When to Identify Nonpregnant Lactating Dairy Cows Using Transrectal Ultrasonography and Why

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Ultrasonography.jpg [1]

1. 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 someday replace transrectal palpation as the method of choice for nonpregnancy diagnosis in the dairy industry. Two events must transpire before this transition occurs. First, a technology must be developed that exceeds transrectal palpation in one or more of the attributes of the ideal early nonpregnancy 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 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 counter intuitive notion that delaying pregnancy diagnosis may improve reproductive efficiency when combining ultrasonography with 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.

2. 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 on-farm conditions, and 5) able to determine pregnancy status at the same time the test is administered. Most currently available methods for pregnancy diagnosis exhibit one or more of these attributes, but none exhibit all of them. A final attribute of an ideal nonpregnancy test would be the ability to determine pregnancy status without the need to physically handle the animal to administer the test.

3. Methods for Nonpregnancy Diagnosis in Cattle

3.1. Transrectal Palpation

Transrectal palpation of the uterus for pregnancy diagnosis in cattle was first described in the 1800's (Cowie, 1948) and, with the exception of detecting cows that return to estrus after a breeding, is the oldest method for early nonpregnancy diagnosis in dairy cattle today. 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 continue to train their students in the art of transrectal palpation for diagnosis of pregnancy in dairy cattle. 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.

3.2. 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). 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, 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 ultrasonography 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 d 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 d after estrus (Kastelic et al., 1991). For lactating dairy cows, ultrasonographic detection of uterine fluid as well as embryonic membranes from 28 to 35 d after AI was an accurate estimation of the presence of an embryo at the time of observation (Nation et al., 2003). In a field study using transrectal ultrasonography 27 d after AI to determine pregnancy status in cows, sensitivity and specificity were 93.8 and 96.2 %, respectively (Romano et al., 2006). Although ultrasound conducted at = 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).

3.3. 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).

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 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 a1 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). 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. 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). Wathes 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 PG 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 10 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. 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).

4. 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.

4.1. 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) and Presynch/Ovsynch (Moreira et al., 2001), and use of transrectal ultrasonography for early identification of nonpregnant cows (Fricke, 2002). We conducted a field trial to compare three intervals from first timed artificial insemination (TAI) to initiation of resynchronization of ovulation on a dairy incorporating transrectal ultrasonography as a method for early pregnancy diagnosis (Fricke et al., 2003). At first TAI, cows were randomly assigned to each of three treatment groups for resynchronization of ovulation (Resynch) using Ovsynch. 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.

Table 1. Pregnancies per artificial insemination (P/AI) and pregnancy loss after timed artificial insemination (TAI) to Ovsynch (Fricke et al., 2003).

Overall fertility to Ovsynch was 40% and was greater for D19 and D26 cows than for D33 cows (Table 1). 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 P/AI to Ovsynch was 31% and did not differ among treatments (Table 1). Thus, differences in P/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 P/AI to Resynch was 32% and was greater for D26 and D33 cows than for D19 cows (Table 2).

Table 2. Pregnancies per artificial insemination (P/AI) after timed artificial insemination (TAI) to Resynch beginning 19, 26, or 33 d after first TAI (Fricke et al., 2003).

In summary, the strategy incorporating 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 likely 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.

4.2. 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 (BioTracking, LLC, Moscow, ID). The objective of this field trial was to compare the accuracy of a

plasma PAG ELISA test now under development and not yet commercialized (i.e., not the BioPRYN test) to transrectal ultrasonography for determining pregnancy status of lactating dairy cows 27 d after timed AI (Silva et al., 2007). 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 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.

To determine which outcome was correct when the transrectal ultrasonography and PAG ELISA outcomes disagreed, we developed a category designation for pregnancy outcomes using transrectal ultrasonography (Table 3). The frequency distribution of pregnancy outcomes for each transrectal ultrasonography category is summarized in Table 4. The percentage of cows diagnosed pregnant based on visualization of an embryo using transrectal ultrasonography (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 the transrectal ultrasonography outcomes that disagreed with the PAG ELISA, the percentage of incorrect transrectal ultrasonography outcomes was less for not-pregnant than for pregnant outcomes (26.5%, n=83 vs. 70.4%, n= 98) mainly due to the incorrect transrectal ultrasonography outcomes classified as either QP1 or QP2. An important consideration when interpreting data from this study is that transrectal ultrasonography 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 transrectal ultrasonography skill among practitioners could result in different outcomes among farms and practitioners.

Table 3. Category definitions used to classify pregnancy outcomes based on transrectal ultrasonography (TU) examinations conducted 27 d after timed AI (Silva et al., 2007).

A portion of cows in the QP1 and QP2 categories misdiagnosed as pregnant were likely undergoing pregnancy loss at the time of the transrectal ultrasonography examination.

Differences in these categories based on the amount of chorioallantoic fluid detected using transrectal ultrasonography 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 and Ginther, 1989; Kastelic et al., 1991). This delay in expulsion of the conceptus from the uterus may have produced false positive results when using transrectal ultrasonography in the present study. 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.

Table 4. 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 reevaluation using TU 32 d after timed AI (Adapted from Silva et al., 2007).

Results from the present study and those of others support the notion that pregnancy outcomes based on transrectal ultrasonography before 29 d after TAI can lead to errors which may substantially reduce the benefit of early pregnancy diagnosis. With transrectal ultrasonography, cows treated with GnRH 7 d before pregnancy diagnosis to initiate Resynch can be diagnosed not-pregnant and be immediately treated with PGF2a during the same cowhandling 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 PGF2a 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.

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 method (93.7 to 97.8%). Results from this study support that pregnancy diagnosis using transrectal ultrasonography 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 PGF2a during the resynchronization protocol.

5. Conclusion

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 transrectal ultrasonography 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.

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