# Use of Chemical Tests for Pregnancy Diagnosis in a Reproductive Management Program

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## Introduction

Early identification of nonpregnant dairy cows post breeding can improve reproductive efficiency and pregnancy rate by decreasing the interval between AI services and increasing AI service rate. Thus, new technologies to identify nonpregnant dairy cows early after artificial insemination (**AI**) may play a key role in systematic management strategies to improve reproductive efficiency and profitability on commercial dairy farms. Transrectal palpation is the oldest and most widely used method for early nonpregnancy diagnosis in dairy cattle (Cowie, 1948); however, a newer technology may emerge to replace transrectal palpation as the method of choice for nonpregnancy diagnosis in the dairy industry. One such technology is the development of commercially available tests to detect pregnancy-associated glycoproteins (**PAGs**) in maternal serum; however, this new technology must be practically integrated into a systematic on-farm reproductive management strategy for it to succeed. Research reviewed in this paper support use of PAGs as a method for early nonpregnacy diagnosis in dairy cattle; however, there are some caveats and limitations with regard to their incorporation into a systematic reproductive management program that must be considered to determine the appropriate timing of nonpregnancy diagnosis.

## Pregnancy-Associated Glycoproteins (PAGs)

Pregnancy-associated glycoproteins constitute a family of inactive aspartic proteinases (Xie et al., 1991) comprising 22 genes located on chromosome 29 in the bovine (Telugu et al., 2009) with different patterns of expression throughout pregnancy and produced mainly by the binucleate cells of the placenta (Xie et al., 1991; Green et al., 2000; Patel et al., 2004) but also by the trophectoderm (Xie et al., 1991). Placentation in ruminants is noninvasive and is classified as synepitheliochorial cotyledonary, which describes the fetal-maternal syncytium formed by the fusion of trophoblast binucleate cells and uterine epithelial cells (Wooding, 1992). The giant binucleate cells are large cells containing two nuclei and are the invasive component of the trophoblast representing 15 to 20 % of the total cellular population within the mature placenta. Mature chorionic binucleate cells at all stages of bovine pregnancy migrate into the uterine epithelium and release the contents of cytosolic granules containing PAG's through exocytosis where they enter the maternal circulation (Wooding and Whates, 1980; Wooding, 1983; Zoli et al., 1992b).

### *Pregnancy-Associated Glycoproteins (PAGs) vs. Pregnancy Specific Protein-B (PSPB)*

Initial studies to determine the presence of pregnancy-associated proteins in sheep and cattle detected the presence of proteins related to pregnancy in uterine flushings

around 7 to 14 d of gestation (Roberts and Parkers, 1976; Roberts et al., 1976). Butler et al. (1982) determined the presence of two pregnancy-specific proteins in extracts of bovine placental membranes. One of these proteins was identified as $\alpha_1$ fetoprotein, whereas the second protein was identified as pregnancy specific protein-B (**PSPB**) and was considered to be secreted by the trophoblast. A double antibody radioimmunoassay (RIA) for PSPB was subsequently developed as a specific serological test for pregnancy in cattle (Sasser et al. 1986). In addition, a pregnancy serum protein purified from extracts of bovine cotyledons was also developed as a pregnancy test, and this protein was named PSP60 (based on its molecular weight of 60 kDA) and is now considered to be a form of PSPB (Mialon et al., 1993). Zoli et al. (1991) purified a bovine pregnancy associated glycoprotein (bPAG) from ovine and bovine cotyledons that could be detected in maternal blood near the time that the trophoblast forms a definitive attachment to the uterine endometrium. Zoli et al. (1991) determined that bPAG was similar in molecular weight to PSPB. In a second study, an assay was developed that allowed measurement of bPAG in placental extracts, fetal serum, fetal fluids, and serum or plasma of pregnant cows (Zoli et al., 1992a). Similar to the work from Sasser (1986), bPAG was detectable at 22 d of pregnancy in some cows and by 30 d in all cows.

*Temporal PAG Profiles*

We recently assessed circulating PAG and PSPB concentrations in lactating dairy cows before and after induced pregnancy loss (Giordano et al., 2012). After insemination, serum PAG is detectable as early as 22 to 24 d after AI (Sasser et al., 1986; Zoli et al., 1992a; Green et al. 2005) which is supported by our data in Figure 1 in which both PAG and PSPB concentrations for pregnant cows differed from nonpregnant cows by 25 d after AI.

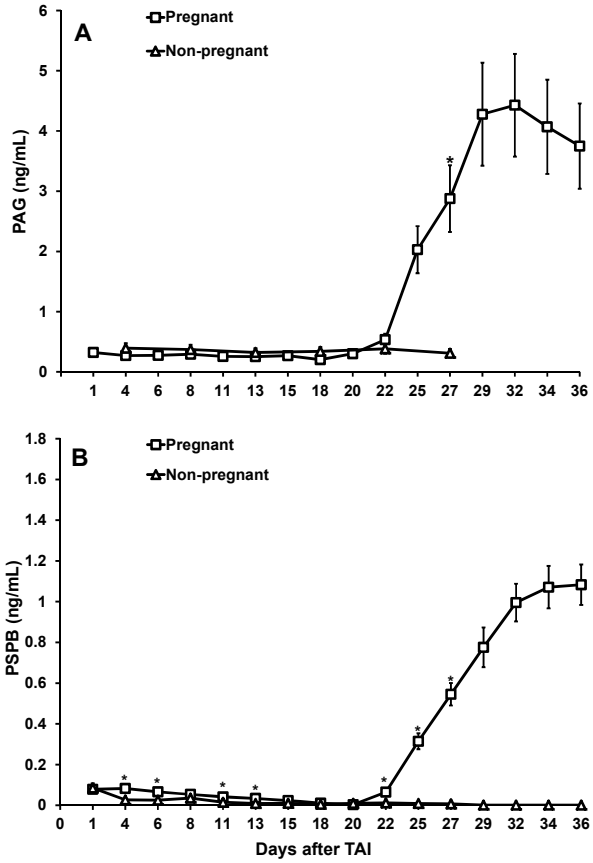


**Figure 1**. Pregnancy associated glycoprotein (PAG; A) and Pregnancy specific protein B (PSPB; B) concentrations for cows diagnosed pregnant vs. non-pregnant 29 d after TAI using transrectal ultrasonography (Giordano et al., 2012). Blood samples were analyzed for PAG concentrations from 1 to 36 d after TAI for cows diagnosed pregnant (n = 29) and at 4, 8, 13, 18, 22, and 27 d after TAI for cows diagnosed non-pregnant (n = 31) . Blood samples were analyzed for PSPB from 1 to 36 d after TAI for cows diagnosed pregnant (n = 29) and from 1 to 27 d after TAI for cows diagnosed non-pregnant (n = 31). Statistical comparison of PAG and PSPB between cows diagnosed pregnant and non-pregnant were analyzed for time points represented by both groups of cows. Concentrations of PAG tended to be affected by pregnancy status ($P = 0.098$), and were affected by time ($P < 0.0001$), and the pregnancy status by time interaction ($P < 0.0001$). Pregnancy specific protein B (PSPB) was affected by pregnancy status ($P < 0.0001$), time ($P < 0.0001$), and the pregnancy status by time interaction ($P < 0.0001$). *Pregnant different from non-pregnant.

Whates and Wooding (1980) described the changes occurring in bovine uterine and chorionic epithelia between 18 and 28 d of gestation, and the areas of attachment were first observed at 20 d. Release of PAG from the binucleate cells to the maternal circulation only occurs after attachment, therefore, PAG is not detectable in maternal circulation before this period. Concentration of PAG was determined in 20 beef and dairy cows once daily from 20 to 35 d after conception and at 2 wk intervals until 100 d postpartum (Zoli et al., 1992a). Serum PAG concentration increased continually as pregnancy advanced, and this increase was greater during the last 10 d prepartum. In this study, undetectable PAG levels occurred by 100 d postpartum. In another study, Green et al. (2005) analyzed PAG concentration from 42 heifers and cows that delivered a live

calf. Serum was collected beginning on the day of standing estrus, 15 d after AI, daily from 22 to 28 d after AI, and weekly throughout the remainder of pregnancy and for 10 wk after parturition. Circulating PAG concentration peaked during the last week of pregnancy, and PAG was undetectable by 6 wk after parturition in most of the cows. After parturition, PAG concentration decreases until it is undetectable around 56 to 100 d postpartum (Zoli et al., 1992a; Mialon et al., 1993; Green et al., 2005; Haugejorden et al., 2006). In our experiment, PSPB concentrations were high immediately after AI for five cows in which blood sampling began before 60 d after AI; three of these cows were diagnosed pregnant and two were diagnosed non-pregnant (Figure 2). Thus, because of the peak in PSPB concentration after parturition, circulating PSPB in maternal blood will yield false positives when the BioPRYN assay is used too early after parturition.

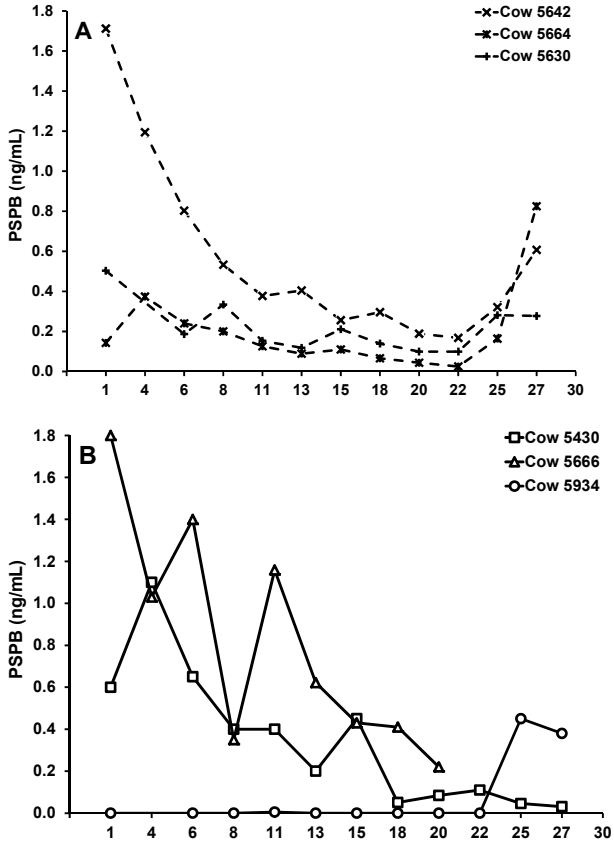


**Figure 2**. Pregnancy specific protein B (PSPB) concentrations from 1 to 27 d after TAI for three cows diagnosed pregnant (A) and three cows diagnosed non-pregnant (B) 29 d after TAI using transrectal ultrasonography (Giordano et al., 2012). All five cows were <60 d after AI when blood sampling began.

An ideal method for determination of pregnancy status in cows would not only be accurate for early identification of pregnant cows, but would also identify cows that underwent pregnancy loss or present a higher risk for loss at the time of diagnosis. This is important because the incidence of pregnancy loss in dairy cows from an early diagnosis (~25 to 30 d after AI) to term is high in lactating dairy cows. To assess PAG, PSPB, and progesterone profiles after induced pregnancy loss (Giordano et al., 2012), cows diagnosed pregnant 39 d after TAI were randomly assigned to one of three treatments to

receive: 1) an i.m. injection of saline (**CON**, n = 10); 2) an i.m. injection of $PGF_{2\alpha}$ (**PGF**, n = 10); or 3) an intrauterine infusion of 120 ml hypertonic saline (**INF**, n = 9). Time from treatment to cessation of the embryonic heart beat was greater for PGF than for INF cows (36.0 ± 5.7 vs. 0.2 ± 0.1 h, respectively), and time from treatment to conceptus disappearance was greater for INF than for PGF cows (7.1 ± 3.3 vs. 1.9 ± 0.3 d, respectively). Overall, P4 concentration was greater for CON and INF than for PGF cows (8.7 ± 2.8, 8.2 ± 3.1, and 1.0 ± 2.3 ng/ml, respectively) due to luteal regression for PGF cows and CL maintenance for CON and INF cows. Serum PAG and PSPB concentrations differed among CON cows and PGF and INF cows beginning 1 and 2.5 d after treatment for PAG and PSPB, respectively. By 9.5 d after treatment, PAG and PSPB concentrations were similar or greater compared to non-pregnant cows or pregnant cows before the initial increase around 22 d after TAI. Thus, although timing of conceptus expulsion occurred 5.2 d later for INF than for PGF cows, serum PAG and PSPB concentrations decreased at a similar rate from the onset of treatment for both models of pregnancy loss evaluated. Based on these data, commercial tests that measure PAGs or PSPB may more reliably detect pregnancy loss compared to use of transrectal ultrasonography when the non-pregnancy test is conducted before 30 d after AI (Giordano et al., 2012).

**On-Farm Incorporation of PAG Testing for Reproductive Management**

Synergies between new reproductive management technologies hold the key to maximizing reproductive efficiency on dairy farms; however, reproductive management protocols that allow for synchronization of ovulation and subsequent identification and resynchronization of nonpregnant cows must be practical to implement within the day to day operation of a dairy farm or the protocol will fail due to lack of compliance (Fricke et al., 2003). This is especially true for larger farms that must schedule and administer artificial inseminations, hormone injections, and pregnancy tests for a large number of animals on a daily or weekly basis. Identification of nonpregnant cows early post breeding can only improve reproductive efficiency when coupled with a management strategy to rapidly submit nonpregnant cows for a subsequent AI service. Thus, any method for early nonpregnancy diagnosis must be integrated as a component of the overall reproductive management strategy in place on the farm.

Currently, three non-pregnancy tests based on detection of PAGs in maternal serum are commercially available for use by dairy farmers:

1) BioPRYN
   BioTracking, LLC, Moscow, ID
   http://www.biotracking.com/
2) DG29
   Conception Animal Reproduction Technologies, Beaumont, QC
   http://www.conception-animal.com/test_an.html
3) IDEXX Bovine Pregnancy Test
   IDEXX Laboratories, Inc., Westbrook, ME,
   http://www.idexx.com/view/xhtml/en_us/livestock-poultry/ruminant/lpd-bovine-pregnancy-test.jsf

*3.1. Field Trial: Accuracy of Pregnancy Outcomes using PAG and Transrectal Ultrasonography 27 d after a Timed AI (Silva et al., 2007).*

The three commercial tests recommend that the earliest a non-pregnancy test can be conducted is either 28 d after AI (for BioPRYN and the IDEXX Bovine Pregnancy Test) or 29 d after AI (DG29). Thus, PAG testing competes directly with transrectal ultrasonography for early identification of non-pregnant cows. The objective of this field trial was to compare the accuracy of a plasma PAG ELISA test that has now been commercialized by IDEXX Laboratories, Inc. (Westbrook, ME) and marketed as the IDEXX Bovine Pregnancy Test to transrectal ultrasonography for determining pregnancy status of lactating dairy cows 27 d after timed AI (Silva et al., 2007). Ultrasound was used as a gold standard to determine the accuracy of an early PAG ELISA to determine pregnancy status after timed artificial insemination (**TAI**). Blood samples were collected from lactating Holstein cows (n = 1079) 27 d after their first, second, and third postpartum TAI services. Pregnancy diagnosis using transrectal ultrasonography was performed immediately after blood sample collection, and pregnancy outcomes using ultrasonography served as a gold standard to test the accuracy of the PAG ELISA. Pregnancy outcomes based on the PAG ELISA and transrectal ultrasonography that agreed were considered correct, whereas pregnancy status of cows in which pregnancy outcomes disagreed between PAG and transrectal ultrasonography were re-assessed using transrectal ultrasonography 5 d later.

Tables 1 and 2 show the sensitivity, specificity, positive predictive value, negative predictive value, and accuracy for determination of pregnancy status 27 d after TAI for transrectal ultrasonography and the PAG ELISA, respectively.

**Table 1**. Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and accuracy of transrectal ultrasonography (TU) for determination of pregnancy status 27 d after timed AI (TAI) by TAI number (Silva et al., 2007).

| TAI | Sensitivity[1] % (no./no.) | Specificity[2] % (no./no.) | PPV[3] % (no./no.) | NPV[4] % (no./no.) | Accuracy[5] % (no./no.) | Kappa |
|---|---|---|---|---|---|---|
| 1 | 96.8 (367/379) | 91.7 (461/503) | 89.7 (367/409) | 97.5 (461/473) | 93.9 (828/882) | 0.87 |
| 2 | 94.2 (145/154) | 93.5 (303/324) | 87.3 (145/166) | 97.1 (303/312) | 93.7 (448/478) | 0.85 |
| 3 | 98.9 (91/92) | 97.3 (215/221) | 93.8 (91/97) | 99.5 (215/216) | 97.8 (306/313) | 0.94 |

[1]Proportion of pregnant cows with a positive TU outcome.
[2]Proportion of not-pregnant cows with a negative TU outcome.
[3]Proportion of cows diagnosed pregnant using TU that truly were pregnant.
[4]Proportion of cows diagnosed as not-pregnant using TU that truly were not-pregnant.
[5]Proportion of pregnancy status, pregnant and not-pregnant, that was correctly classified by TU.

**Table 2**. Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and accuracy of the PAG ELISA 27 d after timed AI (TAI) by TAI number (Silva et al., 2007).

| TAI | Sensitivity[1] % (no./no.) | Specificity[2] % (no./no.) | PPV[3] % (no./no.) | NPV[4] % (no./no.) | Accuracy[5] % (no./no.) | Kappa |
|---|---|---|---|---|---|---|
| 1 | 96.3 (365/379) | 91.7 (461/503) | 89.7 (365/407) | 97.1 (461/475) | 93.7 (826/882) | 0.87 |
| 2 | 93.5 (144/154) | 96.3 (312/324) | 92.3 (144/156) | 96.9 (312/322) | 95.4 (456/478) | 0.89 |
| 3 | 94.6 (87/92) | 96.8 (214/221) | 92.6 (87/94) | 97.7 (214/219) | 96.2 (301/313) | 0.90 |

[1]Proportion of samples from pregnant cows with a positive PAG ELISA.
[2]Proportion of samples from not-pregnant cows with a negative PAG ELISA.
[3]Proportion of PAG ELISA with a pregnant outcome that truly were pregnant.
[4]Proportion of PAG ELISA with a not-pregnant outcome that truly was not-pregnant.
[5]Proportion of pregnancy status, pregnant and not-pregnant, that was correctly classified.

In summary of this field trial, the PAG ELISA used for determination of PAG concentration in cows had an accuracy of 93.7 to 96.2 % 27 d after TAI and is similar to the accuracy of transrectal ultrasonography (93.7 to 97.8%). Determination of pregnancy status based on plasma PAG concentration 27 d after TAI resulted in acceptable sensitivity and specificity. The negative predictive value of the PAG ELISA was high (96.9 to 97.7 %) indicating that few cows would be subjected to induced pregnancy loss due to administration of $PGF_{2\alpha}$ during the resynchronization protocol. Our results agree with those of Romano and Larson (2010) who evaluated the PSPB test and concluded that the PSPB ELISA was a sensitive, specific, and accurate test for pregnancy diagnosis (relative to transrectal ultrasonography) at Days 28, 30, and 35 after AI.

### *3.2. Field Trial: Effect of interval to resynchronization of ovulation on fertility of lactating Holstein cows when using transrectal ultrasonography or a pregnancy-associated glycoptorein (PAG) ELISA to diagnose pregnancy status (Silva et al., 2009).*

The objective of this field trial was to compare two strategies for resynchronization of ovulation based on non-pregnant diagnoses using transrectal ultrasonography or a pregnancy-associated glycoprotein (PAG) ELISA (IDEXX Laboratories, Inc., Westbrook, ME). Figure 3 shows a schematic of the experimental design. Briefly, lactating Holstein cows (n = 1038) were submitted for first postpartum TAI using a Presynch - Ovsynch protocol. After the initial breeding, cows were randomly assigned to initiate resynchronization 25 d (D25) or 32 d (D32) later. Pregnancy status of cows initiating Resynch 25 d after TAI was determined 27 d after TAI by using a PAG ELISA, whereas pregnancy status of cows initiating Resynch 32 d after TAI was determined 39 d after TAI using transrectal ultrasonography. Cows diagnosed not pregnant continued the Resynch protocol by receiving an i.m. injection of PGF2α 7 d after the initial GnRH injection and a second GnRH injection 54 h after the PGF2α injection. Cows in both treatments were inseminated approximately 16 h after the second GnRH injection. Blood samples for analysis of progesterone (P4) were collected at the first GnRH injection of each Resynch protocol.

Overall, D25 cows had fewer (P < 0.0001) DIM at TAI than D32 cows for first (113.8 ± 0.23 vs. 119.2 ± 0.25) and second resynchronization (147.2 ± 0.31 vs. 161.2 ± 0.31). The D25 cows also had fewer (P < 0.0001) total days open after the initial TAI (77.4 ± 0.9; pregnant + open cows) compared to D32 cows (91.9 ± 1.0). By design, there were fewer days between TAI for D25 than D32 cows (37.0 ± 0.24 vs. 42.0 ± 0.00). Pregnancies per AI at 27 d for D25 cows were 35.9 % and 29.4 % for RES1 and RES2, respectively. Effect of treatment on P/AI was based on pregnancy examinations conducted 39 and 62 d after TAI. Pregnancies per AI of nonpregnant cows initiating Resynch 25 vs. 32 d after first postpartum TAI did not differ 39 d after TAI and were 28.3 vs. 30.9 % for D25 vs. D32 cows, respectively (Table 3).

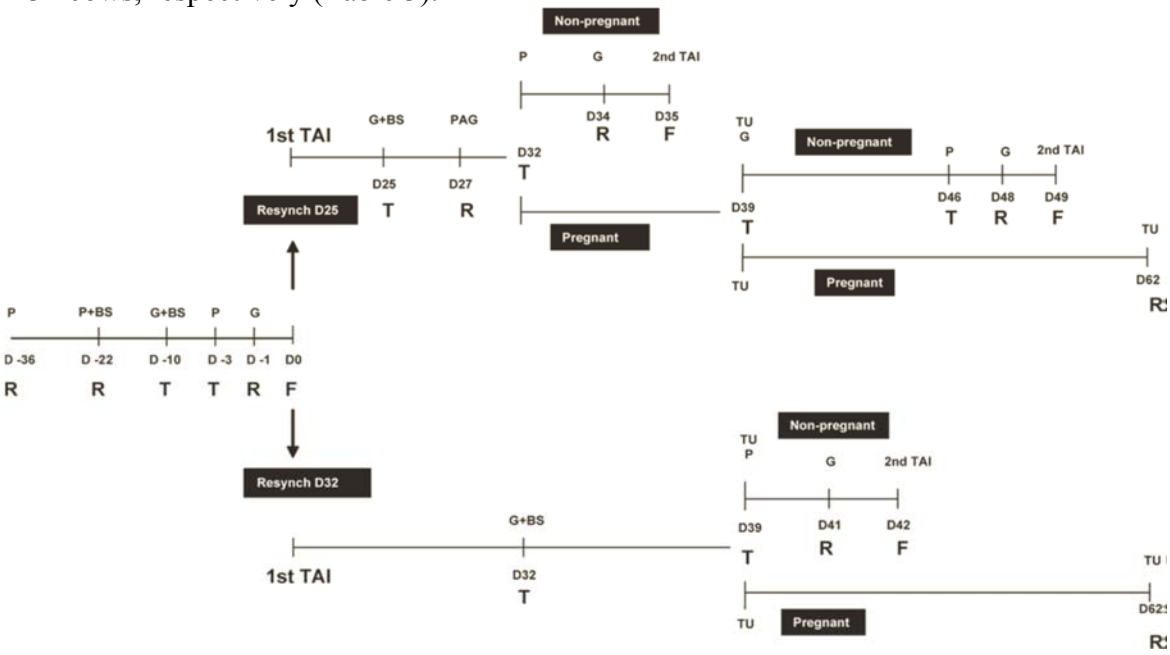


**Figure 3**. Protocol for blood sample collection and ultrasound examination for determination of pregnancy status in each Resynch treatment (Silva et al., 2009).

**Table 3**. Effect of treatment, parity, and resynch number on pregnancies per AI (P/AI) and pregnancy loss after timed AI (Silva et al., 2009).

| Item | Treatment | | | | P value | |
|---|---|---|---|---|---|---|
| | D25 | | D32 | | | |
| | 1st Resynch | 2nd Resynch | 1st Resynch | 2nd Resynch | Treatment | Resynch no. |
| P/AI 39 d (%) | 32.8 | 23.3 | 32.7 | 29.1 | | |
| (no./no.) | (86/262) | (34/146) | (74/226) | (41/141) | | |
| LS mean | 33.8 | 23.5 | 31.7 | 30.0 | 0.487 | 0.094 |
| P/AI 62 d (%) | 27.7 | 21.7 | 31.0 | 25.7 | | |
| (no./no.) | (69/249) | (31/143) | (70/226) | (36/140) | | |
| LS mean | 29.2 | 21.9 | 30.8 | 26.7 | 0.350 | 0.666 |
| Loss (%) 39 to 62 d | 5.5 | 0.0 | 5.4 | 10.0 | | |
| (no./no.) | (4/73) | (0/31) | (4/74) | (4/40) | | |

Results from this field trial showed that initiation of resynchronization 25 d after an initial TAI resulted in similar fertility to initiation of resynchronization 32 d after TAI in lactating Holstein cows. Thus, early resynchronization reduced DIM at TAI and total days open after the initial TAI.

## Conclusion

Data from our first field trial (Silva et al., 2007) supported that use of a commercial PAG assay for detecting non-pregnant cows 27 d after AI yielded acceptable sensitivity and specificity, had a high negative predictive value indicating that few cows would be subjected to iatrogenic pregnancy loss during a resynchronization protocol, and had a similar accuracy when compared to transrectal ultrasonography. Furthermore, commercial PAG and PSPB tests may more reliably detect cows undergoing pregnancy loss compared to use of transrectal ultrasonography when the non-pregnancy test is conducted before 30 d after AI (Giordano et al., 2012). Data from our second field trial (Silva et al., 2009) showed that initiation of resynchronization 25 d after an initial TAI resulted in similar fertility to initiation of resynchronization 32 d after TAI in lactating Holstein cows thereby decreasing DIM at TAI and total days open after the initial TAI. Based on our results (Giordano et al., 2012), it is important to follow the manufacturer's instructions when using these commercial tests so that cows are not subjected to non-pregnancy testing too early postpartum to avoid false positive results and that cows are not tested too early post-insemination to avoid poor sensitivity, specificity, and accuracy of the test.

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