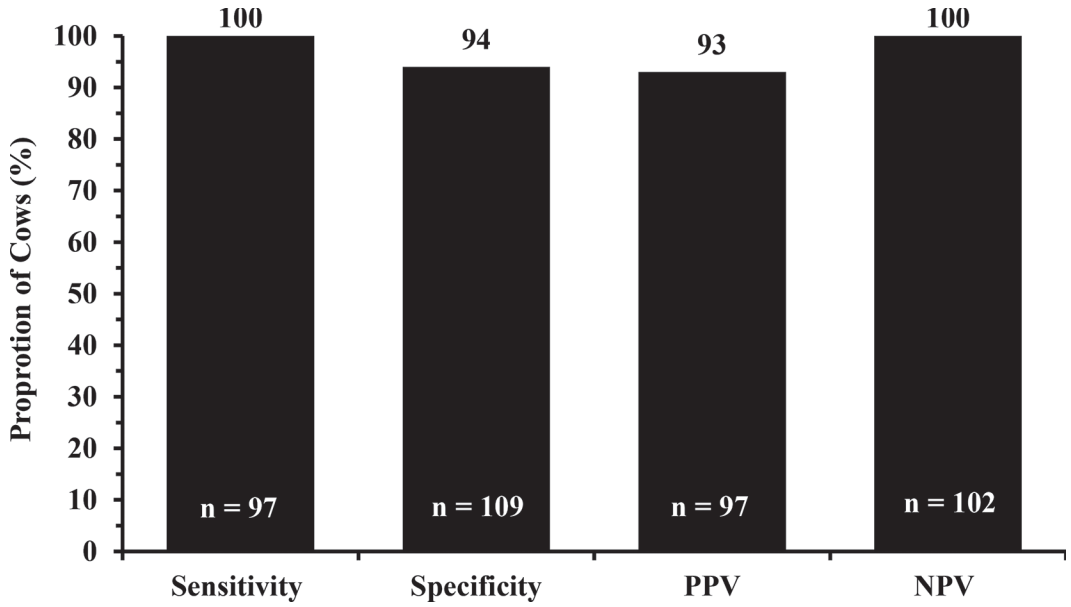

Figure 1. Sensitivity [proportion of cows diagnosed non-pregnant by ultrasound and by percentage change in serum pregnancy-specific protein B (PSPB) optical density (OD)], specificity (proportion of cows diagnosed pregnant by ultrasound and by percentage change in serum PSPB OD), positive predictive value (PPV; proportion of cows diagnosed pregnant by percentage change in serum PSPB OD that were actually pregnant), and negative predictive value (NPV; proportion of cows diagnosed non-pregnant by percentage change in serum PSPB OD that were actually non-pregnant) of a non-pregnancy diagnosis based on a decrease or less than 10% increase in serum levels of PSPB from basal level to d 24 post-AI in lactating dairy cows. Cows with a 10% or greater increase in serum levels of PSPB OD from basal level to d 24 post-AI were diagnosed pregnant. The veterinarian's pregnancy diagnosis with ultrasound on d 34 post-AI was the benchmark reference.

percentage change in serum PSPB from basal level to d 24 post-AI accurately identified non-pregnant cows. The GLIMMIX procedure of SAS was used for evaluation of continuous data. Pearson correlation coefficients were calculated using the CORR procedure of SAS.

A 10% increase in PSPB OD values from d 17 to 24 was the factor used as a cutoff to identify non-pregnant cows. This conservative value was based on the lowest percentage increase in PSPB OD in cows (n = 102) diagnosed pregnant 34 d post-AI using US. Sensitivity, specificity, and positive and negative predicted values are described in Figure 1. Mean PSPB OD values were 0.080 (±0.001 SEM) in non-pregnant cows and 0.083 (±0.001 SEM) in pregnant cows on d 17 post-AI. Mean PSPB OD values on d 24 were 0.082 (±0.001 SEM) in non-pregnant cows and 0.183 (±0.005 SEM) in pregnant cows. Pregnant cows ranged from a 13 to 306% increase, with a mean increase of 122% ± 0.06. Cows (n = 104) with a decrease or less than 10% increase in serum PSPB OD from d 17 to 24 post-AI were determined non-pregnant and had 100% sensitivity and 94% specificity. Cows diagnosed non-pregnant (n = 104) by US ranged from a 14% decrease to an 86% increase in serum PSPB OD from basal level to d 24, with a mean increase of 2% ± 0.01 (Figure 2). There was a greater incidence of pregnancy loss between 34 and 62 d post-AI in cows, with the lowest levels of PSPB OD on d 24 post-AI (Table 1). Cows in the lowest quartile of percentage change of PSPB OD between d 17 and 24 (n = 102) had the greatest pregnancy losses ($P = 0.02$).

The purpose of this experiment was to test whether the BioPRYN commercial assay can be an accurate predictor of non-pregnancy. The commercial laboratory did not calculate the sensitivity of the assay, so it is unclear what the d 17 samples are measuring. There are several reasons why it seems likely that serum OD levels on d 17 are measuring PSPB and thus are

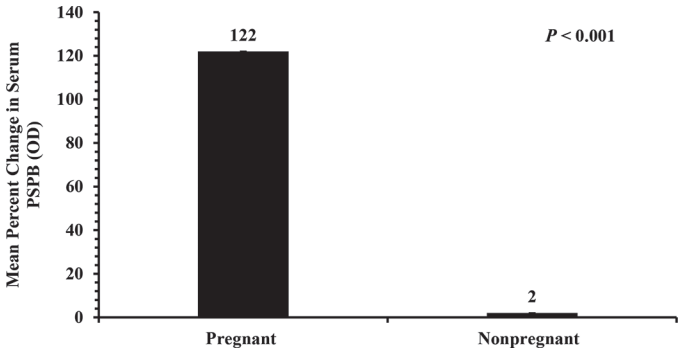

Figure 2. Mean percentage change in serum pregnancy-specific protein B (PSPB) optical density (OD) levels from d 17 to 24 post-AI in cows diagnosed pregnant (n = 102) and cows diagnosed non-pregnant (n = 104) via ultrasound. Data are shown as mean ± SEM.

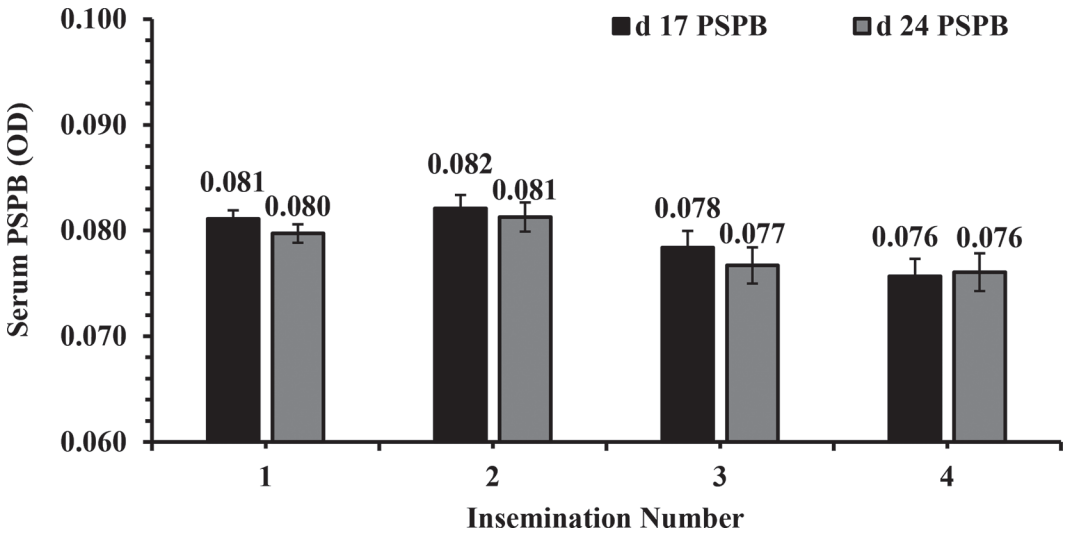

Figure 3. Serum pregnancy-specific protein B (PSPB) optical density (OD) levels on d 17 and 24 post-AI in non-pregnant cows at first (n = 50), second (n = 20), third (n = 13), and fourth (n = 12) insemination. Cows with a fifth insemination (n = 2) were excluded from the analysis. Data are shown as mean ± SEM. Significance between insemination numbers for serum PSPB OD levels on d 17 post-AI is as follows: first versus second ($P = 0.52$), first versus third ($P = 0.13$), first versus fourth ($P = 0.004$), second versus third ($P = 0.07$), second versus fourth ($P = 0.003$), and third versus fourth ($P = 0.24$). Significance between insemination numbers for serum PSPB OD levels on d 24 post-AI is as follows: first versus second ($P = 0.34$), first versus third ($P = 0.12$), first versus fourth ($P = 0.07$), second versus third ($P = 0.04$), second versus fourth ($P = 0.02$), and third versus fourth ($P = 0.8$). There was no effect of parity between first- and ≥second-parity cows at d 17 ($P = 0.39$) and d 24 ($P = 0.37$). Days in milk range at first, second, third, and fourth AI was 75 to 81, 131 to 137, 187 to 193, and 243 to 249.

not an assay sensitivity problem. First, previous data (Arnold et al., 2012) indicated that non-pregnant primiparous and multiparous cows (0.095 ± 0.001 OD; n = 21) had greater OD levels of PSPB compared with non-pregnant nulliparous heifers (0.080 ± 0.001 OD; n = 8). Second, data from Figure 3 indicate that PSPB decreased from first to fourth AI (75 to 250 DIM). This was supported by a negative correlation (Pearson's r = −0.22) between DIM at insemination and d 17 post-AI PSPB OD levels in all cows (n = 206). Third, there is low variability in the percentage change in PSPB from d 17 to 24 in non-pregnant cows (Figure 2; CV = 0.02) but high variability between cows on d 17 (OD values ranged from 0.069 to 0.1; CV = 0.08). Fourth, Sasser et al. (1986) reported that some cows, but not all, had detectable PSPB on d 15 post-breeding. Last, even though data from Kiracofe et al. (1993) indicated that PSBP levels in beef cows decreased, it appeared, to near-basal concentrations at 90 d postpartum, it was not clear why there were basal levels at that point.

There was a positive correlation (Pearson's r = 0.13) between d 17 post-AI PSPB OD levels and M305M

Table 1. Difference in percentages of pregnancy loss from 24 to 34 d post-AI and 34 to 62 d post-AI among quartiles of serum pregnancy-specific protein B (PSPB) optical density (OD) levels on d 24 post-AI in cows diagnosed pregnant from the percentage change in serum PSPB OD level from d 17 to 24 post-AI

Quartile	Range of serum PSPB OD level on d 24 post-AI	Pregnancy loss 24 to 34 d post-AI,[1] % (no./no.)	Pregnancy loss 34 to 62 d post-AI,[2,3] % (no./no.)
Q1	0.083–0.142	18.52 (5/27)	15.00 (3/20)
Q2	0.144–0.170	3.57 (1/28)	7.41 (2/27)
Q3	0.172–0.213	3.70 (1/27)	0.00 (0/25)
Q4	0.213–0.328	0.00 (0/27)	0.00 (0/25)
P-value	—	0.01	0.01

[1]Cows were considered to have undergone pregnancy loss if diagnosed pregnant from the percentage change in serum PSPB OD from basal level to d 24 post-AI and then diagnosed non-pregnant via ultrasound on d 34 post-AI.

[2]Cows were considered to have undergone pregnancy loss if confirmed pregnant at 34 d post-AI via ultrasound and then diagnosed non-pregnant at the next confirmation 62 d post-AI.

[3]Reduced numbers are a result of pregnancy loss 24 to 35 d post-AI (n = 2, n = 1, and n = 2 cows culled from Q1, Q3, and Q4, respectively) before pregnancy confirmation 62 d post-AI.

(n = 206). There was no correlation between d 24 post-AI PSPB OD levels and M305M in pregnant cows (Pearson's r = 0.01; n = 109). Both López-Gatius et al. (2007) and Ricci et al. (2015) reported a negative relationship between milk production and pregnancy-associated glycoprotein levels in pregnant cows. There was no relationship between M305M and pregnancy (P = 0.50; n = 206).

Determining non-pregnancy with percentage change in serum PSPB from basal level to d 24 post-AI was 100% accurate with a conservative percentage change cutoff that purposely included only non-pregnant cows. This favored accuracy of determining non-pregnant cows over pregnant cows. A quantitatively derived cutoff would not have allowed this conservative cutoff due to the variation in percentage change of pregnant cows. It is critical for an early non-pregnancy diagnosis method to not incorrectly diagnose pregnant cows as non-pregnant. Non-pregnant cows may be resynchronized with $PGF_{2\alpha}$, which can cause termination of the pregnancy (Fricke et al., 2016). The lowest cutoff on d 24 for the current data set (0.1054 OD) was only 94% accurate in a much larger data set (n = 734 pregnant cows) in which samples were evaluated at the same laboratory with the same assay. This would mean that approximately 43 pregnant cows would have received $PGF_{2\alpha}$ and would possibly have aborted if using a single OD cutoff from the current study. Approximately 7% of the cows diagnosed pregnant with percentage change in serum PSPB OD from d 17 to 24 post-AI were diagnosed non-pregnant by US 10 d later. This may be due to early pregnancy loss or inaccuracies in using the BioPRYN assay in this novel way. Cows that were diagnosed non-pregnant by the veterinarian on d 34 post-AI had a wide range of percentage differences (−14 to 86%) in serum PSPB OD levels from basal level to d 24 post-AI. The 10% cutoff was conservative enough to not diagnose pregnant cows as non-pregnant while limiting the proportion of cows falsely diagnosed pregnant. Using the BioPRYN assay in this manner may allow for non-pregnant cows to be reinseminated sooner and have more chances for pregnancy by 130 DIM.

Martins et al. (2018) also found a difference in serum levels of PSPB at d 23 and 28 post-AI between cows with versus without pregnancy losses between d 28 and 35 of gestation. There was no difference, however, in serum levels of PSPB at d 23 and 28 post-AI between cows with versus without pregnancy losses between d 35 and 56 in gestation (Martins et al., 2018). The inverse relationship between serum OD levels on d 24 and pregnancy loss agrees with Gábor et al. (2016). Pregnancy loss affects reproductive success on dairy farms (Fricke, 2002). Pregnancy loss rates are greatest during early pregnancy (Santos et al., 2004). The ability to identify cows at risk for losing a pregnancy could prove useful to producers and veterinarians.

Using within-cow PSPB samples at d 17 and 24 post-AI provided a robust and very accurate determination of early non-pregnancy diagnosis. This could lead to development of synchronization methods for early re-synchronization of ovulation for timed AI. It appears to be critical for cows to become pregnant before 130 DIM to maintain high fertility from lactation to lactation. Identifying non-pregnant cows as early as possible is important to allow for this process to happen. After further research, this method of early pregnancy diagnosis may eventually be able to alert producers and veterinarians to cows that are at risk for losing a pregnancy. To our knowledge, no other method can diagnose non-pregnancy with 100% accuracy. Utilizing this method of non-pregnancy diagnosis can detect non-pregnant cows earlier, allowing the cows to be re-synchronized and reinseminated sooner and ultimately decreasing DIM to conception, although the labor and cost of 2 blood samples may not be practical for most dairy operations. In addition, a careful revision of interpretation of results of this use of BioPRYN assay must be made before it is ready for field conditions.

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REFERENCES

Arnold, H. J., P. N. Martins, L. Z. Oliveira, R. K. Policelli, K. M. Stomack, G. Sasser, and J. R. Pursley. 2012. Effectiveness of pregnancy-specific protein B in pregnancy diagnosis of dairy cows and heifers. J. Dairy Sci. 95(Suppl. 1):128. (Abstr.)

Bello, N. M., J. P. Steibel, and J. R. Pursley. 2006. Optimizing ovulation to first GnRH improved outcomes to each hormonal injection of Ovsynch in lactating dairy cows. J. Dairy Sci. 89:3413–3424.

Fricke, P. M. 2002. Scanning the future—Ultrasonography as a reproductive management tool for dairy cattle. J. Dairy Sci. 85:1918–1926.

Fricke, P. M., A. Ricci, J. O. Giordano, and P. D. Carvalho. 2016. Methods for and implementation of pregnancy diagnosis in dairy cows. Vet. Clin. North Am. Food Anim. Pract. 32:165–180.

Gábor, G., J. P. Kastelic, Z. Abonyi-Tőth, P. Gábor, T. Endrődi, and O. G. Balogh. 2016. Pregnancy loss in dairy cattle: Relationship of ultrasound, blood pregnancy-specific protein B, progesterone, and production variables. Reprod. Domest. Anim. 51:467–473.

Giordano, J. O., P. Fricke, and V. E. Cabrera. 2013. Economics of resynchronization strategies including chemical tests to identify nonpregnant cows. J. Dairy Sci. 96:949–961.

Giordano, J. O., P. M. Fricke, M. C. Wiltbank, and V. E. Cabrera. 2011. An economic decision-making support system for selection of reproductive management programs on dairy farms. J. Dairy Sci. 94:6216–6232.

Kiracofe, G. H., J. M. Wright, R. R. Schalles, C. A. Ruder, S. Parish, and R. G. Sasser. 1993. Pregnancy-specific protein B in serum of postpartum beef cows. J. Anim. Sci. 71:2199–2205.

López-Gatius, F., J. M. Garbayo, P. Santolaria, J. Yániz, A. Ayad, N. M. de Sousa, and J. F. Beckers. 2007. Milk production correlates negatively with plasma levels of pregnancy-associated glycoprotein (PAG) during the early fetal period in high producing dairy cows with live fetuses. Domest. Anim. Endocrinol. 32:29–42.

Martins, J. P. N., D. Wang, N. Mu, G. F. Rossi, A. P. Martini, V. R. Martins, and J. R. Pursley. 2018. Level of circulating concentrations of progesterone during ovulatory follicle development affects timing of pregnancy loss in lactating dairy cows. J. Dairy Sci. 101:10505–10525.

Moreira, F., C. Orlandi, C. A. Risco, R. Mattos, F. Lopes, and W. W. Thatcher. 2001. Effects of presynchronization and bovine somatotropin on pregnancy rates to a timed artificial insemination protocol in lactating dairy cows. J. Dairy Sci. 84:1646–1659.

NRC. 2001. Nutrient Requirements of Dairy Cattle. 7th rev. ed. Natl. Acad. Sci., Washington, DC.

Piechotta, M., J. Bollwein, M. Friedrich, T. Heilkenbrinker, C. Passavant, J. Branen, G. Sasser, M. Hoedemaker, and H. Bollwein. 2011. Comparison of commercial ELISA blood tests for early pregnancy detection in dairy cows. J. Reprod. Dev. 57:72–75.

Ricci, A., P. D. Carvalho, M. C. Amundson, R. H. Fourdraine, L. Vincenti, and P. M. Fricke. 2015. Factors associated with pregnancy-associated glycoprotein (PAG) levels in plasma and milk of Holstein cows during early pregnancy and their effect on the accuracy of pregnancy diagnosis. J. Dairy Sci. 98:2502–2514.

Roberts, R. M., S. Xie, and N. Mathialagan. 1996. Maternal recognition of pregnancy. Biol. Reprod. 54:294–302.

Santos, J. E. P., W. W. Thatcher, R. C. Chebel, R. L. A. Cerri, and K. N. Galvão. 2004. The effect of embryonic death rates in cattle on the efficacy of estrus synchronization programs. Anim. Reprod. Sci. 82–83:513–535.

Sasser, R. G., C. A. Ruder, K. A. Ivani, J. E. Butler, and W. C. Hamilton. 1986. Detection of pregnancy by radioimmunoassay of a novel pregnancy-specific protein in serum of cows and a profile of serum concentrations during gestation. Biol. Reprod. 35:936–942.

Souza, A. H., H. Ayres, R. M. Ferreira, and M. C. Wiltbank. 2008. A new presynchronization system (Double-Ovsynch) increases fertility at first postpartum timed AI in lactating dairy cows. Theriogenology 70:208–215.

Wooding, F. B. P. 1992. Current topic: The synepitheliochorial placenta of ruminants: Binucleate cell fusions and hormone production. Placenta 13:101–113.