Past, present and future of pregnancy detection methods

K. G. Pohler, G. A. Franco, S.T. Reese, F. G. Dantas, M. D. Ellis, and R. R. Payton
Department of Animal Science, University of Tennessee, Knoxville, TN

Introduction

Pregnancy diagnosis is an important part of reproductive management in productive and efficient cattle operations. The oldest, simplest and most definitive method of pregnancy diagnosis is to wait until the cow gives birth. At some hobby farms and other small operations, where biotechnologies are not often adopted, this is still done. With the move towards more efficient farms and inclusion of artificial insemination (AI) and other reproductive technologies in cattle production, abstaining from pregnancy diagnosis may no longer be economically viable or practical anymore. Keeping a non-pregnant cow on the farm for an entire year has negative economic implications because she accrues the same cost of a pregnant cow without producing anything. Establishing a pregnancy diagnosis program on the farm detects cows that are not pregnant, the "problem animals," and allows the producer to make management decisions to increase reproduction efficiency.

The ideal pregnancy test should have high sensitivity (i.e., correctly identify pregnant animals), high specificity (i.e., correctly identify non pregnant animals), and be simple and inexpensive to conduct under field conditions. Tests that provide additional information, such as embryonic or fetal viability and sex of the fetus, are increasingly beneficial. There are two categories of pregnancy detection tests: direct and indirect. Direct methods allow the pregnancy to be felt or seen while indirect methods rely on markers that indicate the presence of a pregnancy. Rectal palpation and ultrasonography are the most widely used, direct methods for early pregnancy diagnoses. Both techniques are efficient but require a skilled technician, specific instruments and provide static information on pregnancy status at the specific moment of diagnosis. In some areas, veterinary support is limited and alternative approaches of pregnancy detection are needed. Recent research has aimed to develop indirect methods of pregnancy diagnoses that use qualitative or quantitative measures of hormones or conceptus-specific markers in maternal body fluids as indicators of a viable pregnancy. A series of molecules/markers associated with pregnancy in cattle including early pregnancy factor (EPF), interferon-stimulated genes (ISGs), progesterone, and pregnancy-associated glycoproteins (PAGs) have been studied for the specific use of early pregnancy diagnosis in cattle.

In the past: The foundation of pregnancy diagnosis

Estrus detection

One of the simplest methods of pregnancy detection is observation of estrus after AI or natural service. Around 15 to 17 days after ovulation, bovine embryos signal their presence to the maternal environment, suspending the maternal estrous cycle, maintaining the corpus luteum and production of progesterone. Thus, if a cow does not return to estrus 18 to 24 days after breeding, it is suggested that conception has occurred (Zemjanis, 1969). Although observation is a simple method that does not require specialized skill or instruments, there are many factors that limit its accuracy. For example, undernutrition or lactation are both common causes of beef cows being anestrus (failure to cycle). Producers may observe only a few cows return to estrus after the first few weeks of the breeding season and may be misled to believe cows have become pregnant when they are, in fact, not cycling. If utilizing the observation method for pregnancy detection it is important to be properly trained to recognize estrus behavior, there are some technologies available on the market, such as heat patches, paints or chalks, that can help visually mark cows that were mounted, the principal signal of estrus behavior.

Rectal palpation

The desire for an accurate pregnancy diagnosis led to the routine use of rectal palpation of the uterine contents. Although traditionally examined no earlier than 40 to 60 days of gestation, depending upon the skill of the examiner and the age and size of the dam, pregnancy diagnosis can be performed as early as day 30 and can be utilized thereafter until term. Due to a number of changes in the size, texture, location, and content of the uterus during pregnancy, the examiner must find at least one of these four signs before declaring the cow pregnant: palpation of the amniotic vesicle, the fetal membrane slip, placentomes (cotyledons and caruncles) or the fetus (Zemjanis, 1962). Rectal palpation also allows for estimates of the embryonic/fetal age and detection of ovarian structures such as a corpus luteum or follicle.

Conflicting evidence has been published regarding the effect of rectal palpation for pregnancy diagnosis on the embryo/fetus. Some authors suggest that it had little or no effect on pregnancy loss (Thurmond and Picanso, 1993; Thompson et al., 1994; Alexander et al., 1995; Romano et al., 2007; Romano et al., 2016) while other reports suggest that rectal palpation during early gestation increased pregnancy loss (Paisley, 1978; Franco et al., 1987). In light of the information currently available, it seems reasonable to conclude that the incidence of fetal death due to manipulation of the uterus is low and the value of the information gained is greater than the risk of fetal loss (Abbitt et al., 1978; Paisley, 1978; Vaillancourt et al., 1979; Alexander et al., 1995). However, clinicians and producers must be aware of potential negative effects associated with this method on early pregnancies and conduct examinations meticulously, cautiously, and with awareness.

Current direct pregnancy detection methods

Ultrasonography

The adoption of transrectal real time ultrasonography represents a significant development in the study of bovine reproduction. It is a valuable tool for production, research, field veterinarians and producers that permits detection of pregnancy as early as 25 days of gestation. Pregnancy status, fetal sex, ovarian function and uterine morphology can all be assessed with the use of ultrasound. Fetal heartbeat can be detected at approximately day 21 in cattle (Kastelic et al., 1988) but is more easily detected after day 25 of pregnancy and is the "gold standard" for proof of presence of a viable conceptus along with uterine morphological examinations and embryo measurements (crown rump length). Death of the embryo can be clearly diagnosed by no detectable heartbeat, membrane detachment/disorganization, reduced amount of intrauterine fluid or echoic floating structures, including remnants of the conceptus (Lopez-Gatius F.; Garcia-Ispierto, 2010). The disadvantage of ultrasound is that accuracy is limited before 28 to 30 days of gestation and pregnancy status is only guaranteed at the time of diagnosis, different from some chemical tests (i.e., PAGs) that have been studied as possible predictors of pregnancy maintenance.

Doppler ultrasonography

The incorporation of new ultrasound technologies, such as Doppler ultrasound, enable a more detailed assessment of the uterus, ovarian follicles, and corpus luteum. Color-flow mode (CFM) permits visualization of blood flow within tissues and structures based on the principles of the Doppler effect (Singh et al., 2003; Ginther, 2007; Matsui and Miyamoto, 2009) and indirectly enables inferences to be made on the functional status of the tissue (Herzog et al., 2010).

The establishment and maintenance of pregnancy in cattle is dependent on the presence of a functional, active corpus luteum (CL) and the production of a sufficient level of progesterone (P4; (Mann, 1999; Lucy, 2001; Parr et al., 2012). Thus, measuring blood flow to the CL may be useful for a more accurate early diagnosis of pregnancy in cattle particularly if performed at 19 to 21 days of gestation (Matsui and Miyamoto, 2009; Quintela et al., 2012). Siqueira et al. (2013) evaluated the efficacy of Doppler ultrasound as an early pregnancy detection method. This study showed that visual evaluation of CL blood flow offered

98.5% accuracy in predicting non-pregnant females at 20 days after TAI and 64.8% in predicting pregnant ones, but the operator's experience and definition of clear criteria for positive and negative diagnoses are of great importance for successful outcomes.

Chemical pregnancy diagnosis methods

Chemical based pregnancy tests are attractive to producers for many reasons, including earlier diagnosis of non-pregnant females and ease of testing. However, the effectiveness of individual chemical tests varies greatly based on the biomarker used for diagnosis. There have been a number of markers investigated as possible pregnancy detection methods: progesterone, interferon stimulated genes (ISGs), early pregnancy factor (EPF) and pregnancy associated glycoproteins (PAGs). One important consideration is whether the marker is pregnancy specific, meaning only produced by a pregnant animal, or non-pregnancy specific, which can be produced under other physiological conditions.

Non pregnancy specific markers

One of the most common, non-pregnancy specific tests uses progesterone, a steroid hormone which is produced by the corpus luteum (CL) to maintain pregnancy. However, one major disadvantage that limits its use as a pregnancy detection method is that it is produced during the normal estrous cycle of the cow, specifically during the luteal phase. This limits progesterone use as a pregnancy detection method to just a few days between CL regression and formation of a new CL post ovulation. The major problem with progesterone testing is the prevalence of false positives, identifying a non-pregnant cow as pregnant. According to a series of studies conducted in the 1980s, accurate positive pregnancy diagnosis varied between 60 to 100% for milk progesterone, however, detection of non-pregnant animals varied between 81 to 100% (Nebel et al., 1987; Sasser and Ruder, 1987; Nebel, 1988). Progesterone pregnancy diagnosis has not been heavily adopted in research or production agriculture due to these inconsistencies.

Early pregnancy factor (EPF) was initially a popular candidate as a pregnancy diagnostic test due to its target sampling time of 2 to 7 days post conception. In cattle, EPF is thought to suppress the maternal immune system and prepare for uterine implantation beginning 48 hours after breeding (Morton, 1998; Cordoba et al., 2001). Similar to progesterone, commercial EPF tests are unreliable at identifying non-pregnant animals which limits its use in pregnancy detection. Combined with the high incidence of embryonic loss that occurs after day 2 of gestation, EPF is generally regarded as not an option for pregnancy diagnosis in cattle.

During embryo development, it is critical that the embryo secrete the proper signals at the correct time to the maternal environment in order to successfully establish a pregnancy. In cattle, interferon tau (INFT) is the maternal recognition of pregnancy signal and occurs around day 15 to 17 of gestation. Due to the difficulty of directly assaying INFT, research has focused on the response of leukocytes to INFT which express increased interferon stimulated genes (ISGs). Genes including ISG15, Mx1 and Mx2 are more abundant in blood samples of pregnant cows compared to non-pregnant cows (Han et al., 2006; Gifford et al., 2007). However, viral infections can increase expression leading to false positive results, as ISGs are not pregnancy specific (Yang et al., 2010; Weng et al., 2015). Response of ISGs is more prominent in heifers than cows, which have highly variable responses, that contribute to its effectiveness in pregnancy diagnosis (Green et al., 2010). Despite the challenges of positively diagnosing pregnancy, ISGs are effective at identifying non-pregnant cows which may be useful in some scenarios. There is no commercial pregnancy test utilizing this marker, only research labs using quantitative PCR. Overall, available non-pregnancy specific markers do not provide a reliable tool for specific, accurate pregnancy diagnosis.

Pregnancy specific markers

Pregnancy associated glycoproteins (PAGs) were identified by researchers looking for a pregnancy specific marker that could be used for pregnancy diagnosis in cattle. While PAGs have proven to be a reliable marker for pregnancy, their physiological role is unknown although it has been hypothesized that PAGs may help process growth factors found at the placental-uterine interface or assist with adhesion actions between the uterus and placenta (Wallace et al., 2015). Produced by binucleate trophoblast cells of the placenta, PAGs enter the maternal circulation as early as day 22 to 24 and reach levels currently acceptable for accurate pregnancy diagnosis at day 28.

Pregnancy specific protein B (PSPB) or PAG-1 was the first identified member of the PAG family, which encompass more than 20 individual proteins and two dozen genes. The discovery of PAG-1 led to the development of a radioimmunoassay (RIA) for PAG detection and the validating study concluded that PAGs were secreted into the maternal system and were unique to pregnant animals. Some commercial PAG-based pregnancy diagnostic tests still utilize PAG-1 as a target PAG. A study by Green et al. (2005) validated an ELISA that specifically targeted PAGs secreted early in gestation that had a shorter half-life (4.3 days vs 8.4 days) than the previous targets to reduce the potential for false positives in postpartum cows (Zoli et al., 1992; N. M. de Sousa et al., 2003; Green et al., 2005). The ELISA was demonstrated to detect pregnant or non-pregnant cows via PAGs at day 28 post insemination. Studies comparing the efficacy of the PAG ELISA, PAG RIA and transrectal ultrasonography revealed comparable results for the diagnosis of pregnancy in cattle at day 28 of gestation although some differences were identified in the ability of certain assays to detect non-pregnant animals (Szenci et al., 1998; Karen et al., 2015) . Today, commercial PAG testing is extremely accurate providing 95 to 99% true positive (pregnant) reading and false positive (reported as pregnant but actual non-pregnant) rates from 1-5%; however, some false positives may be due to embryonic mortality. Tests currently available include BioPRYN (BioTracking LLC. Moscow, ID USA), IDEXX Bovine pregnancy test (IDEXX Laboratories Inc. Westbrook, ME USA) and DG29 pregnancy test (Genex Cooperative Inc. Shawano, WI USA). BioPRYN accepts blood samples from heifers 25 days post breeding and cows 28 days post breeding, IDEXX recommends day 28 blood or milk samples and DG29 has been validated using day 29 blood samples.

The future of pregnancy diagnosis

Circulating microRNAs

One of the most promising candidates in the search for an easily accessible biomarker for pregnancy diagnosis is circulating microRNAs (miRNA). Between 18 to 22 nucleotides in length, miRNAs play important roles in regulation of gene expression and have been found in biological fluids ranging from serum and amniotic fluid to urine and milk (Reid et al., 2011; Pohler et al., 2015). MicroRNAs are released from cells of most tissue types in plasma membrane bound extracellular vesicles (Parr et al., 2012), specifically exosomes. MicroRNAs are being used to evaluate many physiological systems, particularly disease research from cancer to heart disease and many others. Evidence indicates that specific miRNAs in maternal serum may have an impact on gene expression and regulation which identifies them as potential biomarkers of pregnancy. Studies have identified miRNAs produced by pregnant animals in horses, sheep and cattle (Cameron et al., 2011; Burns et al., 2014; Pohler et al., 2016). A recent study by Fiandanese et al. (2016) identified a potential miRNA, bta-mir 140, as an early biomarker for pregnancy detection. At day 13, bta-mir 140 was increased in abundance in circulation of pregnant, non-lactating cows and at day 19 it was upregulated in both lactating and non-lactating pregnant cows. Multiple groups, including our lab, have identified specific miRNAs differentially regulated in pregnant versus non-pregnant animals. However, additional research is needed to identify specific miRNA most effective for pregnancy diagnosis as well as methods of detection.

Early gestation diagnosis

Research in early pregnancy diagnosis is focused in two areas: increasing sensitivity of current assays and methods and new markers of pregnancy detection. While PAG testing is confined to the time period of physiological availability (not before day 17 of gestation), a recent study in dairy heifers undergoing embryo transfer has shown potential for a day 24 pregnancy test (Reese et al., 2016). Serum PAG concentrations greater than 1.39 ng/mL at day 24 were 95% accurate in diagnosing a pregnant heifer. However, heifers have a higher PAG concentration than mature cows and recent research predicts that embryo mortality between day 24 and 30 may be higher than originally expected (Pohler et al., 2016). Thus, early pregnancy diagnosis using PAG is possible; however, more work is needed in this area to refine methods and detection assays.

Forecasting embryonic loss

The ability to predict and detect pregnancy loss or failure is the next frontier in pregnancy diagnosis. Both PAG and miRNA data suggest that embryo loss can be predicted using these biomarkers. Recent PAG testing studies have shown a strong correlation between successful pregnancies and elevated serum PAG concentrations early in gestation. In comparison to progesterone concentrations, which exhibit no difference between heifers or cows that undergo embryo mortality and those that maintain pregnancy, PAG concentrations are significantly different between the two groups (Reese et al., 2016). In a study by Pohler et al. (2013), cows that maintained pregnancy had significantly higher serum PAG concentration than cows that underwent pregnancy loss after a viable fetal heartbeat was detected at day 28. As serum concentrations increase, the probability of embryo mortality decreases. In a more recent study, late embryo mortality was predicted with 95% accuracy if PAG levels were less than 1.4 ng/ml at day 31 following timed artificial insemination (Pohler et al., 2016).

Preliminary data from our lab suggests miRNA may provide additional insight to embryo viability. Cows that experience embryo mortality have a significantly increased abundance of specific miRNAs at days 17 and 24 of gestation compared to cows that have a successful pregnancy. Future studies will be needed to assess the repeatability of these findings and to determine precise miRNA most applicable for embryo viability analysis.

Practical applications

Although current research aims to develop earlier methods of diagnosing pregnancy in cattle, there are producers who do not regularly assess the pregnancy status of their herd in any way. According to the USDA cow-calf management practices survey in 2008, only 20% of United States beef producers evaluate pregnancy status of their cows, 18% use palpation and only 2.2% use ultrasound for pregnancy diagnosis. About 38% of producers say that labor/time is the principal obstacle preventing them from diagnosing pregnancy in their herd, another 16% believe it is just too complicated, others blame the cost, lack of facilities or believe that it does not work. Assessing pregnancy in a herd is an essential component of an efficient operation, necessary for making management decisions and minimizing wasted resources on unproductive cows. No matter which method is chosen, a producer that knows the pregnancy status of their cows will be better off than a producer that does not.

There are many important factors to consider when implementing a pregnancy detection program. Timing is the first one. Diagnosing pregnancy early in gestation (2 to 17 days) may provide disappointing end results as some cows diagnosed pregnant will later be found non-pregnant due to embryo mortality that occurs within the first 30-45 days of gestation. Most embryonic loss occurs before the placenta is fully formed at approximately 60 days of gestation (Santos et al., 2004). Pregnancy diagnosis that occurs after day 70 is more accurate compared to the end calving rate however, it may be too late in the breeding season for non-pregnant cows to get rebred.

Table 1. Advantages and disadvantages for three methods of pregnancy testing

	When Pregnancy can be Detected	Age of Calf	Sex of Calf	Experienced Technician Needed?	Cost/Cow	When Results Known
Palpation	35-50 days	Yes	No	Yes	$3-$10	Immediately
Ultrasound	30 days	Yes	Potentially	Yes	$7-$15	Immediately
Blood Test (PAG)	28-30 days	No	No	No	$3-$5	1-4 days

Each method of pregnancy diagnosis has challenges to its uses and may not be the best fit for every production setting (see Table 1). Transrectal ultrasound or palpation can detect pregnancy early as 28 days after AI; however, this technique requires a skilled technician or veterinarian, and may require an additional diagnosis around day 60 of gestation. Using blood based PAG testing on day 30 of gestation may be able to detect embryonic loss and provide a better estimate of calving rate. These two factors increase the cost of pregnancy diagnosis by ultrasound. Transrectal palpation may be more cost effective; however, accurate diagnosis even by a trained technician is limited before day 45 of gestation.

Among several blood tests available for pregnancy diagnosis, PAG testing has a significant advantage as it is pregnancy specific. From a simple blood or milk sample taken on day 28-30, producers can determine pregnancy status in 1 to 4 days for around US$3.00. Chemical based testing may be a good option for many producers due to the low cost, early gestation sampling and ease of blood or milk collection.

Although PAG testing at day 28 may have lower accuracy than the transrectal ultrasound, some discrepancy may be eliminated when final calving rates are recorded. Some serum PAG tests may predict late embryonic loss while ultrasounds cannot. When a fetal heartbeat of an embryo is viewed on an ultrasound, there is no indication whether or not embryo mortality will occur. On the other hand, cows that appeared pregnant on the ultrasound, but will later experience embryo mortality often show up as non-pregnant on serum PAG tests. Pregnancy diagnosis using PAGs may be a better indicator of final calving rate.

Conclusions

Waiting for the end of calving season to determine pregnancy rate is not economically viable; however, a limited percentage of United States beef producers evaluate pregnancy status of their herd. Early pregnancy detection is very important to detect non-pregnant cows as soon as possible. There are several different tests and methods available to provide diagnostic information. Choosing a method is entirely dependent on what fits into the farm's production system. In the future, we expect that blood-based pregnancy test will gain popularity due to its accuracy and ability to predict embryo loss. As technology develops and will look into the future, one might predict that blood based pregnancy test results will be available in minutes, chute side for a relatively low cost.

References

Abbitt, B. et al. 1978. Effect of three methods of palpation for pregnancy diagnosis per rectum on embryonic and fetal attrition in cows. J Am Vet Med Assoc 173: 973-977.

Alexander, B. M. et al. 1995. Embryonic loss from 30 to 60 days post breeding and the effect of palpation per rectum on pregnancy. Theriogenology 43: 551-556.

Burns, G. et al. 2014. Extracellular vesicles in luminal fluid of the ovine uterus. PLoS One 9: e90913.

Cameron, A., J. C. da Silveira, G. Bouma, and J. E. Bruemmer. 2011. Evaluation of exosomes containing miRNA as an indicator of pregnancy status in the mare. Journal of Equine Veterinary Science 31: 2.

Cordoba, M. C., R. Sartori, and P. M. Fricke. 2001. Assessment of a commercially available early conception factor (ECF) test for determining pregnancy status of dairy cattle. J Dairy Sci 84: 1884-1889.

de Sousa, N. M. et al. 2003. Pregnancy-associated glycoprotein concentrations during pregnancy and the postpartum period in Azawak Zebu cattle. Theriogenology 59: 11.

Fiandanese, N. et al. 2016. Circulating microRNAs as potential biomarkers of early pregnancy in high producing dairy cows Proc. International Embryo Technology Society. p 165, Louisville, KY.

Franco, O. J., M. Drost, M. J. Thatcher, V. M. Shille, and W. W. Thatcher. 1987. Fetal survival in the cow after pregnancy diagnosis by palpation per rectum. Theriogenology 27: 631-644.

Gifford, C. A. et al. 2007. Regulation of interferon-stimulated genes in peripheral blood leukocytes in pregnant and bred, nonpregnant dairy cows. J Dairy Sci 90: 274-280.

Ginther, O. J. 2007. Ultrasonic imaging and animal reproduction: color-Doppler ultrasonography. Equiservices Publishing, Cross Plains, WI.

Green, J., C. Okamura, S. Poock, and M. Lucy. 2010. Measurement of interferon-tau (IFN-τ) stimulated gene expression in blood leukocytes for pregnancy diagnosis within 18–20d after insemination in dairy cattle. Animal reproduction science 121: 24-33.

Green, J. A. et al. 2005. The establishment of an ELISA for the detection of pregnancy-associated glycoproteins (PAGs) in the serum of pregnant cows and heifers. Theriogenology 63: 1481-1503.

Han, H., K. J. Austin, L. A. Rempel, and T. R. Hansen. 2006. Low blood ISG15 mRNA and progesterone levels are predictive of non-pregnant dairy cows. J Endocrinol 191: 505-512.

Herzog, K. et al. 2010. Luteal blood flow is a more appropriate indicator for luteal function during the bovine estrous cycle than luteal size. Theriogenology 73: 691-697.

Karen, A. et al. 2015. Comparison of a commercial bovine pregnancy-associated glycoprotein ELISA test and a pregnancy-associated glycoprotein radiomimmunoassay test for early pregnancy diagnosis in dairy cattle. Anim Reprod Sci 159: 31-37.

Kastelic, J. P., R. A. Pierson, S. Curran, and O. J. Ginther. 1988. Ultrasonic evaluation of the bovine conceptus. Theriogenology 29: 16.

Lopez-Gatius F.; Garcia-Ispierto, I. 2010. Ultrasound and endocrine findings that help to assess the risk of late embryo/early foetal loss by non-infectious cause in dairy cattle. Reprod Domest Anim 45: 9.

Lucy, M. C. 2001. Reproductive loss in high-producing dairy cattle: where will it end? J Dairy Sci 84: 1277-1293.

Mann, G. E., Lamming, G. E. 1999. Influence of progesterone during early pregnancy in cattle. Reprod Domest Anim 34: 6.

Matsui, M., and A. Miyamoto. 2009. Evaluation of ovarian blood flow by colour Doppler ultrasound: practical use for reproductive management in the cow. Vet J 181: 232-240.

Morton, H. 1998. Early pregnancy factor: an extracellular chaperonin 10 homologue. Immunol Cell Biol 76: 483-496.

Nebel, R. L. 1988. On-farm milk progesterone tests. J Dairy Sci 71: 1682-1690.

Nebel, R. L., W. D. Whittier, B. G. Cassell, and J. H. Britt. 1987. Comparison of on-farm laboratory milk progesterone assays for identifying errors in detection of estrus and diagnosis of pregnancy. J Dairy Sci 70: 1471-1476.

Paisley, L. G., Mickelson, W. D., Trost, O. L. 1978. A survey of the incidence of prenatal mortality in cattle following pregnancy diagnosis by rectal palpation. Theriogenology 9: 11.

Parr, M. H. et al. 2012. Relationship between pregnancy per artificial insemination and early luteal concentrations of progesterone and establishment of repeatability estimates for these traits in Holstein-Friesian heifers. J Dairy Sci 95: 2390-2396.

Pohler, K. G. et al. 2013. Circulating bovine pregnancy associated glycoproteins are associated with late embryonic/fetal survival but not ovulatory follicle size in suckled beef cows. J Anim Sci 91: 4158-4167.

Pohler, K. G. et al. 2015. Predicting Embryo Presence and Viability. Adv Anat Embryol Cell Biol 216: 253-270.

Pohler, K. G. et al. 2016. Circulating concentrations of bovine pregnancy-associated glycoproteins and late embryonic mortality in lactating dairy herds. J Dairy Sci 99: 1584-1594.

Quintela, L. A. et al. 2012. Use of ultrasound in the reproductive management of dairy cattle. Reprod Domest Anim 47 Suppl 3: 34-44.

Reese, S. T., M. C. Pereira, J. L. Vasconcelos, and K. G. Pohler. 2016. Pregnancy Associated Glycoprotein (PAG) concentrations in early gestation from dairy heifers undergoing embryo transfer ASAS-ADAS Joint Annual Meeting, Salt Lake City, Utah.

Reid, G., M. B. Kirschner, and N. van Zandwijk. 2011. Circulating microRNAs: Association with disease and potential use as biomarkers. Crit Rev Oncol Hematol 80: 193-208.

Romano, J. E., K. Bryan, R. S. Ramos, J. Velez, and P. Pinedo. 2016. Effect of early pregnancy diagnosis by per rectum amniotic sac palpation on pregnancy loss, calving rates, and abnormalities in newborn dairy calves. Theriogenology 85: 419-427.

Romano, J. E. et al. 2007. Early pregnancy diagnosis by palpation per rectum: influence on embryo/fetal viability in dairy cattle. Theriogenology 67: 486-493.

Santos, J. E., W. W. Thatcher, R. C. Chebel, R. L. Cerri, and K. N. Galvao. 2004. The effect of embryonic death rates in cattle on the efficacy of estrus synchronization programs. Anim Reprod Sci 82-83: 513-535.

Sasser, R. G., and C. A. Ruder. 1987. Detection of early pregnancy in domestic ruminants. J Reprod Fertil Suppl 34: 261-271.

Singh, J., G. P. Adams, and R. A. Pierson. 2003. Promise of new imaging technologies for assessing ovarian function. Anim Reprod Sci 78: 371-399.

Siqueira, L. G. et al. 2013. Color Doppler flow imaging for the early detection of nonpregnant cattle at 20 days after timed artificial insemination. J Dairy Sci 96: 6461-6472.

Szenci, O. et al. 1998. Comparison of ultrasonography, bovine pregnancy-specific protein B, and bovine pregnancy-associated glycoprotein 1 tests for pregnancy detection in dairy cows. Theriogenology 50: 77-88.

Thompson, J. A. et al. 1994. Pregnancy attrition associated with pregnancy testing by rectal palpation. J Dairy Sci 77: 3382-3387.

Thurmond, M. C., and J. P. Picanso. 1993. Fetal loss associated with palpation per rectum to diagnose pregnancy in cows. J Am Vet Med Assoc 203: 432-435.

Vaillancourt, D. et al. 1979. Correlation between pregnancy diagnosis by membrane slip and embryonic mortality. J Am Vet Med Assoc 175: 466-468.

Wallace, R. M., K. G. Pohler, M. F. Smith, and J. A. Green. 2015. Placental PAGs: gene origins, expression patterns, and use as markers of pregnancy. Reproduction 149: R115-126.

Weng, X. G. et al. 2015. Genetic characterization of bovine viral diarrhea virus strains in Beijing, China and innate immune responses of peripheral blood mononuclear cells in persistently infected dairy cattle. Journal of veterinary science 16: 491-500.

Yang, L. et al. 2010. Up-regulation of expression of interferon-stimulated gene 15 in the bovine corpus luteum during early pregnancy. Journal of dairy science 93: 1000-1011.

Zemjanis, R. 1962. Diagnostic and therapeutic techniques in animal reproduction. The Williams and Wilikins Company, Baltimore, MD.

Zemjanis, R. F., M.; Schultz, R.H. 1969. Anestrus: the practitioner's dilemma. Vet Scope: 1.

Zoli, A. P., L. A. Guilbault, P. Delahaut, W. B. Ortiz, and J. F. Beckers. 1992. Radioimmunoassay of a bovine pregnancy-associated glycoprotein in serum: its application for pregnancy diagnosis. Biol Reprod 46: 83-92.