Strategies for Nonpregnancy Diagnosis

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Introduction

Identification of nonpregnant dairy cows early after AI improves reproductive efficiency and the 21-day pregnancy rate by decreasing the interval between AI services thereby increasing the AI service rate (Fricke, 2002). Thus, new technologies to identify nonpregnant dairy cows early after AI may play a key role in management strategies to improve reproductive efficiency and profitability on dairy farms. Assays for detecting pregnancy-associated glycoprotein (**PAG**) levels in maternal circulation originating from mononucleated and binucleated cells of the embryonic trophoblast have been developed and commercialized to determine pregnancy status in cattle (Sasser et al., 1986; Zoli et al., 1992; Green et al., 2000).

Pregnancy-associated glycoproteins belong to a large family of inactive aspartic proteinases expressed by the placenta of domestic ruminants including cows, ewes, and goats (Haugejorden et al., 2006). In cattle, the PAG gene family comprises at least 22 transcribed genes as well as some variants (Prakash et al., 2009). Mean PAG concentrations in cattle increase from 15 to 35 d in gestation; however, variation in plasma PAG levels among cows precludes PAG testing as a reliable indicator of pregnancy until about 26 to 30 d after AI (Zoli et al., 1992; Humblot, 2001). Assessment of pregnancy status through detection of placental PAG levels in maternal blood (Sasser et al.,1986; Zoli et al 1992; Green et al 2005) is now used to evaluate pregnancy status within the context of a reproductive management scheme on commercial dairies (Silva et al., 2007, 2009; Sinedino et al., 2014). A commercial test for detecting PAG levels in milk (The IDEXX Milk Pregnancy Test, IDEXX Laboratories, Westbrook, ME) has been developed and marketed to the dairy industry and is now being assessed in field trials (LeBlanc, 2013).

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. This paper overviews results from an experiment conducted to assess factors associated with PAG levels in plasma and milk during early gestation in Holstein cows and to determine the accuracy of pregnancy outcomes based on PAG levels in plasma and milk compared to pregnancy outcomes based on transrectal ultrasonography (Ricci et al., 2015).

Factors affecting PAGs in Blood and Milk

Lactating Holstein cows (n = 141) were synchronized for first timed AI using a Double Ovsynch protocol (Souza et al., 2008). Pregnancy diagnosis was initially performed 32 d after timed AI for all cows using transrectal ultrasonography. Pregnant cows diagnosed with singletons (n = 48) based on transrectal ultrasonography 32 d after timed AI continued the experiment in which pregnancy status was assessed weekly using transrectal ultrasonography from 39 to 102 d after timed AI. Blood and milk samples were collected weekly from 25 to 102 d after timed AI. From 32 to 102 d after timed AI, blood and milk samples were collected from cows on the same day that pregnancy status was assessed using transrectal ultrasonography once a week.

After completion of sample collection at the end of the experiment, frozen plasma samples were shipped overnight in a cooled container by courier from the University of Wisconsin to IDEXX laboratories for analysis of plasma PAG levels using a commercial ELISA kit (the IDEXX Bovine Pregnancy Test, IDEXX Laboratories, Westbrook, ME). Milk samples were delivered weekly to AgSource headquarters (Verona, WI) on the day of collection throughout the experiment and then to AgSource Laboratories (Menomonie, WI) for analysis of milk PAG levels using a commercial ELISA kit (The IDEXX Milk Pregnancy Test, IDEXX Laboratories, Westbrook, ME). Results 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

subtraction of the reference wavelength OD of the negative control), which resulted in an S-N value. Each microplate included negative and positive controls.

Pregnancy outcomes were determined based on cutoff values determined by the PAG ELISA manufacturer. For the plasma PAG ELISA, when the S-N value was < 0.300, the cow was classified "not pregnant"; when the S-N value was > 0.300 to < 1.000, the cow was classified "recheck"; and when the S-N value was ≥ 1.000, the cow was classified "pregnant." For the milk PAG ELISA, when the S-N value was < 0.100, the cow was classified "not pregnant"; when the S-N value was > 0.100 to < 0.250, the cow was classified as "recheck"; and when the S-N value was ≥ 0.250, the cow was classified "pregnant."

Plasma and Milk PAG Profiles

Overall, the weekly PAG profile in both plasma and milk from 25 to 102 d after timed AI for pregnant cows was similar (Figure 1); however, plasma PAG levels were approximately 2-fold greater compared to milk PAG levels. Temporal PAG profiles from the present study are similar to other studies reporting PAG profiles in serum. In the first study to evaluate PAG-1 concentrations throughout gestation in Holstein cows (Sasser et al., 1986), serum PAG-1 concentrations were detectable in some but not all cows 15 d after AI, increased to about 40 d after AI and stayed constant until about 70 d, then steadily increased until the end of gestation. A study that evaluated the same commercial PAG ELISA test kits evaluated in the present experiment reported similar relative PAG profiles (S-N values) in both plasma and milk (Lawson et al., 2014).

Plasma and milk PAG levels were affected by both week after TAI and parity (Figure 1). When all cows that maintained pregnancy from 25 to 102 d after TAI were analyzed, plasma and milk PAG levels increased from 25 d after TAI to an early peak 32 d after TAI. Plasma and milk PAG levels then decreased from 32 d after TAI to a nadir from 53 to 60 d after TAI for the plasma PAG ELISA and from 46 to 67 d after TAI for the milk PAG ELISA followed by a gradual increase in PAG levels from 74 to 102 d after TAI. Primiparous cows had greater plasma and milk PAG levels compared to multiparous cows.
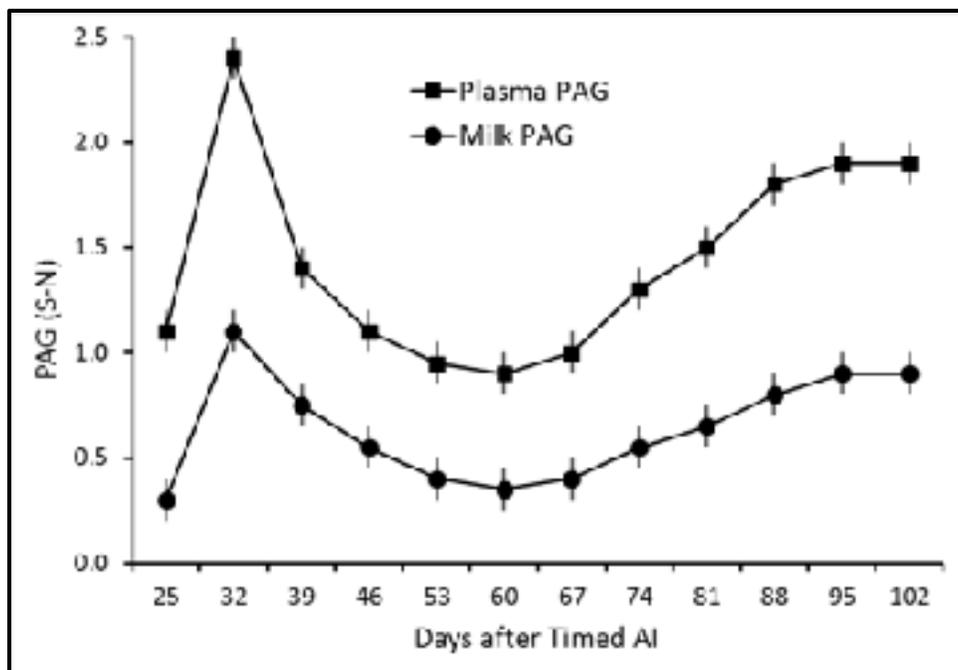

Figure 1. Plasma and milk pregnancy-associated glycoprotein (PAG) profiles for Holstein cows (n = 48) that maintained pregnancy from 25 to 102 d after AI. ELISA outcomes were calculated from the optical density (OD) of the sample (corrected by subtraction of the reference wavelength

OD of the sample (S) minus the OD of the negative control (N) at 450 nm with both values corrected by subtraction 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 < 0.01). Adapted from Ricci et al., 2015.

Accuracy of Pregnancy Outcomes 32 d after TAI

To evaluate pregnancy outcomes from the plasma and milk PAG ELISA tests in cows of unknown pregnancy status, 2 × 2 contingency tables were constructed to calculate sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy of the pregnancy outcomes for the plasma and milk PAG ELISA tests 32 d after TAI, and these outcomes were compared to those based on transrectal ultrasonography 32 d after TAI (Table 1).

Sensitivity for both the plasma and milk PAG ELISA tests in the present experiment was high (100% and 98%, respectively), compared to specificity (87% and 83%, respectively). As a result, the NPV for the plasma and milk PAG ELISA tests in the present experiment was high (100% and 99%, respectively) compared to the PPV of both tests (84% and 79%, respectively). The overall accuracy of the plasma and milk PAG ELISA tests 32 d after TAI 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 d after AI is high, and our results agree with others who have conducted similar analyses from 27 to 39 d in gestation when PAG levels in both plasma and milk are at early peak levels (Silva et al., 2007; Lawson et al., 2014; Sinedino et al., 2014).

Table 1. Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and accuracy of plasma and milk pregnancy-associated glycoprotein (PAG) ELISA tests for determination of pregnancy status 32 d after AI. Adapted from Ricci et al., 2005.

PAG ELISA	PPV[1] % (no./no.)	NPV[2] % (no./no.)	Sensitivity[3] % (no./no.)	Specificity[4] % (no./no.)	Accuracy[5] % (no./no.)
Plasma	84 (57/68)	100 (73/73)	100 (57/57)	87 (73/84)	92 (130/141)
Milk	79 (52/66)	99 (68/69)	98 (52/53)	83 (68/82)	89 (120/135)

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

From an economic perspective, the sensitivity of an early nonpregnancy test (i.e., correct identification of pregnant cows) is more important than the specificity (i.e., correct identification of nonpregnant cows) based on two economic simulations (Galligan, 2011; Giordano et al., 2013). Further, to obtain a positive economic value for an early chemical nonpregnancy test, the sensitivity had to be greater than 96% when the test is used 31 d and greater than 94% when used 24 d after AI (Giordano et al., 2013). The sensitivity of both the plasma and the milk PAG ELISA tests evaluated in the present study (Table 1) as well as the sensitivity reported by others (Silva et al., 2007; Romano and Larson, 2010) exceed those criteria and support that use of these commercial tests to diagnose pregnancy status 32 d after AI would economically benefit a dairy farm.

Results from the present study support use of plasma PAG testing around 32 d after TAI and milk PAG testing 32 to 39 d after TAI when PAG levels in pregnant cows are at an early peak and pregnancy outcomes for pregnant cows approach 100% accuracy. By contrast, the advantages of the plasma and milk PAG ELISA tests are diminished when conducted during the temporal nadir in plasma and milk PAG levels from 46 to 74 d after TAI due to an increase in pregnant cows with outcomes of not pregnant or recheck. Pregnant cows incorrectly diagnosed not pregnant ultimately may undergo iatrogenic pregnancy loss if they continue the resynchronization protocol and are treated with $PGF_{2\alpha}$ thereby resulting in an economic loss (Galligan, 2009; Giordano et al., 2013).

Accuracy of Pregnancy Outcomes during the First Trimester of Gestation

To determine the accuracy of plasma and milk PAG ELISA outcomes during the first trimester of gestation, pregnancy outcomes from cows that maintained a singleton pregnancy from 25 to 102 d after TAI (n = 48) were analyzed. Cows diagnosed pregnant 32 d after TAI based on transrectal ultrasonography continued the experiment in which pregnancy outcomes based on PAG levels in plasma and milk were classified based on cutoff levels specified by the manufacturer. Overall, pregnancy outcomes for all pregnant cows based on both plasma and milk PAG ELISA tests were a reflection of PAG levels in plasma and milk (Figure 1). Plasma and milk PAG ELISA outcomes of "not pregnant" and "recheck" occurred 25 d after TAI for pregnant cows. Plasma PAG ELISA outcomes for pregnant cows, however, were 100% pregnant 32 d after TAI, whereas the milk PAG ELISA exceeded 98% pregnant outcomes 32 d and 39 d after TAI. Plasma and milk PAG ELISA outcomes of "not pregnant" and "recheck" increased concomitant to the temporal decrease in plasma and milk PAG levels during the nadir and then decreased as plasma and milk PAG levels increased as gestation ensued.

In a study to assess aggressive early nonpregnancy diagnosis with a strategy for resynchronization of ovulation, pregnancy status of cows initiating the first GnRH injection of an Ovsynch protocol 25 d after TAI was determined 27 d after TAI by using a PAG ELISA test (Silva et al., 2009). Cows diagnosed not pregnant continued the Resynch protocol by receiving an injection of $PGF_{2\alpha}$ 7 d after the initial GnRH injection and a second GnRH injection 54 h after the $PGF_{2\alpha}$ injection. Cows received TAI approximately 16 h after the second GnRH injection 35 d after AI. The authors concluded that earlier detection of nonpregnant cows using the PAG ELISA in conjunction with a protocol for resynchronization of ovulation and TAI increased the rate at which cows became pregnant in a dairy herd compared with transrectal ultrasonography conducted at a later stage after TAI. This agrees with an economic simulation of use of chemical tests for identification of nonpregnant cows early after AI in conjunction with a protocol for resynchronization of ovulation and TAI which concluded that the major economic advantage of using a chemical test was to decrease the interbreeding interval (Giordano et al., 2013).

Pregnancy Loss

The incidence of pregnancy loss in the present study for cows diagnosed with singleton pregnancies 32 d after TAI during the experiment was 13% (7/55) which agrees with the 13% loss reported to occur from 27 to 31 and 38 to 50 d of gestation based on transrectal ultrasonography in a summary of 14 studies (Santos et al., 2004). For the plasma PAG ELISA, all but one cow that underwent pregnancy loss tested positive, 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 to 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 d delay in identification of cows undergoing pregnancy loss based on plasma or milk PAG levels compared to transrectal ultrasonography. Because PAG levels are high during late gestation, it takes up to 60 d for residual PAG to be cleared from maternal circulation after parturition in cows (Sasser et al., 1986; Zoli et al., 1992) and other ruminants (Haugejorden et al., 2006). Because of the PAG half-life in circulation, cows submitted for a pregnancy diagnosis before 60 d postpartum can test positive due to residual PAG levels from the previous pregnancy (Giordano et al., 2012), and the manufacturer of the plasma and milk PAG ELISA tests evaluated in this experiment recommends that cows be > 60 d after parturition when tested.

Based on serum samples assayed using the same PAG ELISA test evaluated in the present experiment to determine how rapidly PAG concentrations decrease after an induced pregnancy loss in dairy cows at 39 d in gestation (Giordano et al., 2012), approximately 5 to 7 d elapsed before PAG levels returned to basal levels when luteal regression was induced with PGF2α or when the embryo died. Thus, most cows undergoing pregnancy loss will test pregnant or recheck at an early pregnancy diagnosis conducted using either the plasma or the milk PAG ELISA test. Because it is impossible to distinguish between the pregnancy outcomes of cows undergoing pregnancy loss and those of pregnant cows that test as "recheck" or "not pregnant" during the temporal PAG nadir, it is important that all cows with "pregnant" or "recheck" outcomes at an early test be retested at a later time. Based on temporal PAG profiles in the present study, the best time to conduct a first pregnancy test is around 32 d after TAI with all pregnant cows submitted for a pregnancy recheck 74 d after AI or later when PAG levels in plasma and milk of pregnant cows are rebounding from their nadir.

Effect of Milk Production on Plasma and Milk PAG Levels

Plasma PAG levels in pregnant cows were negatively correlated with milk production for both primiparous (P = 0.002; R^2 = 0.05) and multiparous (P < 0.01; R^2 = 0.18) cows. Similarly, milk PAG levels in pregnant cows were negatively correlated with milk production for both primiparous (P < 0.01; R^2 = 0.14) and multiparous (P < 0.01; R^2 = 0.23) cows. López-Gatius et al (2007) first reported a negative association between plasma PAG levels and milk production in dairy cows. Because relative PAG concentrations decreased in both plasma and milk with increasing milk production, the negative association between PAG levels and milk production is not a result of dilution of PAG levels in milk with increasing production. One possible explanation not tested in this experiment is that PAG production by the conceptus decreases with increasing milk production. If PAG production by the conceptus is a proxy for embryonic growth and development during early pregnancy, the decrease in plasma and milk PAG levels with increasing milk production might suggest that cows with greater milk production may have had slower growing embryos during early development. Further experiments are needed to fully understand the relationship between increased milk production and decreased PAG levels in plasma and milk and what, if any, implications this may have on the health of the developing embryo.

Which pregnancy test is Better - Blood or Milk?

Based on the sensitivity analysis in this experiment (Table 1), both the plasma and milk PAG ELISA tests are accurate for pregnancy diagnosis when conducted 32 d after AI based on the temporal plasma and milk PAG profiles (Figure 1). Further, several economic analyses support the use of early nonpregnancy tests for improving reproduction within a dairy herd (Galligan et al., 2009; Giordano et al., 2013). Thus, the choice of whether to use the blood or the milk test to diagnose pregnancy is determined by the availability of the test, and the ability to collect the samples.

From a practical perspective, neither the plasma nor the milk PAG tests are cow-side or on-farm tests. Cows must be identified and restrained to collect a blood or a milk sample, and the samples must be sent to an off-farm laboratory that can run the ELISA test. Within several days and after receiving the pregnancy outcome, cows diagnosed not pregnant must again be identified and restrained to submit them to a strategy for rapidly returning them to AI. This is best achieved as part of an aggressive resynchronization strategy for nonpregnant cows as we have described in a number of experiments (Fricke et al., 2003; Sterry et al., 2006; Silva et al., 2009; Bilby et al., 2013; Lopes et al., 2013). It is important to note that no matter what method of pregnancy testing you use (i.e., transrectal palpation, transrectal ultrasonography, or chemical testing) that there are three possible outcomes: 1) pregnant; 2) not pregnant; and 3) recheck. For the plasma and milk PAG tests evaluated in this experiment, the proportion of recheck outcomes is highly dependent on when after AI blood or milk samples are collected (Figure 1); however, a few cows will test recheck even at 32 d after AI due to the occurrence of pregnancy loss and the variation in PAG levels among pregnant cows.

Depending on the farm, milk samples may be easier to collect than blood samples. The only commercially available milk PAG ELISA (IDEXX Laboratories, Westbrook, ME) is marketed through regional DHIA testing centers throughout the United States making the test widely accessible to most farms. A pregnancy diagnosis can be easily conducted on the same milk samples sent for DHIA testing

on a monthly basis; however, monthly pregnancy examinations are not frequent enough to drive the reproductive program on a dairy farm. This makes it necessary to conduct additional tests on a weekly or bi-weekly basis. By contrast, many farms can easily collect blood samples, and three commercial blood pregnancy tests are available in North America (BioPRYN, BioTracking, LLC, Moscow, ID; DG29, Conception Animal Reproduction Technologies, Beaumont, QC; IDEXX Bovine Pregnancy Test, IDEXX Laboratories, Inc, Westbrook, ME). The blood ELISA tests are run in regional laboratories located around North America and should be accessible to most farms. Care should be taken, however, to make sure samples are labeled correctly.

Conclusions

The experiment described herein (Ricci et al., 2015) is one of the first studies to directly compare factors associated with plasma and milk PAG levels during the first trimester of gestation in Holstein cows. Stage of gestation, parity, pregnancy loss, and milk production were associated with relative PAG levels in both plasma and milk in a similar manner; however, milk PAG levels were about 2-fold lower than plasma PAG levels. Based on PAG profiles in plasma and milk samples collected weekly, the optimal time to conduct a first pregnancy diagnosis is around 32 d after TAI when plasma and milk PAG levels are at an early peak, whereas conducting either the plasma or milk PAG test during the temporal nadir in plasma and milk PAG levels would result in poor overall accuracy. Because of the occurrence of pregnancy loss, all pregnant cows should be submitted for a pregnancy recheck 74 d or later after AI when relative PAG levels in plasma and milk of pregnant cows have rebounded from their nadir.

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