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KEY POINTS

Although coupling a nonpregnancy diagnosis with a management strategy to quickly re-initiate artificial insemination (AI) may improve reproductive efficiency by decreasing the interval between AI services, early pregnancy loss limits the accuracy of many direct and indirect methods for early pregnancy diagnosis currently under development. These limitations make the benefits of many currently available methods for early preg-nancy diagnosis questionable and require that all cows diagnosed pregnant early after insemination be scheduled for pregnancy reconfirmations at later times during gestation to identify cows experiencing pregnancy loss. Although research and development efforts are being made toward development of an in-direct pregnancy test for dairy cows, it remains to be seen whether these indirect tests will replace transrectal palpation or transrectal ultrasonography as the primary methods used for pregnancy diagnosis in dairy cows or whether veterinarians will combine these methods in a reproductive management program. Future technologies for pregnancy diagnosis in dairy cows may someday overcome cur-rent limitations of direct and indirect methods for pregnancy diagnosis, thereby improving reproductive performance.

ATTRIBUTES OF THE IDEAL PREGNANCY TEST

An ideal early pregnancy test for dairy cows would fulfill the following criteria:

1. High sensitivity (ie, correctly identify pregnant animals)
2. High specificity (ie, correctly identify nonpregnant animals)
3. Inexpensive to conduct
4. Simple to conduct under field conditions
5. Ability to determine pregnancy status at the time the test is performed

A final attribute of an ideal early pregnancy test would be the ability to determine pregnancy status without the need to physically handle the cow to conduct the test. Such a test may overcome the inherent limitations of current tests caused by preg-nancy loss and may make pregnancy diagnosis before 28 to 35 days postpartum in dairy cows an economically viable reproductive management strategy. Although all of the methods described in this article require physical handling of individual cows to administer the test, future technologies for early pregnancy diagnosis may someday realize all of these criteria.

From an economic perspective, the sensitivity of an early nonpregnancy test (ie, correct identification of pregnant cows) is more important than the specificity (ie, cor-rect identification of nonpregnant cows) based on an economic simulation.1 Inaccu-rate diagnosis of nonpregnancy (ie, false negatives), however, increases the rate of iatrogenic pregnancy loss when prostaglandin F2a (PGF2a) or one of its analogues is administered to synchronize estrus or ovulation to reduce the interval to the next arti-ficial insemination (AI) service. The economic loss incurred because of pregnancy loss depends on many factors and has been estimated to range from $462 to $300.3 Because a management intervention can only be implemented for nonpregnant cows, it is critical that a pregnancy test accurately identify nonpregnant cows to avoid iatrogenic pregnancy loss. Nonetheless, a high rate of false-positive results diminishes the usefulness and cost-effectiveness of an early pregnancy test by failing to present a management opportunity to return nonpregnant cows to AI service early after AI and potentially increasing the interval to the subsequent AI.

RETURN TO ESTRUS AS A DIAGNOSTIC INDICATOR OF PREGNANCY STATUS

Accurate identification of cows returning to estrus from 18 to 32 days after AI is the easiest and least costly method for determining nonpregnancy early after insemina-tion. 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.4 Only 51.5% of the eligible cows were detected in estrus and inseminated in a recent study in which detection of estrus was performed through continuous monitoring with activity tags after a previ-ous insemination until pregnancy diagnosis 32 3 days after AI.5 Second, estrous cy-cle duration varies widely with a high degree of variability among individual cows.6 Finally, the high rate of pregnancy loss in dairy cows can increase the interval from insemination to return to estrus for cows that establish pregnancy early then undergo pregnancy loss later during gestation.7

PREGNANCY LOSS IN LACTATING DAIRY COWS

Pregnancy loss contributes to reproductive inefficiency because fertility assessed at any point during pregnancy is a function of both conception rate and pregnancy loss.8 Pregnancy loss can be monitored using a variety of methods, including measurement of milk progesterone concentration or pregnancy-specific proteins, transrectal ultrasonography, and transrectal palpation. Since the widespread applica-tion of transrectal ultrasonography for reproductive research in cattle,[9] many studies have reported rates of pregnancy loss during early gestation under field conditions. In a summary of 14 studies,[10] pregnancy loss from 27 to 31 and 38 to 50 days of gesta-tion averaged 13% based on transrectal ultrasonography. Vasconcelos and col-leagues[11] characterized pregnancy loss at various stages of gestation using transrectal ultrasonography and reported pregnancy losses of 11% from 28 to 42 days, 6% from 42 to 56 days, and 2% from 56 to 98 days after AI (Fig. 1), support-ing that the rate of loss is greater early during gestation and then decreases as gesta-tion proceeds. It has long been accepted that pregnancy status should be determined in dairy cows as soon as possible after insemination but without having the diagnosis confounded by subsequent pregnancy loss.[12,13] Pregnancy loss diminishes the benefit of early pregnancy diagnosis. Because of the high rate of pregnancy loss that occurs around the gestational period that most direct and indirect pregnancy tests are performed, the magnitude of pregnancy loss observed is greater the earlier after breeding that a positive diagnosis is made. Thus, the earlier that pregnancy is diagnosed after insemination, the fewer nonpregnant cows are identified to which a management strategy can be implemented to reinseminate them. If left unidentified, cows diagnosed pregnant early after insemination that subsequently undergo preg-nancy loss decreases reproductive efficiency by extending the interval from calving to the insemination that results in a full-term pregnancy. To compensate for pregnancy loss, cows diagnosed pregnant early after insemina-tion must undergo one or more subsequent pregnancy examinations to identify and reinseminate cows that experience pregnancy loss. Most dairy farms conduct an early nonpregnancy diagnosis around 28 to 35 days after AI and then reconfirm pregnancies.

for cows diagnosed pregnant around 4 to 6 weeks later and around dry off to identify cows that have lost pregnancies. For many herds, particularly those with low estrus detection efficiency, pregnancy reconfirmation is critical to reinseminate cows that un-dergo pregnancy loss. Problems caused by pregnancy loss apply to all currently avail-able methods for assessing pregnancy status early after breeding and may make pregnancy testing before 25 days after insemination impractical unless pregnancy di-agnoses can be made continually and cost-effective 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.

DIRECT METHODS FOR PREGNANCY DIAGNOSIS

By definition, direct methods for early pregnancy diagnosis involve direct detection of the tissues and/or associated fluids of the conceptus either manually or via electronic instrumentation. Currently used direct methods for diagnosis of pregnancy include transrectal palpation and B-mode ultrasonography. Technical expertise, operator pro-ficiency, and the stage after insemination that the technique is performed can affect the specificity and sensitivity of the test; however, experienced bovine practitioners can achieve high sensitivity and specificity with either method.

Transrectal palpation

Transrectal palpation of the uterus for pregnancy diagnosis in cattle was first described in the 1800s[14] and is the oldest and most widely used direct method for early pregnancy diagnosis in dairy cows. Transrectal palpation of the amniotic vesicleas an aid in determining pregnancy status in dairy cows was described by Wisnickyand Cassida,[15] whereas slipping of the chorioallantoic membranes between the thumb and forefinger beginning on about 30 days in gestation was described by Zemjanis.[16]Because pregnancy in cows can be intentionally terminated by manual rupture of the amnionic vesicle,[17,18] several studies have investigated the extent of iatrogenic pregnancy loss induced by transrectal palpation. Examining pregnant cows early in gestation by transrectal palpation has been reported to increase the risk of iatrogenic pregnancy loss in some studies,[19–23] whereas other studies have reported that cows submitted for transrectal palpation had a decreased risk for pregnancy loss or that palpation had no effect on subsequent pregnancy loss.[12,24] Although controversy still exists regarding the extent of iatrogenic pregnancy loss induced by transrectal palpa- tion, other factors have a greater influence on calving rates than pregnancy examina- tion using transrectal palpation.[25]Because of its widespread use and the number of bovine practitioners trained to perform the procedure, transrectal palpation will likely remain a popular method for pregnancy diagnosis in dairy cows until newer direct or indirect methods for preg-nancy diagnosis are developed and adopted. Furthermore, because of its widespread use, high accuracy, and low cost per cow, transrectal palpation is the standard that newer direct and indirect methods for pregnancy diagnosis in dairy cows 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 extensively reviewed and described elsewhere.[8,9,26] Although early pregnancy diagnosis is among the most practical application for repro-ductive management using transrectal ultrasonography, additional informationgathered 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.[8] Recently, changes in endometrial thickness using transrectal ultrasonography near the time of AI were reported to be a good indicator of ovulation failure and pregnancy success.[27] Transrectal ultrasonography has not been implicated as a direct cause of pregnancy loss in cows,[28,29] and ultrasound is a less invasive technique for early pregnancy diagnosis than is transrectal palpation.[21,22]As a pregnancy diagnosis method, transrectal ultrasonography is accurate and rapid; the outcome of the test is known immediately at the time the test is conducted. Transrectal ultrasonography has begun to displace transrectal palpation as the direct method of choice by veterinarians for pregnancy diagnosis.[30] Because many experi-enced bovine practitioners can accurately diagnose pregnancy as early as 35 days af-ter insemination using transrectal palpation, pregnancy examination using transrectal ultrasonography 28 to 34 days after insemination only reduces the interval from insem-ination to pregnancy diagnosis by a few days. Although ultrasound conducted at 45 or more days after breeding did not increase the accuracy of pregnancy diagnosis for an experienced palpator, it may improve diagnostic accuracy of a less experienced one.[31] The rate of pregnancy loss and the efficacy of strategies to reinseminate cows at various stages after breeding also play a role in determining the advantages and disadvantages on the timing of pregnancy diagnosis and resynchronization.[32] Another potential benefit of transrectal ultrasonography over transrectal palpation is the opportunity to more accurately determine the ovarian status of cows at a nonpreg-nancy diagnosis facilitating the assignment of cows to different treatment alternatives. For example, use of an Ovsynch protocol for resynchronization of cows identified not pregnant 32 days after AI resulted in greater conception rates when cows were iden-tified with a corpus luteum (CL) compared with cows without a CL at the first gonadotropin-releasing hormone (GnRH) treatment of the protocol.[33,34]

Treatment of cows without a CL at the first GnRH treatment of an Ovsynch protocol with exog-enous progesterone (ie, a intravaginal progesterone insert) increased fertility at first as well as resynch timed AI in lactating dairy cows.[35,36] Treatment of cows with a CL of 20 mm or greater at nonpregnancy diagnosis with a PGF2a injection increased the overall proportion of cows inseminated after a detected estrus for second and subse-quent AI services.[5] Based on these data, many veterinarians now use the presence or absence of a CL at a nonpregnancy diagnosis to improve outcomes to timed AI pro-tocols used to resynchronize nonpregnant cows or to increase the proportion of cows inseminated in estrus after a previous insemination.

Problems with Early Pregnancy Diagnosis Using Transrectal Ultrasonography

Early studies in which transrectal ultrasonography was used to assess embryonic development in vivo reported that a fetal heartbeat could be visualized at around 21 days in gestation under controlled experimental conditions and using a high-quality scanner and transducer.[37] Several studies reported that pregnancy diagnosis can be rapidly and accurately diagnosed using ultrasound as early as 26 days after AI.[38,39] A recent report evaluated using transrectal ultrasonography as early as 18 to 21 days after insemination in Irish Holstein Friesian dairy cows.[40] Because of these re-ports, many bovine practitioners focused on pushing the lower limit of early pregnancy diagnosis to conduct pregnancy diagnosis using transrectal ultrasonography. Use of transrectal ultrasonography before about 30 days after insemination under field con-ditions on a commercial dairy, however, can negatively affect the accuracy of preg-nancy diagnosis outcomes.[41].

To determine the accuracy of early pregnancy diagnosis using transrectal ultraso-nography, we conducted a field trial on a commercial dairy farm milking approximately 2000 cows.[41] Pregnancy status was determined 29 days after timed AI using transrec-tal ultrasonography (Easi-scan, BCF Technology Ltd, Rochester, MN) based on the following criteria: presence or absence of a CL; presence, absence, volume, and appearance of uterine fluid typical for a 29-day conceptus; presence or absence of an embryo with a heartbeat. Cows were classified as (1) not pregnant: presence or absence of a CL, absence of uterine fluid or insufficient uterine fluid, and absence of an embryo; (2) pregnant: CL present, normal uterine fluid, and no embryo; (3) preg-nant embryo: CL present, normal uterine fluid, and at least one embryo visualized; and (4) questionable pregnant: CL present and one or more of the following: uterine fluid, insufficient uterine fluid, and either no embryo or a nonviable embryo. At 39 and 74 days after timed AI, pregnancy status was determined using transrectal palpation and pregnancy loss occurring between each pregnancy examination was calculated. Results from this experiment are shown in Table 1. Overall, 802 cows were classi-fied as not pregnant 29 days after timed AI, whereas 799 cows were classified as not pregnant 39 days after timed AI resulting in a not-pregnant misdiagnosis rate of 0.5% (4 of 802) for transrectal ultrasonography 29 days after timed AI. At 29 days after timed AI, 1116 cows were classified as either pregnant with an embryo visualized (68%), pregnant based on uterine fluid alone (29%), or questionable pregnant (3%). Among questionable pregnant cows, 69% were classified as not pregnant 39 days after timed AI and an additional 46% were classified as not pregnant 74 days after timed

AI. For cows classified pregnant 29 days after timed AI, more (P<0.01) cows diagnosed based on uterine fluid only than fluid and the presence of an embryo were classified as not pregnant using transrectal palpation 39 days after timed AI. Similarly, more (P<0.01) cows diagnosed pregnant based on uterine fluid alone than cows diagnosed pregnant based on visualization of an embryo with a heartbeat were classified as not pregnant

Table 1

Pregnancy loss by pregnancy classification for lactating Holstein cows diagnosed pregnant using ultrasonography 29 days after timed AI

Item	Pregnancy Classification[b]		
	Pregnant	Uterine Fluid	Questionable
	% (n/n)		
29 d after timed AI	68 (758 of 1116)	29 (322 of 1116)	3 (36 of 1116)
Pregnancy loss			
29–39 d	4[a] (30 of 758)	18[a] (57 of 322)	69[a] (25 of 36)
39–74 d	5[a] (39 of 728)	12[a] (32 of 265)	46[a] (5 of 11)

Total loss	9[a] (69 of 758)	28[a] (89 of 322)	83[a] (30 of 36)

[a]Within a row, proportions with different superscripts differ (P<.001).

[b]Lactating Holstein cows diagnosed pregnant were classified based on the following criteria using transrectal ultrasonography: pregnant: visualization of a CL ipsilateral to the gravid uterine horn, visualization of an amount of nonechogenic uterine fluid in accordance to stage of pregnancy, and visualization of an embryo with a heartbeat; uterine fluid: visualization of a CL ipsilateral to the gravid uterine horn, visualization of an amount of nonechogenic uterine fluid in accordance to stage of pregnancy but without visualization of the embryo; questionable: visualization of a CL ipsilateral to the gravid uterine horn with insufficient uterine fluid for the stage of pregnancy.

Adapted from Giordano JO, Fricke PM. Accuracy of pregnancy diagnosis outcomes using trans-rectal ultrasonography 29 days after artificial insemination in lactating dairy cows. J Dairy Sci 2012;95(Suppl 2):75; with permission.

using transrectal palpation 74 days after timed AI. From the initial pregnancy examina-tion at 29 days to the last examination 74 days after timed AI, more (*P*<0.01) cows diagnosed pregnant based on uterine fluid alone than cows diagnosed pregnant based on visualization of an embryo with a heartbeat were classified as not pregnant using transrectal palpation 74 days after timed AI. Cows classified pregnant based on uterine fluid alone 29 days after timed AI were 3.8 (95% confidence interval 5 2.7–5.4) times more likely to be classified as not pregnant 74 days after timed AI than cows diagnosed pregnant based on visualization of an embryo with a heartbeat. Based on these data, the authors concluded that the accuracy of pregnancy out-comes using transrectal ultrasonography increase dramatically when an embryo with a heartbeat is visualized compared with outcomes based only on the presence of a CL and the volume of uterine fluid in the absence of a visualized embryo with a heartbeat. The presence of a large proportion of cows with a CL and fluid was visual-ized in the absence of an embryo with a heartbeat is likely due to a high degree of early pregnancy loss in dairy cows. In 2 experiments, 35% to 44% of dairy cows diagnosed not pregnant 32 days after timed AI had extended luteal phases.[7,42] Based on the authors' results, early pregnancy diagnosis should not be conducted earlier than an embryo with a heartbeat can be rapidly and reliably detected in pregnant cows under on-farm conditions using transrectal ultrasonography (w30 days after AI) to reduce the negative impact of false-positive results.

INDIRECT METHODS FOR PREGNANCY DIAGNOSIS IN DAIRY COWS

Indirect methods for early pregnancy diagnosis use qualitative or quantitative mea-sures of hormones or conceptus-specific substances in maternal body fluids as indi-rect indicators of the presence of a viable pregnancy. Commercially available indirect methods for pregnancy diagnosis in dairy cows include milk progesterone tests and tests for pregnancy-associated glycoproteins (PAGs) in blood or milk.

Progesterone

Progesterone is the most biologically active progestagen in cattle and is primarily pro-duced and secreted by the corpus luteum during the estrous cycle and the placenta during pregnancy. Quantification of progesterone in blood or milk can be achieved in a laboratory using radioimmunoassay (RIA) or enzyme-linked immunosorbent assay (ELISA) methods. The biology of early pregnancy and maintenance of the CL results in distinct progesterone profiles for pregnant compared with nonpregnant cows. Lactating dairy cows were synchronized for first timed AI, and resulting progesterone profiles based on thrice weekly (Monday, Wednesday, Friday) blood sampling are shown in Fig. 2. The upper panel of Fig. 2 indicates a cow that failed to become preg-nant and had a normal luteal phase followed by a subsequent estrous cycle. By contrast, the middle panel of Fig. 2 indicates a cow that maintained pregnancy. The lower panel of Fig. 2 is representative of cows that fail to maintain a pregnancy and had an extended luteal phase. Extended luteal phases are common in dairy cows after AI. In one experiment, 35% of dairy cows diagnosed not pregnant 32 days after timed AI had extended luteal phases[42]; in another experiment, the proportion of cows with extended luteal phases was 44%.[7] Unfortunately, sequential sampling of milk or blood for determination of progesterone using RIA or ELISA methods is not practical or cost-effective for use on commercial dairy farms. Future technologies to monitor milk progesterone profiles of individual cows on a daily or even a weekly basis could revo-lutionize reproductive management strategies for dairy cows. 172

Rapid on-farm qualitative tests for assessing progesterone levels in milk were commercialized for pregnancy diagnosis in dairy cows in the 1980s,[43] and a few remain commercially available today. Manufacturers recommended these tests be conducted 18 to 24 days after insemination to determine pregnancy status. Based on the proges-terone profiles in Fig. 2, cows with low progesterone 18 to 24 days after AI would be classified as not pregnant, whereas cows with high progesterone 18 to 24 days after AI would be classified as pregnant. Although not-pregnant outcomes are highly accu-rate for identifying cows that truly are not pregnant, the accuracy of high progesterone 18 to 24 days after AI for accurately diagnosing pregnant cows is poor. This poor accu-racy is due to the biology associated with pregnancy loss that confounds early preg-nancy diagnosis using transrectal ultrasonography discussed previously. Most of these extended luteal phases may be explained by cows that establish a pregnancy early by signaling maternal recognition of pregnancy and maintenance of the CL past the normal time of luteal regression but then subsequently undergo pregnancy loss.7 Thus, although future technologies may allow for on-farm sampling of milk progester-one, the use of cow-side milk progesterone tests conducted 18 to 24 days after insem-ination should focus on identifying not-pregnant cows rather than pregnant cows.

Pregnancy-Associated Proteins

Proteins produced and secreted by the placenta early during pregnancy are obvious candidates for development of an early pregnancy test; however, proteins produced by the placenta vary widely among eutherotic mammals. For example, only the higher primates produce a chorionic gonadotropin homologous to the human protein (human chorionic gonadotropin) required for luteal support early during pregnancy, whereas only ruminant ungulates are known to produce type I interferon as an antiluteolytic hor-mone.44 Because cattle do not produce a chorionic gonadotropin, research has focused on discovery and characterization of pregnancy-specific proteins suitable for determining pregnancy status in cattle early after insemination. Some pregnancy-associated factors, such as the early conception factor, have not proven to be accurate in dairy cows.45 It is now possible to detect a viable conceptus between 15 to 22 days after AI by measuring the expression of interferon-stimulated genes in circulating white blood cells46–48; however, this method has not yet been commercialized. The most recent advance in this area has been made in the commercialization of tests for PAGs.

Pregnancy-Associated Glycoproteins

Bovine PAGs were discovered through attempts to develop indirect early pregnancy tests in dairy cows.44 In 1982, two proteins, pregnancy-specific protein (PSP) A and B, were isolated from bovine fetal membrane extracts.49 Development of a specific RIA for PSPB50 allowed for quantification of PSPB in maternal serum as an indirect method for pregnancy diagnosis and pregnancy loss in dairy cows.51,52 Molecular cloning and sequencing studies revealed that PAGs belong to a large family of inactive aspartic proteinases expressed by the placenta of domestic ruminants, including cows, ewes, and goats.53

In cattle, the PAG gene family comprises at least 22 transcribed genes as well as some variants.54 Bovine PAGs have been immunologically localized to trophoblast binucleate cells present in fetal cotyledonary villi and to a lesser extent to caruncular epithelium.55 Migration of binucleate cells from the trophectoderm to the uterine epithelium allows for exocytosis of granules containing PAG into the maternal circula-tion.56 Because cellular products of binucleate cells are released into maternal circu-lation, the ideal antigen for an indirect early pregnancy test in dairy cows would be a PAG expressed in binucleate cells around the time of implantation.[57] Mean PAGconcentrations in cattle increase from 15 to 35 days in gestation[42]; however, variation in plasma PAG levels among cows precludes PAG testing as a reliable indicator of pregnancy until about 26 to 30 days after AI.[58,59] Several experiments have evaluated the use of commercial PAG tests to determine pregnancy status in dairy cows and heifers (Table 2). The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy obtained using PAG tests are summarized based on several experiments[58,60–66] in Table 2. Few studies have compared factors associated with PAG levels in blood and milk of dairy cows early in gestation and the impact these factors may have on the accuracy of pregnancy diagnosis. The authors recently conducted an experiment to determine the factors affecting PAG levels in blood and milk of dairy cows during early gesta-tion.[66] Lactating Holstein cows (n 5 141) were synchronized to receive their first timed artificial AI. Blood and milk samples were collected 25 and 32 days after timed AI (TAI), and pregnancy status was determined 32 days after TAI using transrectal ultrasonog-raphy. Cows diagnosed pregnant with singletons (n 5 48) continued the experiment in which blood and milk samples were collected, and pregnancy status was assessed weekly using transrectal ultrasonography from 39 to 102 days after TAI. Plasma and milk samples were assayed for PAG levels using commercial ELISA kits.

To evaluate pregnancy outcomes from the plasma and milk PAG tests in cows of unknown pregnancy status, 2 by 2 contingency tables were constructed to calculate sensitivity, specificity, PPV, NPV, and accuracy of the pregnancy outcomes for the plasma and milk PAG tests 32 days after timed AI; these outcomes were compared with those based on transrectal ultrasonography 32 days after timed AI (Table 3). Sensitivity for both the plasma and milk PAG tests in the present experiment was high (100% and 98%, respectively) compared with specificity (87% and 83%, respectively). As a result, the NPV for the plasma and milk PAG tests in the present experi-ment was high (100% and 99%, respectively) compared with the PPV of both tests (84% and 79%, respectively). The overall accuracy of the plasma and milk PAG tests 32 days after timed AI was 92% and 89%, respectively. Results from this sensitivity analysis support that the accuracy of using plasma or milk PAG levels as an indicator of pregnancy status in dairy cows 32 days after AI is high, and the authors' results agree with others who have conducted similar analyses from 27 to 39 days in gestation when PAG levels in both plasma and milk are at early peak levels.[61,64,65]

The incidence of pregnancy loss in the present study for cows diagnosed with singleton pregnancies 32 days after TAI during the experiment was 13% (7 of 55), which agrees with the 13% loss reported to occur from 27 to 31 and 38 to 50 days of gestation based on transrectal ultrasonography in a summary of 14 studies.[10] For the plasma PAG ELISA, all but one cow that underwent pregnancy loss tested posi-tive, whereas all cows undergoing pregnancy loss tested positive at one or more time points for the milk PAG test. Similarly, 5 of 7 cows tested recheck based on the plasma PAG test before the loss occurred compared with 3 of 7 cows based on the milk PAG test. Thus, PAG levels detected by these ELISA tests in the present study have a half-life in maternal circulation resulting in a 7- to 14-day delay in identification of cows undergoing pregnancy loss based on plasma or milk PAG levels compared with transrectal ultrasonography. Profiles of PAG in plasma and milk of cows that maintained pregnancy from 25 to 102 days in gestation are shown in Fig. 3. Compared with transrectal ultrasonography, accuracy was 92% for the plasma PAG test and 89% for the milk PAG test 32 days after timed AI. Factors associated with PAG levels in dairy cows included stage of gestation, parity, pregnancy loss, and milk production. Based on plasma and milk PAG profiles, the optimal time to conduct a first pregnancy diagnosis is around 32 days after AI coin-ciding with an early peak in PAG levels. The authors concluded that because of the

Adapted from Ricci A, Carvalho PD, Amundson MC, et al. 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 2015;98:2502–14; with permission

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 2015;98:2502–14; with permission.) occurrence of pregnancy loss, all pregnant cows should be retested 74 days after AI or later when plasma and milk PAG levels in pregnant cows have rebounded from their nadir.

FUTURE TECHNOLOGIES FOR PREGNANCY DIAGNOSIS

A novel approach to the problem of early pregnancy diagnosis in dairy cows would be to monitor a pregnancy-specific substance or hormone secreted in milk in sufficient quantities to be detected by an in-line milk-sensing device during normal milking pe-riods on a dairy. Obviously, this pregnancy-specific substance must first be discov-ered or a known marker must be used and the in-line milk sampling technology developed to accurately detect and monitor this substance. If sensitive and specific, such a system would have a minimal marginal cost per test once the initial capital outlay was made to install the equipment on the dairy. By using such a system, a preg-nancy diagnosis would be conducted during each milking period for all lactating cows on a dairy so that nonpregnancy, pregnancy, and pregnancy loss could be continually monitored and tracked on a daily basis. Integration of this information into a comput-erized dairy management software system would allow dairy managers to review the pregnancy status of individual cows in the herd on a daily or weekly basis so that reproductive management strategies could be implemented to establish, maintain, or attempt to reinitiate a pregnancy. Finally, such a system would achieve the heretofore-unrealized characteristic of conducting the pregnancy test without having to handle the cow to administer the test. Limitations imposed by pregnancy loss dur-ing early gestation will not be overcome until such a system is developed

SUMMARY

Although coupling a nonpregnancy diagnosis with a management strategy to quickly reinitiate AI may improve reproductive efficiency by decreasing the interval between AI services, early pregnancy loss limits the accuracy of many direct and indirect methods for early pregnancy diagnosis currently under development. These limitations make the benefits of many currently available methods for early pregnancy diagnosis questionable and require that all cows diagnosed pregnant early after insemination be scheduled for pregnancy reconfirmations at later times during gestation to identify cows experiencing pregnancy loss. Although much research and development efforts are being made toward development of an indirect pregnancy test for dairy cows, it remains to be seen whether these indirect tests will replace transrectal palpation or transrectal ultrasonography as the primary method used for pregnancy diagnosis in dairy cows or whether veterinarians will combine these methods in a reproductive management program. Future technologies for pregnancy diagnosis in dairy cows may someday overcome current limitations of direct and indirect methods for preg-nancy diagnosis, thereby improving reproductive performance.

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2.5

Plasma PAG

Milk

PAG

2.0

PA G (S -N)

1.5

1.0

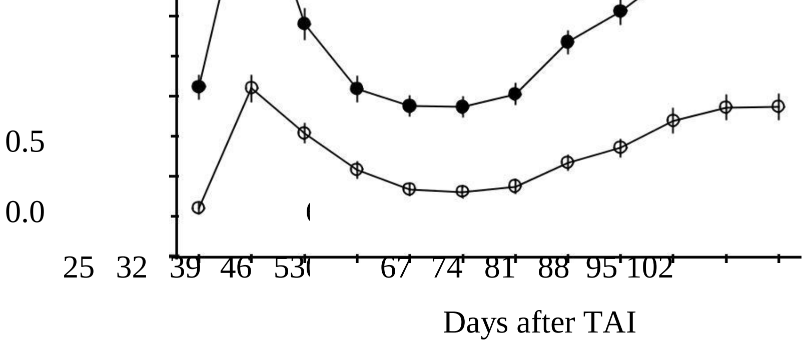


Fig. 3. Plasma and milk PAG profiles for Holstein dairy cows (n 5 48) that maintained preg-nancy from 25 to 102 days after AI. ELISA outcomes were calculated from the optical density (OD) of the sample (corrected by subtraction of the reference wavelength OD of the sample [S] minus the OD of the negative control [N] at 450 nm with both values corrected by subtrac-tion of the reference wavelength OD of the negative control), which resulted in an S-N value. Plasma and milk PAG levels were affected by week after AI (P<.01). (Adapted from Ricci A, Car-valho PD, Amundson MC, et al.)

Table 3

Sensitivity, specificity, PPV, NPV, and accuracy of plasma and milk PAG ELISA tests for determination of pregnancy status 32 days after AI

PAG ELISA	PPV[a] % (No./No.)	NPV[b] % (No./No.)	Sensitivity[c] % (No./No.)	Specificity[d] % (No./No.)	Accuracy[e] % (No./No.)
Plasma	84 (57 of 68)	100 (73 of 73)	100 (57 of 57)	87 (73 of 84)	92 (130 of 141)
Milk	79 (52 of 66)	99 (68 of 69)	98 (52 of 53)	83 (68 of 82)	89 (120 of 135)

a Proportion of cows diagnosed pregnant using the PAG ELISA that truly were pregnant.

b Proportion of cows diagnosed as not pregnant using the PAG ELISA that truly were not pregnant.

c Proportion of pregnant cows with a positive PAG ELISA outcome.

d Proportion of not-pregnant cows with a negative PAG ELISA outcome.

eProportion of pregnancy status outcomes, pregnant and not pregnant, that were correctly clas-sified by the PAG ELISA.

Table 2

Sensitivity, specificity, PPV, NPV, and accuracy for RIA and ELISA PAG test results

Reference	Days After AI	Test	Sensitivity[a]	Specificity[b]	PPV[c]	NPV[d]	Accuracy[e]
Zoli et al,[58] 1992	22–30	RIA (blood PAG)	98.8	87.5	93.0	97.9	94.5
Szenci et al,[60] 1998	26–58	RIA (PSPB)	75–100	85–92	81–91	80–100	80–96
		RIA (blood PAG)	81–100	57–71	62–74	78–100	69–84
Silva et	27	ELISA	94–96	92–97	90–	97–98	94–96

al,[61] 2007		(blood PAG)			98		
Romano & Larson,[62] 2010	28–35	ELISA (PSPB)	94–97	94–96	92–95	95–98	95–96
Piechotta et al,[63] 2011	26–58	ELISA (PSPB)	98.0	97.1	99.3	91.9	97.8
		ELISA (blood PAG)	97.8	91.2	97.8	91.2	96.4
Sinedino et al,[64] 2014	28	ELISA (blood PAG)	95	89	90	95	92
Lawson et al,[65] 2014	30–95	ELISA (milk PAG)	98–100	98–100	99–100	83–100	98–100
Ricci et al,[66] 2015	32	ELISA (blood PAG)	100	87	84	100	92
		ELISA (milk PAG)	98	83	79	99	89

Abbreviations: NPV, negative predictive value; PAG, pregnancy-associated glycoprotein; PPV, pos-itive predictive value; PSPB, pregnancy specific protein B.
[a]Proportion of serum samples from pregnant cows with a positive PAG test result.
[b]Proportion of serum samples from nonpregnant cows with a negative PAG test result.
[c]Probability that a positive PAG test result is from a pregnant cow.
[d]Probability that a negative PAG test result is from a nonpregnant cow.
[e]Probability of correctly identifying pregnancy status.

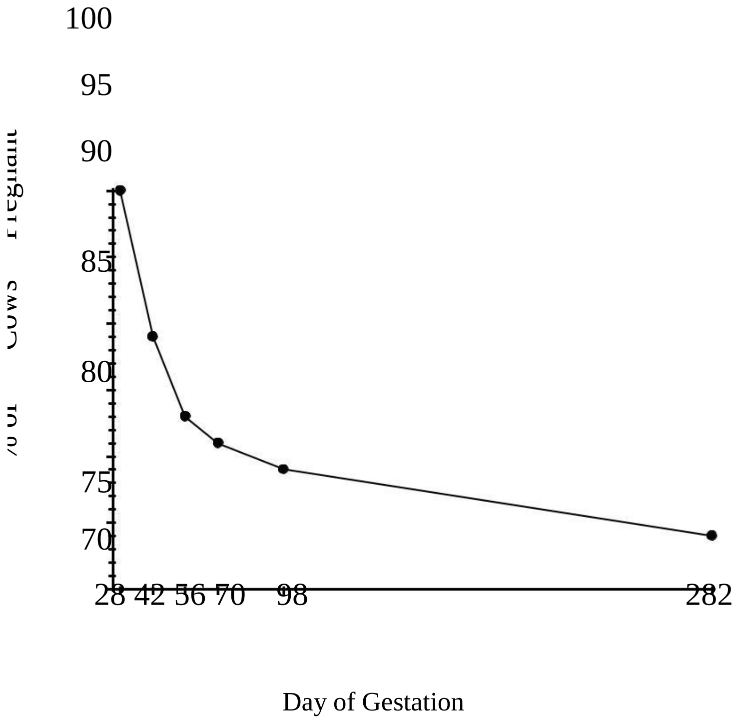
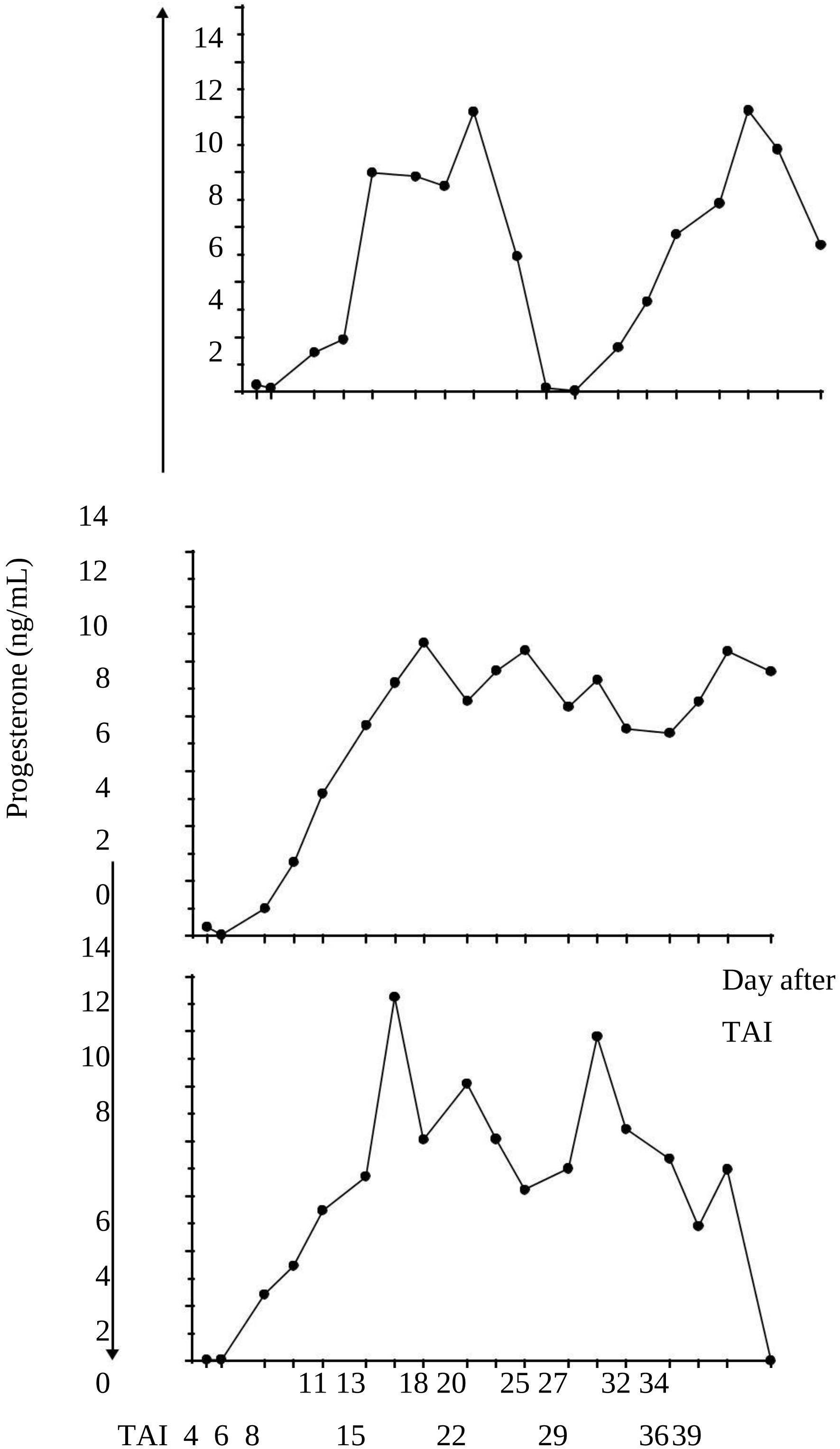


Fig. 1. Pregnancy loss in lactating Holstein cows assessed using transrectal ultrasonography from 28 days after AI to calving. (Adapted from Vasconcelos JLM, Silcox RW, Lacerda JA, et al. Pregnancy rate, pregnancy loss, and response to heat stress after AI at two different times from ovulation in dairy cows [abstract]. Biol Reprod 1997;56(Suppl 1):140; with permission.



Progesterone (ng/mL)
14
12
10
8
6
4
2
0
Day after
TAI
11 13 18 20 25 27 32 34
TAI 4 6 8 15 22 29 36 39

Fig. 2. Representative progesterone profiles from blood samples collected thrice weekly (Monday, Wednesday, Friday) from Holstein dairy cows after synchronization of ovulation and timed AI. Upper panel: a cow that failed to become pregnant after timed AI and had a normal luteal phase; middle panel: a cow that became pregnant after timed AI; lower panel: a cow that failed to become pregnant after timed AI and had an extended luteal phase.