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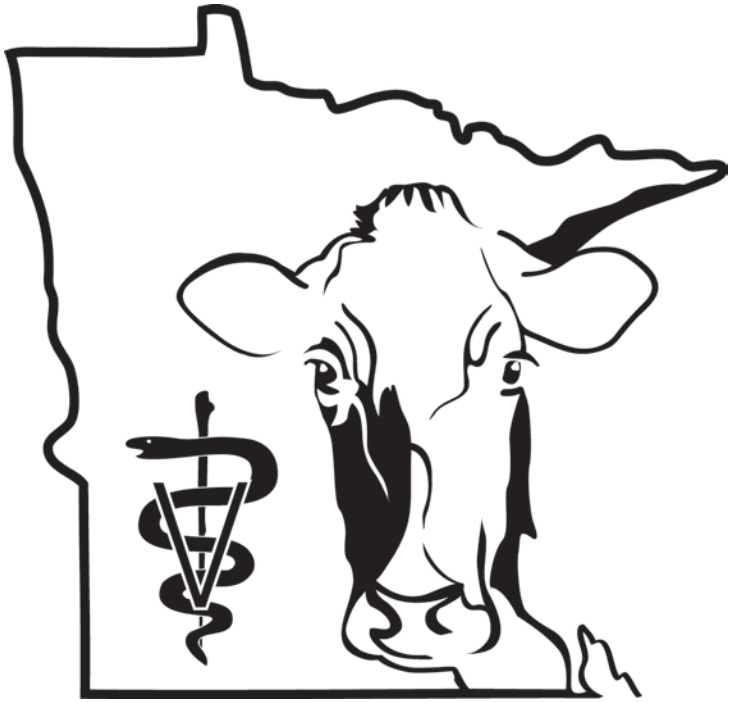

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When to Preg Check and Why

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ABSTRACT

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 pregnancy diagnosis in dairy cattle (Cowie, 1948). However, a newer technology may someday replace transrectal palpation as the method of choice for pregnancy diagnosis in the dairy industry. Before this transition can occur, two events must transpire. First, a technology must be developed that exceeds transrectal palpation in one or more of the characteristics of the ideal early pregnancy test. Second and no less important, this new technology must be practically integrated into a systematic on-farm reproductive management strategy and empirically demonstrated to exceed the status quo of the industry (i.e., transrectal palpation) in reproductive performance. Results from several recent studies indicate that positive pregnancy outcomes diagnosed by transrectal ultrasonography conducted 26 or 27 d after timed AI may be inflated due to pregnancy loss and/or diagnostic errors compared to pregnancy outcomes conducted 32 to 39 d after timed AI. Furthermore, fertility to timed AI after resychronization of ovulation was greater when initiated 33 d after timed AI compared to 19 or 26 d after timed AI. Taken together, these results support the counterintuitive notion that delaying pregnancy diagnosis may improve reproductive efficiency when using a hormonal protocol for timed AI to program nonpregnant cows for rebreeding due to the high rate of pregnancy loss and errors occurring too early post TAI. Finally, new strategies being developed and tested for resynchronizing cows failing to conceive to a previous TAI may require a nonpregnancy diagnosis as early as 29 d after TAI.

Return to Estrus as a Diagnostic Indicator of Pregnancy Status

Return to estrus from 18 to 24 days after AI is often considered by dairy farmers the easiest and least costly method for determining nonpregnancy in dairy cattle early post breeding. This assumption, however, is being challenged by new research and long-recognized reproductive problems. First, estrous detection efficiency is estimated to be less than 50% on most dairy farms in the United States (Senger, 1994). This is likely a result of the short duration of estrus behavior reported for lactating cows (Dransfield et al., 1998) and because cows display estrus behavior poorly when housed on concrete flooring (Vailes and Britt, 1990), a common housing situation for dairy cattle in many regions of the U.S. Second, estrous cycle duration varies widely among lactating dairy cows from the standard 21-day interval and averaged around 23 days with a high degree of variability among animals lactating dairy cows (Sartori et al., 2004). This variability makes it difficult to target detection of return to estrus for groups of animals receiving AI on the same day. Finally, the high rate of pregnancy loss in dairy cows can increase the interval from insemination to return to estrus for cows that maintain a pregnancy then loose that pregnancy later during gestation (Fricke et al., 2003). The rate of pregnancy loss occurring during the period of gestation when dairy cattle are submitted for pregnancy examinations using

ultrasonography or rectal palpation is high and, therefore, is a key factor for understanding the implementation and implications of methods for early nonpregnancy diagnosis.

Pregnancy Loss in Dairy Cattle

Pregnancy loss contributes to reproductive inefficiency because fertility assessed at any point during pregnancy is a function of both conception rate and pregnancy loss (Fricke, 2002). Since the widespread implementation of transrectal ultrasonography for reproductive research in cattle (Griffin and Ginther, 1992), several studies have reported rates of pregnancy loss during early gestation under field conditions. Table 1 summarizes reported rates of pregnancy loss in lactating dairy cows from an initial pregnancy diagnosis conducted 27 to 30 days post breeding to a subsequent pregnancy reassessment 14 to 42 days later. Taken together, average pregnancy loss reported in these studies exceeded 15%. Vasconcelos et al. (1997) characterized pregnancy loss at various stages of gestation using transrectal ultrasonography and reported pregnancy losses of 11% from 28 to 42 d, 6% from 42 to 56 d, and 2% from 56 to 98 d post AI, suggesting that the rate of loss is greater early during gestation, then decreases as gestation proceeds. In a recent study by Sterry et al., (2006), pregnancy loss was 3.7% (13/353) from 33 to 40 d after TAI and 3.3% (11/337) from 40 to 61 d after TAI for a total loss of 6.8% (24/353) from 33 to 61 d after TAI.

Early pregnancy diagnosis can improve reproductive performance by decreasing the interval between successive AI services and coupling a nonpregnancy diagnosis with an aggressive strategy to rapidly rebreed these animals (Fricke, 2002). Conversely, it has long been accepted that pregnancy status should be determined in dairy cattle as soon as possible after insemination but without having the diagnosis confounded by subsequent pregnancy loss (Melrose, 1979; Thurmond and Picanso, 1993). Pregnancy loss diminishes the benefit of early pregnancy diagnosis in two ways. First, because of the high rate of pregnancy loss that occurs around the time during gestation that most direct and indirect pregnancy tests are performed (Table 1); the magnitude of pregnancy loss detected is greater the earlier post breeding that a positive diagnosis is made. Thus, the earlier that pregnancy is diagnosed post breeding, the fewer nonpregnant cows are identified to which a management strategy can be implemented to rebreed them. Second and more important, cows diagnosed pregnant earlier post breeding have a greater risk for pregnancy loss compared to cows diagnosed later post breeding. If left unidentified, cows diagnosed pregnant early post breeding that subsequently loose that pregnancy reduce reproductive efficiency by extending the interval from calving to the conception that results in a full-term pregnancy. In addition, diagnostic errors which inflate the proportion of cows diagnosed pregnant can lead to dramatically high rates of pregnancy losses (Silva et al., 2007), and this issue will be discussed in detail under the second field trial.

To compensate for pregnancy loss, cows diagnosed pregnant early post breeding must undergo one or more subsequent pregnancy reconfirmations to identify and rebreed cows that experience pregnancy loss. Thus, dairy mangers who have implemented early pregnancy diagnoses must consider the timing and frequency of subsequent pregnancy examinations to maintain the reproductive performance of the herd. Problems caused by pregnancy loss apply to all currently available methods for assessing pregnancy status early post breeding, and may relegate pregnancy testing before 30 to 40 days post breeding an untenable management strategy unless pregnancy diagnoses can be made continually on a daily basis or at each milking until the rate of

pregnancy loss decreases, or until the underlying causes of pregnancy loss are understood and mitigated.

Attributes of the Ideal Nonpregnancy Test

For successful integration into a reproductive management system, an ideal early nonpregnancy test for dairy cattle would be 1) sensitive (i.e., correctly identify pregnant animals) 2) specific (i.e., correctly identify nonpregnant animals), 3) inexpensive, 4) simple to conduct under field conditions, and 5) able to determine pregnancy status at the time the test is performed. Most currently available methods for pregnancy diagnosis exhibit one or more of these attributes, but none currently available or under development exhibit all of them. A final attribute of an ideal test would be the ability to determine pregnancy status without the need to physically handle the animal to administer the test. Such a test may overcome the inherent limitations of current tests caused by pregnancy loss and may make pregnancy diagnosis before 30 to 40 days postbreeding in dairy cattle an economically viable reproductive management strategy. Although rectal palpation and transrectal ultrasonography both require animal handling to administer the test, new technologies such as measurement of pregnancy-associated factors for early nonpregnancy diagnosis may someday realize this goal.

Methods for Nonpregnancy Diagnosis in Cattle

Transrectal Palpation

Transrectal palpation of the uterus for pregnancy diagnosis in cattle was first described in the 1800's (Cowie, 1948) and is the oldest and most widely used method for early pregnancy diagnosis in dairy cattle today. Palpation technique can vary among practitioners. Transrectal palpation of the amniotic vesicle as an aid in determining pregnancy status in dairy cattle was described by Wisnicky and Cassida (1948) whereas slipping of the chorioallantoic membranes between the palpator's thumb and forefinger beginning on about day 30 of gestation was described by Zemjanis (1970). Veterinary schools across the US and in other countries continue to train their students in the art of transrectal palpation for diagnosis of pregnancy in dairy cattle.

Because pregnancy in cattle can be terminated by manual rupture of the amnionic vesicle (Ball and Carroll, 1963), many studies have investigated the extent of iatrogenic pregnancy loss induced by transrectal palpation. Several studies have suggested that examining pregnant cows early in gestation by transrectal palpation increases the risk of iatrogenic pregnancy loss (Abbitt et al., 1978; Franco et al., 1987; Paisley et al., 1978; Vaillancourt et al., 1979; White et al., 1989) whereas other studies have suggested that cows submitted for transrectal palpation earlier during gestation had a decreased risk for abortion or that palpation had no effect on subsequent embryonic losses (Studer, 1969; Thurmond and Picanso, 1993). Although controversy still exists regarding the extent of iatrogenic pregnancy loss induced by transrectal palpation, other factors have a greater influence on calving rates than pregnancy examination by transrectal palpation (Thompson et al., 1994). Furthermore, because the risk of pregnancy loss is high during the period of gestation when cows are diagnosed pregnant by transrectal palpation (Table 1), and because most cows within a herd are submitted for pregnancy examination, it is impossible for dairy producers and veterinarians to distinguish between iatrogenic losses occurring due to transrectal palpation and spontaneous losses that would normally have occurred in these cows.

Because of its widespread use and the number of bovine practitioners trained to perform the procedure, transrectal palpation will likely remain a mainstay for pregnancy diagnosis in dairy cattle until a newer method for pregnancy diagnosis is developed that exceeds the technique in one or more of the attributes of the ideal nonpregnancy test. Furthermore, because of its widespread use, high accuracy, and relatively low cost per animal, transrectal palpation is the industry standard that newer methods for pregnancy diagnosis in dairy cattle must displace as the method of choice for pregnancy diagnosis.

B-Mode Ultrasonography

Applications of and detailed methods for performing transrectal ultrasonography for reproductive research have been reviewed and described in detail (Ginther, 1998; Griffin and Ginther, 1992). Most veterinary students continue to be taught that ultrasound is a secondary technology for bovine reproductive work; however, the information-gathering capabilities of ultrasonic imaging far exceed those of transrectal palpation (Ginther, 1995). Although early nonpregnancy diagnosis is among the most practical application for reproductive management using transrectal ultrasonography, additional information gathered using the technology that may be useful for reproductive management include evaluation of ovarian structures, identification of cows carrying twin fetuses, and determination of fetal sex (Fricke, 2002). A fetal heartbeat can be visualized at around 21 d of gestation under controlled experimental conditions and using a high-quality scanner and transducer (Curran et al., 1986), and represents the definitive characteristic for positive confirmation of a viable pregnancy using transrectal ultrasonography. Although the rate of pregnancy loss is significant in studies using ultrasound to assess the rate of loss (Table 1), the technique itself has not been implicated as a direct cause of pregnancy loss in cattle (Ball and Logue, 1994; Baxter and Ward, 1997). Ultrasound is a less invasive technique for early pregnancy diagnosis than is transrectal palpation (Paisley et al., 1978; Vallancourt et al., 1979), and may minimize the rare incidence of palpation-induced abortions.

Under most on-farm conditions, pregnancy diagnosis can be rapidly and accurately diagnosed using ultrasound as early as 26 d post AI (Filteau and DesCoteaux, 1998; Kastelic et al., 1991). When conducted between 21 and 25 d post breeding, sensitivity and specificity of pregnancy diagnosis using ultrasound was 44.8% and 82.3%, respectively, but increased to 97.7% and 87.7%, respectively, when conducted between 26 and 33 d post AI (Pieterse et al., 1990). Sensitivity and specificity of pregnancy diagnosis in lactating dairy cows based on ultrasonographic detection of uterine fluid as well as embryonic membranes from 28 to 35 days after AI was 96% and 97%, respectively (Nation et al., 2003). Pregnancy diagnosis in dairy heifers based on the presence of intraluminal uterine fluid before Day 16, however, is unreliable because small amounts of fluid are present in non-inseminated heifers as early as 10 days after estrus (Kastelic et al., 1991). For lactating dairy cows, ultrasonographic detection of uterine fluid as well as embryonic membranes from 28 to 35 days after AI was an accurate estimation of the presence of an embryo at the time of observation (Nation et al., 2003). Although ultrasound conducted at $\geq$ 45 days post breeding did not increase accuracy of pregnancy diagnosis for an experienced palpator, it may improve diagnostic accuracy of a less experienced one (Galland et al., 1994).

Veterinary-grade ultrasound machines equipped with one rectal transducer are expensive and cost $8,000 to $16,000, and the cost of this technology may limit its practical implementation (Fricke, 2002). Although dairy producers can purchase an ultrasound scanner and conduct pregnancy examinations on their own cows, they generally lack the knowledge, training, and experience required to accurately perform pregnancy examinations (Fricke, 2002). Transrectal ultrasonography is now being incorporated into reproductive management schemes in dairies primarily by bovine practitioners who have adopted this technology. The extent to which transrectal ultrasonography will displace transrectal palpation as the primary direct method for pregnancy diagnosis in dairy cattle remains to be determined. Because many experienced bovine practitioners can accurately diagnose pregnancy as early as 35 days post breeding using transrectal palpation, pregnancy examination using transrectal ultrasonography at 26 to 28 days post breeding only reduces the interval from insemination to pregnancy diagnosis by 7 to 9 days. The rate of pregnancy loss and the efficacy of strategies to rebreed cows at various stages post breeding also play a role in determining the advantages and disadvantages on the timing of pregnancy diagnosis and resynchronization (Fricke et al., 2003).

Pregnancy-Associated Glycoproteins (PAGs)

Pregnancy associated glycoproteins are produced by the binucleate cells of the embryonic trophoblast. 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 specific protein-B. 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 et al., 1976; Roberts and Parkers, 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 α_1 fetoprotein, whereas the second protein was identified as pregnancy specific protein-B (PSP-B) and was considered to be secreted by the trophoblast. A double antibody radioimmunoassay (RIA) for PSP-B 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 PSP-B (Mialon et al., 1993).

Pregnancy-associated glycoproteins (PAGs). 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 PSP-B, however they needed to compare their amino acid sequences to conclude if the two proteins were identical. 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.

Serum PAG profiles. After breeding, serum PAG is detectable as early as 22 to 24 d after AI and increases steadily throughout gestation peaking before parturition (Sasser et al., 1986; Zoli et al., 1992a; Green et al. 2005). 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 in the region of the embryo. 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 days prepartum. In this study, undetectable levels of PAG were reached by day 100 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. PAG concentration peaked during the last week of pregnancy, and PAG was undetectable by 6 wk after parturition in most of the cows. Because of the peak in PAG concentration after parturition, circulating PAG in maternal blood may lead to false positive results if an assay to detect PAG is used too early after parturition. 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).

On Farm Implementation of Early Nonpregnancy Diagnosis

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. The various component technologies of the reproductive management system will in turn determine the timing of the events as they occur on a daily or weekly basis. As stated previously, it has long been accepted that pregnancy status should be determined in dairy cattle as soon as possible after insemination but without having the diagnosis confounded by subsequent pregnancy loss (Melrose, 1979; Studer, 1969). New research on the practical implementation of early pregnancy diagnosis using transrectal ultrasonography into a systematic synchronization and resynchronization system has confirmed this notion and illustrated the pitfalls and limitations of early pregnancy diagnosis (Fricke et al., 2003; Silva et al., 2007).

Field Trial: Integrating Systematic Synchronization and Resynchronization of ovulation with Transrectal Ultrasonography

Two recently adopted technologies for reproductive management of dairy cattle include hormonal protocols such as Ovsynch (Pursley et al., 1995, 1997) and Presynch/Ovsynch (Moreira et al., 2001; Navanukraw et al., 2004), and use of transrectal ultrasonography for early identification of nonpregnant cows (Fricke, 2002). We conducted a field trial to compare three intervals from first TAI to initiation of resynchronization of ovulation on a dairy incorporating transrectal ultrasonography as a method for early pregnancy diagnosis (Fricke et al., 2003). The objective was to compare conception rate to first TAI service after a modified Presynch protocol with conception rates after resynchronization of ovulation using Ovsynch at three intervals post TAI (Resynch) coupled with pregnancy diagnosis using transrectal ultrasonography. Lactating dairy cows on a commercial dairy farm were enrolled into this study on a weekly basis.

All cows received a modified Presynch protocol to receive first postpartum TAI as follows: 25 mg $PGF_{2\alpha}$ (d 32 ± 3; d 46 ± 3); 50 μg GnRH (d 60 ± 3); 25 mg $PGF_{2\alpha}$ (d 67 ± 3) and 50 μg GnRH (d 69 ± 3) postpartum (Navanukraw et al., 2004). All cows received TAI immediately after the second GnRH injection of the Presynch protocol (d 0) as per a Cosynch TAI schedule. At first TAI, cows were randomly assigned to each of three treatment groups for resynchronization of ovulation (Resynch) using Ovsynch [50 μg GnRH (d -9); 25 mg $PGF_{2\alpha}$ (d -2) and 50 μg GnRH + TAI (d -0)] to induce a second TAI for cows failing to conceive to first TAI service. All cows (n=235) in the first group (D19) received a GnRH injection on d 19 post TAI and continued the Ovsynch protocol if diagnosed nonpregnant using transrectal ultrasound on d 26 post TAI. Cows (n=240) in the second (D26) and cows (n=236) in the third (D33) groups initiated the Ovsynch protocol if diagnosed nonpregnant using transrectal ultrasound on d 26 post-TAI or d 33 post-TAI, respectively. Submission of cows for first postpartum TAI service was scheduled so that the first four injections of the Presynch plus Ovsynch protocol occurred on Tuesdays followed by the second GnRH injection and TAI occurring on Thursdays. Initiation times for Resynch for each of the three treatment groups in this study were chosen to occur on Tuesdays so that injection schedules would remain consistent for all cows assigned to weekly breeding groups at any given time. To adhere to the Tuesday/Thursday schedule, all pregnancy examinations were conducted on Tuesdays. To fit the reproductive management system, the first pregnancy examination using transrectal ultrasound was conducted 26 d after TAI for the D19 and D26 cows and 33 d after TAI for the D33 cows.

Implicit to the experimental design, first assessment of pregnancy status was not conducted at the same interval after the Ovsynch TAI among the three treatment groups. Pregnancy status after the Ovsynch TAI was first assessed 26 d after TAI for cows in the D19 and D26 groups, whereas pregnancy status was assessed 33 d post Ovsynch TAI for cows in the D33 group. Overall fertility to Ovsynch was 40% and was greater for D19 and D26 cows than for D33 cows (Table 2). This difference is likely due to a combination of two factors 1) a greater period in which pregnancy loss can occur in the D33 cows due to the increased interval from TAI to pregnancy diagnosis (26 vs. 33 d) and 2) diagnostic errors in which cows diagnosed pregnant are actually undergoing pregnancy loss. When pregnancy status was reassessed for all treatment groups at 68 d after Ovsynch TAI, overall PR/AI to Ovsynch was 31% and did not differ among treatments

(Table 2). Thus, differences in PR/AI at the first pregnancy exam and pregnancy losses between the first and second pregnancy exams among treatment groups likely represent an artifact of time of assessment of pregnancy status after TAI inherent to the experimental design rather than to treatment differences. Overall PR/AI to Resynch was 32% and was greater for D26 and D33 cows than for D19 cows (Table 3).

In summary of this field trial, the system with the most aggressive early nonpregnancy diagnosis and resynchronzation schedule (i.e., the D19 treatment) was not a viable management strategy based on the poor fertility after the Resynch TAI probably due to follicular and luteal dynamics at the stage post breeding that the synchronization protocol was initiated. In addition, the early nonpregnancy diagnoses conducted 26 d after TAI identified fewer nonpregnant cows compared to the 33 d nonpregnancy diagnosis and also lead to a dramatically higher rate of pregnancy loss.

Field Trial: Accuracy of Pregnancy Outcomes using PAG and Transrectal Ultrasonography 27 d after a Timed AI

Another recently commercialized strategy for identifying nonpregnant cows is the use of a serum PAG ELISA test marketed as BioPRYN. The objective of this study was to compare the accuracy of a plasma PAG ELISA test now under development and not yet commercialized to transrectal ultrasonography (TU) for determining pregnancy status of lactating dairy cows 27 d after timed AI (TAI). To determine the accuracy of a pregnancy-associated glycoprotein (PAG) ELISA to identify pregnancy status 27 d after timed AI (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 (TU) was performed immediately after blood sample collection, and pregnancy outcomes using TU served as a gold standard to test the accuracy of the PAG ELISA. Pregnancy outcomes based on the PAG ELISA and TU that agreed were considered correct, whereas pregnancy status of cows in which pregnancy outcomes disagreed between PAG and TU were re-assessed using TU 5 d later.

Accuracy of Transrectal Ultrasonography 27 d after TAI

During the early portion of the experiment, a significant population of cows diagnosed pregnant using TU were diagnosed nonpregnant using the PAG ELISA. To determine which outcome was correct, we developed a category designation for pregnancy outcomes using TI (Table 4). The frequency distribution of pregnancy outcomes for each TU category is summarized in Table 5. The percentage of cows assigned to each category was 17.4, 19.6, 3.4, 0.7, and 58.9 % for PG, QP1, QP2, PL, and NP categories, respectively. The percentage of pregnant (PG, QP1 and QP2) and not-pregnant cows (PL and NP) was 40.5 and 59.5 %, respectively. The percentage of cows diagnosed pregnant based on visualization of an embryo using TU (PG, 17.4%) is similar to the number of cows diagnosed pregnant based solely on the presence of chorioallantoic fluid and a CL but without visualizing an embryo (QP1, 19.6 %). For nonlactating heifers evaluated under optimal experimental conditions and with no time constraints, the embryonic vesicle was first detectable at about 12 d of gestation, and detection of the embryo proper was possible by 20 d (Curran et al., 1986). Circular non-echogenic structures within the uterus were common in non-inseminated and pregnant heifers 10 to 14 d after ovulation, and by 16 d there were more elongated non-echogenic structures in pregnant heifers (Kastelic et al., 1991). For the TU

outcomes that disagreed with the PAG ELISA, the percentage of incorrect TU outcomes was less for not-pregnant than for pregnant outcomes (26.5%, n=83 vs. 70.4%, n= 98) mainly due to the incorrect TU outcomes classified as either QP1 or QP2. An important consideration when interpreting data from the present study is that TU outcomes were made by only one bovine practitioner and all of the pregnancy outcomes were conducted on a single farm. Variation among farms in the rate of embryonic loss as well as variation in TU skill among practitioners could result in different outcomes among farms and practitioners.

A portion of cows in the QP1 and QP2 categories misdiagnosed as pregnant were likely undergoing pregnancy loss at the time of the TU examination. Differences in these categories based on the amount of chorioallantoic fluid detected using TU may be explained by differences in the timing of embryonic death. When embryonic death (spontaneous or induced) in heifers preceded luteal regression, the conceptus fluid and embryonic tissue were retained longer in the uterus than when luteolysis was induced (Kastelic et al., 1989; Kastelic et al., 1991). This delay in expulsion of the conceptus from the uterus may have produced false positive results when using TU in the present study. The number of cows diagnosed as PL (n=11, 0.7%) was low because cows that initiated pregnancy loss before TU examination were probably categorized as either QP1 or QP2 (Table 6). Furthermore, embryonic death is usually diagnosed based on visualization of the embryo proper using TU, and cows in the present study were classified into the QP1 and QP2 categories based on visualization of chorioallantoic fluid alone. Overall, less than half (43.1 %, 295/685) of the pregnant outcomes were based on visualization of an embryo (i.e., PG) probably due to the small mass of the embryo 27 d after TAI and the time constraints for individual cow diagnoses imposed by the cow-flow on the commercial dairy. Four cows classified as PL by TU disagreed with the PAG ELISA outcome (pregnant) and 2 of these cows were found to be pregnant 5 d later based on the recheck using TU.

Sensitivity, specificity, PPV, and NPV of TU 27 d after TAI were calculated based on the assumption that the outcomes that agreed with the PAG ELISA were correct whereas the outcomes that disagreed were readjusted to the correct outcome based on the TU re-evaluation 32 d after TAI (Table 6). In this study, sensitivity ranged from 94.2 to 98.9 % and specificity ranged from 91.7 to 97.3 %. Pieterse et al. (1990) reported a sensitivity and specificity of pregnancy diagnosis using ultrasound of 44.8 and 82.3 % respectively, when conducted between 21 and 25 d after AI and 97.7 and 87.7 % respectively, when conducted between 26 and 33 d after AI. In a second field study using TU 27 d after AI to determine pregnancy status in cows, sensitivity and specificity were 93.8 and 96.2 %, respectively (Romano et al., 2006), similar to results from the present study for pregnancy outcomes after first, second, and third postpartum TAI.

Results from the present study and those of others support the notion that pregnancy outcomes based on TU before 29 d after TAI can lead to errors which may substantially reduce the benefit of early pregnancy diagnosis. From a practical perspective, although there is an advantage of the PAG ELISA over TU with regard to the false positive results at 27 d after TAI associated with embryonic loss, the 2 d delay from the time of blood collection to the establishment of pregnancy diagnosis based on the PAG ELISA has a negative impact on the reproductive management program of a dairy implementing a systematic synchronization and resynchronization program. With TU, cows treated with GnRH 7 d before pregnancy diagnosis to initiate Resynch can be

diagnosed not-pregnant and be immediately treated with $PGF_{2\alpha}$ during the same cow-handling period (Fricke et al., 2003; Sterry et al., 2006). By contrast, an additional cow-handling period is required during Resynch to collect the blood sample for the PAG ELISA at least 2 d before the scheduled $PGF_{2\alpha}$ injection. Development of an on-farm or cow-side form of this PAG assay would improve the management aspects of adopting this technology on a dairy. Furthermore, results of studies evaluating the timing of initiation of Resynch indicate that the most aggressive strategies in which Resynch is initiated 19 or 26 d after a previous TAI result in lower fertility compared to initiation of Resynch 32 or 33 d after TAI (Fricke et al., 2003; Sterry et al., 2006). Thus, both the efficacy of and the need for determining pregnancy status as early as 26 d after a previous TAI need to be questioned when deciding when to position a pregnancy diagnosis within a reproductive management strategy using a systematic synchronization and resynchronization approach.

Accuracy of the PAG ELISA 27 d after TAI

Values for sensitivity, specificity, PPV, NPV and accuracy of the PAG ELISA are summarized in Table 7. In this study sensitivity ranged from 93.5 to 96.3 % and specificity ranged from 91.7 to 96.8 %. Zoli et al. (1992) reported a similar accuracy to the present study for determining pregnancy status 35 d after AI in cows carrying transferred embryos (94.7 %, 407/430) based on a PAG RIA that was compared with rectal palpation 45 d after AI. When comparing PAG ELISA and TU, the rate of false negative results for the PAG ELISA for second postpartum TAI (6.5%) was similar to the rate of false negative results when TU 27 d after TAI was performed in cows (6.2%, Romano et al. 2006), but the rate of false negative results was lower for first and third postpartum TAI (3.7 and 5.4 %, respectively). Moreover, the rate of false positive results was greater for the PAG ELISA for first postpartum TAI than for TU in the study by Romano et al. 2006 (8.3 vs. 3.8 %), but was similar for second and third postpartum TAI (3.7 and 3.2 % respectively). Thus, both the PAG ELISA and TU have similar sensitivity and specificity 27 d after TAI.

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 TU method (93.7 to 97.8%). Results from this study support that pregnancy diagnosis using TU 27 d after TAI based on the presence of chorioallantoic fluid in the uterine horn and a CL alone leads to more false positive results than when an embryo is visualized. 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.

The Challenge of Early Pregnancy Diagnosis

Data from these two field trials illustrate the limitations of integrating early pregnancy diagnosis into a reproductive management program. First, the system with the most aggressive early nonpregnancy diagnosis and resynchronzation schedule (i.e., the D19 treatment) was not a viable management strategy based on the poor fertility after the Resynch TAI probably due to follicular and luteal dynamics at the stage post breeding that the synchronization protocol was initiated.

Furthermore, accuracy of pregnancy diagnosis was less than expected when using TU 27 d after TAI (93.7 to 97.8%), especially when pregnant outcomes were based on visualization of chorioallantoic fluid and a CL but when an embryo was not visualized. Taken together, these results suggest the counterintuitive notion that delaying pregnancy diagnosis may improve reproductive efficiency when using a hormonal protocol for timed AI to program nonpregnant cows for rebreeding due to the high rate of pregnancy loss occurring in cows diagnosed pregnant at 26 vs. 33 days post TAI. Based on current resynchronization strategies, the need for a nonpregnancy diagnosis before 32 to 39 d after TAI is questionable. Development of a new strategy for resynchronizing ovulation in cows failing to conceive may force an earlier nonpregnancy diagnosis, and such a strategy is currently being tested in a field trial.

Conclusion

Although coupling a nonpregnancy diagnosis with a management decision to quickly reinitiate AI service may improve reproductive efficiency by decreasing the interval between AI services, early pregnancy loss and the effectiveness of hormonal ovulation and estrus control protocols initiated at certain physiologic stages post breeding may limit the effectiveness of many methods for early pregnancy diagnosis currently under development, especially when compared to transrectal palpation. These limitations make the benefits of many currently available methods for early pregnancy diagnosis questionable and require that all animals diagnosed pregnant early after insemination be scheduled for rechecks at later times during gestation to identify animals experiencing pregnancy loss. It remains to be seen whether a new test will replace transrectal palpation as the primary method used for pregnancy diagnosis in dairy cattle.

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Table 1. Pregnancy loss in lactating dairy cows occurring from first early pregnancy diagnosis conducted from 27 to 30 days post breeding to a pregnancy recheck conducted 14 to 42 days later.

Number of pregnancies evaluated	Days of gestation at diagnosis		Loss interval, d	Pregnancy loss, %	Reference
	First	Second			
256	28	38-58	~ 20	28.0	Cartmill et al., 2001
195	28	42	14	17.9	Chebel et al., 2003
89	28	56	28	13.5	Fricke et al., 1998
209	26	68	42	27.8	Fricke et al., 2003
77	33	68	35	11.7	Fricke et al., 2003
139	27	45	18	20.7	Moreira et al., 2001
172	28	45	17	9.3	Santos et al., 2001
372	31	45	14	11.4	Santos et al., 2004
215	27	41	14	9.9	Santos et al., 2004
705	28	42	14	3.2	Silke et al., 2002
347	33	61	28	6.6	Sterry et al., 2006

Table 2. Pregnancy rate per artificial insemination (PR/AI) and pregnancy loss after timed artificial insemination (TAI) to Ovsynch (Fricke et al., 2003).

Item	Treatment group			Overall
	D19	D26	D33	
Interval from Ovsynch TAI to 1st pregnancy exam (d)	26	26	33	-
PR/AI at 1st pregnancy exam, % (no./no.)	46[a] (108/235)	42[a] (101/240)	33[b] (77/236)	40 (286/711)
Interval from Ovsynch TAI to 2nd pregnancy exam (d)	68	68	68	-
PR/AI at 2nd pregnancy exam, % (no./no.)	33 (78/235)	30 (73/240)	29 (68/236)	31 (219/711)
Interval between pregnancy exams (d)	42	42	35	-
Pregnancy loss, % (no./no.)	28[a] (30/108)	28[a] (28/101)	12[b] (9/77)	23 (67/286)

[a,b]Within a row, percentages with different superscripts differ ($P < 0.01$) among treatment groups.

Table 3. Pregnancy rate per artificial insemination (PR/AI) after timed artificial insemination (TAI) to Resynch beginning 19, 26, or 33 d after first TAI (Fricke et al., 2003).

	Treatment group			
Item	D19	D26	D33	Overall
Mean (± SEM) interval (d) from Resynch	27.1 ± 0.4	26.6 ± 0.2	33.7 ± 0.4	-
TAI to pregnancy exam (range)	(26 to 54)	(26 to 40)	(26 to 75)	
PR/AI, %	23[a]	34[b]	38[b]	32
(no./no.)	(28/120)	(41/121)	(54/143)	(123/384)

[a,b]Within a row, percentages with different superscripts differ ($P < 0.01$) among treatment groups.

Table 4. Category definitions used to classify pregnancy outcomes based on transrectal ultrasonography (TU) examinations conducted 27 d after timed AI (Silva et al., 2007).

TU category	Category definition
PG	Pregnant – presence of a CL; a normal amount of chorioallantoic fluid in the uterine horn ipsilateral to the ovary with a CL; embryo visualized.
QP1	Questionable Pregnant 1 – presence of a CL; a normal amount of chorioallantoic fluid in the uterine horn ipsilateral to the ovary with a CL; embryo not visualized.
QP2	Questionable Pregnant 2 – presence of a CL; abnormally less than a normal amount of chorioallantoic fluid in the uterine horn ipsilateral to the ovary with a CL, embryo not visualized.
PL	Pregnancy loss – Nonviable embryo lacking a heartbeat and organized structure.
NP	Not-Pregnant – absence of ovarian and uterine signs of pregnancy with enough confidence to administer $PGF_{2\alpha}$.

Table 5. Frequency of pregnancy outcomes based on transrectal ultrasonography (TU) categories 27 d after timed AI and the frequency of incorrect TU outcomes based on pregnancy status re-evaluation using TU 32 d after timed AI (Silva et al., 2007).

TU category	Frequency % (no./no.)	TU outcome disagreements with PAG ELISA % (no./no.)	Missed re-evaluation (n)	Outcomes re-evaluated (n)	Outcomes included (n)	[1]Overall rate of incorrect TU outcomes % (no./no.)
PG	17.4 (295/1692)	5.1 (15/295)	2	13	293	2.4[a] (7/293)
QP1	19.6 (332/1692)	17.5 (58/332)	7	51	325	9.5[b] (31/325)
QP2	3.4 (58/1692)	65.5 (38/58)	4	34	54	57.4[c] (31/54)
Total pregnant	40.5 (685/1692)	16.2 (111/685)	13	98	672	10.3 (69/672)
PL	0.7 (11/1692)	36.4 (4/11)	0	4	11	18.2[b] (2/11)
NP	58.9 (996/1692)	8.5 (85/996)	6	79	990	2.0[a] (20/990)
Total not pregnant	59.5 (1007/1692)	8.8 (89/1007)	6	83	1001	2.2 (22/1001)
Overall	100.0 (1692/1692)	11.8 (200/1692)	19	181	1673	5.4 (91/1673)

[a,b,c] Within a column, values with different superscripts differ ($P < 0.05$).

[1]Overall rate of incorrect TU outcomes was analyzed using a Chi square test in PROC FREQ of SAS. Transrectal ultrasonography was used as a gold standard to determine the accuracy of the PAG ELISA 27 d after TAI. Outcomes between

PAG ELISA and TU that agreed were considered correct. For outcomes that disagreed between TU and the PAG ELISA d 27 after TAI, cows were re-evaluated 5 d later using TU and the incorrect outcomes based on TU 27 d after TAI were determined. The total number of cows is reduced due to missed TU re-evaluations (n=19). Cows classified as PG, QP1, or QP2 were considered to be pregnant, whereas cows classified as PL or NP were considered to be not-pregnant.

Table 6. 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 7. Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and accuracy of 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.