Pregnancy Diagnosis in Cattle: When, Why and How

Ky Pohler, Gessica Franco, Sydney Reese, Rebecca Payton, and Justin Rhinehart,

Department of Animal Science, University of Tennessee, Knoxville, TN

Key Points

Establishing a pregnancy diagnosis program is an essential component for developing strategies to improve reproductive efficiency in beef cattle. Therefore, effort should be directed toward increasing producer awareness about the importance and advantages of pregnancy diagnosis.

When efficiently implemented, knowing pregnancy status of the herd overcomes the costs associated with pregnancy diagnosis.

There are several different technologies available to provide diagnostic information, choosing a method is entirely dependent on what fits into the production system.

In the future, we expect that blood-based pregnancy testing will gain popularity due to its accuracy, ease of use and ability to predict pregnancy loss.

Introduction

According to the (USDA, 2009, 2011) cow-calf management practices survey, only 20% of United States beef producers evaluate pregnancy status of their cows on a yearly basis. This number is correlated to farm size with small scale farmers, which represent 90 % of all US cow/calf operations, being less likely to diagnosis pregnancy in their cowherd compared to larger operations (12.6% compared to 58% for herds with 200+ head) Of these producers, 18% use palpation and only 2.2% use ultrasound for pregnancy diagnosis. Labor and lack of time are the primary reasons cited by producers for not performing pregnancy diagnosis. Another 16% of producers believe it is just too complicated, while others are hesitant due to cost, lack of facilities or belief that it does not work or is not worth the investment . More effort is necessary to change this perspective and spread awareness that assessing pregnancy status is an essential component of an efficient operation; necessary for making management decisions and minimizing wasted resources on unproductive cows.

Several methods of pregnancy diagnosis are available which can be performed at different time points through gestation. They can fit in different management systems and budgets with high efficacy. Each operation should evaluate the options to identify a method to suit its situation. Most of the available methods will be discussed in this paper and a summary of advantages and disadvantages for each will be presented at the end (Table 1). It is also critical to conduct pregnancy diagnosis at time points that allow for effective management decisions to be made. Conducting pregnancy diagnosis without using the resulting information to adjust or make management decisions is not an effective use of time or resources.

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. All methods should strive to 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. Additional information that can be derived at the time of pregnancy diagnosis (such as embryonic or fetal viability and sex of the fetus), are increasingly beneficial.

Rectal palpation and ultrasonography are the most widely used direct methods for early pregnancy diagnosis. Both techniques are efficient but require a skilled technician, specific instruments in the case of ultrasound and provide static information on pregnancy status at the specific moment of diagnosis. In some areas, veterinary support is limited and alternative approaches for pregnancy detection are needed.

Indirect methods of pregnancy diagnosis that use qualitative or quantitative measures of hormones or conceptus-specific substances in maternal body fluids as indicators of a viable pregnancy have been the focus of pregnancy diagnosis research over the last 20 years. Molecules and 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.

Establishing the Pregnancy Diagnosis Schedule

The first question to be answered when implementing pregnancy diagnosis at the farm is “when should I test or check my cows?” Decisions regarding when pregnancy diagnosis should occur are not one size fits all and often vary based on an operation’s management strategies. The ideal time point would be as early as possible while minimizing the number of cows identified as pregnant that go on to lose the pregnancy before calving. Early pregnancy diagnosis often results in high number of false positive due to significant percentage of pregnancy loss that happens later on gestation and is not detected at the moment of the test. Pregnancy diagnosis occurring later (e.g., after day 70 or 100 of gestation) more accurately reflects calving rate because most loss occurs before the placenta is fully formed at approximately 60 days of gestation (Santos et al., 2004). However, it may be too late in the breeding season for non-pregnant cows to get rebred.

For farms utilizing artificial insemination (AI) it is recommended to do the first pregnancy check 28-35 days after AI, when rectal palpation/ultrasonography is easily performed and the earliest that commercial blood tests can accurate detect pregnancy. Detecting non-pregnant cows at this point allows for resynchronization and provides information for the producer on AI versus bull bred pregnancies. A later, second test is recommend to detect cows bred by natural service and cows that may have undergone late embryonic loss. Farms that employ natural service should be encouraged to utilize a set breeding season (no more than 90 days) and diagnose pregnancy 30 days after removal of the bull (Figure 1). In some scenarios, multiple pregnancy diagnoses may be beneficial. However, many operations can identify open cows and allot available resources with a single pregnancy diagnosis.

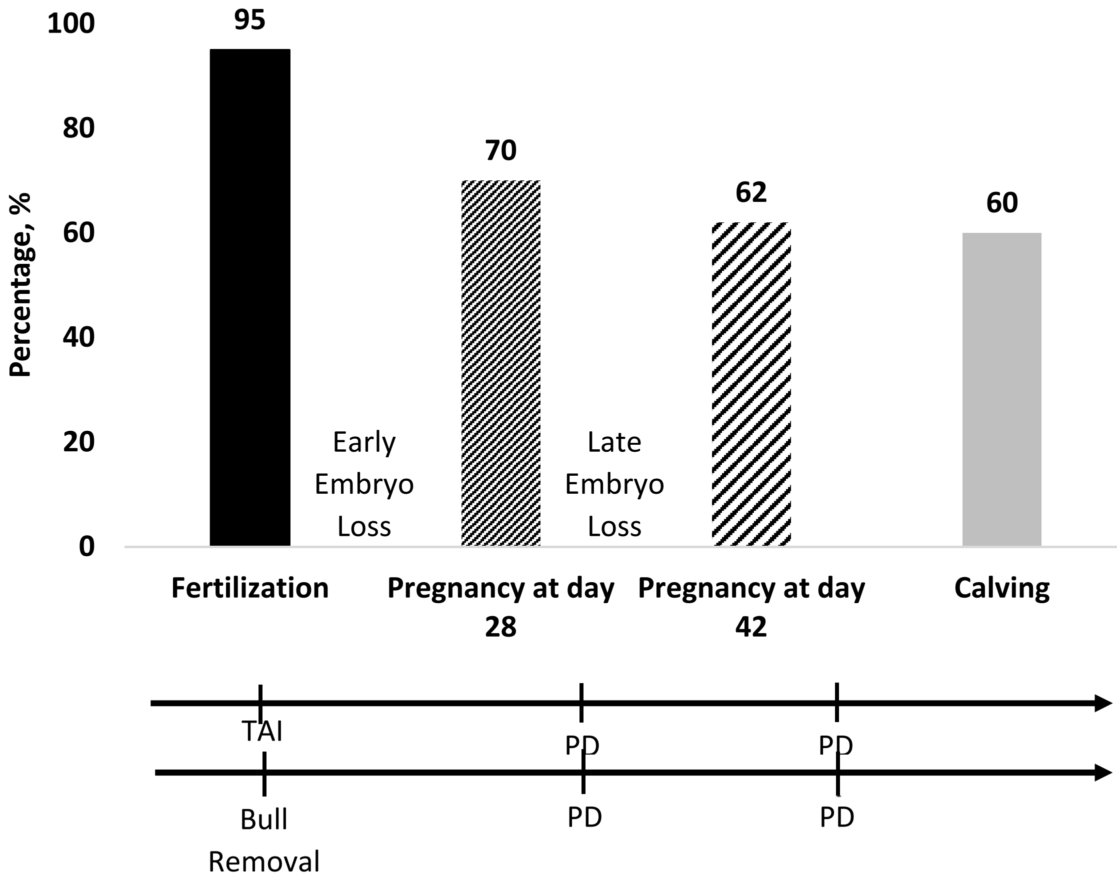

Figure 1. To increase reproductive efficiency of beef herds, it is recommended to perform the first pregnancy diagnosis (PD) as early as possible (~28 days) after timed artificial insemination (TAI) or after bull removal to detect non pregnant cows. A second PD is recommended 42 days after TAI or bull removal or later to detect cows that underwent embryonic loss. Current research focuses on developing an ideal pregnancy test that provides all this information at a single time point early in gestation.

Advantages of Knowing the Pregnancy Status of the Herd

Reproductive efficiency is critical for economic sustainability of beef cow-calf herds. Specifically, the average cost of maintaining a mature beef cow in United States ranges from \$558/year to \$785/year including production costs such as feed and labor as well as fixed costs such as land, facilities and depreciation (Warner et al., 2015). To maintain economic viability in commercial production, each cow has two opportunities to generate a marketable product that offsets its annual carrying cost: 1) a calf or 2) the cow itself. Knowing whether a cow will produce a calf - at least by the time of weaning the previous year's calf – facilitates better decisions for marketing and replacements. Therefore, understanding the pregnancy status of the herd is indispensable.

The primary measure of reproductive success is calving rate - the percentage of cows exposed to a bull or artificially inseminated that give birth to a live calf. However, it is necessary to understand the periods when pregnancy loss occurs: early (<day 28) or late embryonic loss (day 28 to day 60). For researchers, this data is fundamental to elucidate the potential mechanisms

related to fertilization failure. For veterinarians and producers, it is a helpful tool to identify the reproductive performance of the herd, and establish management decisions to improve productivity. At farms where natural service is used, pregnancy diagnosis may be useful to evaluate sire performance, help identify calving groups and assist in planning development of farm activities such as pasture management. Pregnancy diagnosis after AI or ET allows identification of non-pregnant cows as early as possible after insemination and reduces inter-insemination interval for herds performing resynchronization.

Current Available Methods for Pregnancy Diagnosis

Estrus detection

Accurate identification of cows returning to estrus from 18 to 32 days after exposure to bulls or artificial insemination is the easiest and least costly method for determining non-pregnant cows after insemination. Approximately 15 to 17 days after ovulation, bovine embryos signal their presence to the maternal environment, suspending the estrous cycle by maintaining the corpus luteum and production of progesterone. Failure of a cow to return to estrus within 18 to 24 days after breeding suggests that conception has occurred (Zemjanis, 1969). This assumption, however, is challenged by high failure rate in estrus detection, and by cows in anestrus (failure to cycle) due to factors such as undernutrition.

Proper training to recognize estrus behavior is fundamental to increase success in identifying open or pregnant cows. There are some technologies available on the market, such as heat patches, paint or chalks, which can help visually mark cows that were mounted (the principal signal of estrus behavior). Recent studies by University of Missouri evaluated the use of a commercial heat detection patch for pregnancy diagnosis in Nelore beef cows in Brazil. Cows with 50% or more rub-off surface removed were significantly more likely to be pregnant after timed AI. Moreover, another study by South Dakota State University showed that information obtained by heat detector patch had similar or higher efficacy in confirming pregnancy, compared to blood based tests (Estrotect, 2014).

Rectal palpation/Ultrasonography

One of the first and most widely used methods for diagnosis of pregnancy in cattle involves rectal palpation of the reproductive tract. The examiner, through rectal palpation, must find at least one of these five pathognomonic signs of pregnancy: palpation of the amniotic vesicle, fetal membrane slip, placentomes (cotyledons and caruncles), the fetus itself or prominence of the mid-uterine artery (Zemjanis, 1962). Rectal palpation also allows for estimation of the embryonic/fetal age and detection of ovarian structures such as a corpus luteum or follicle. Disadvantages of this method include the necessity of skilled technicians and inability to diagnosis impending pregnancy failure and/or other abnormalities during pregnancy.

The use of transrectal real-time ultrasonography (US) emerged as a quicker and more reliable technique for studying reproductive functions in cattle in the 1980s (Pierson and Ginther, 1984). The first markers of pregnancy (discrete black areas – embryonic vesicle) are visible by US as early as day 12 of gestation (Pierson and Ginther, 1984); however, recent reports have emphasized that it is difficult to distinguish between the fluid accumulation during early pregnancy (< day 18 to 21) and the uterine fluid that accumulates naturally during proestrus and estrus. Thus, examination should be performed at a more advanced stage of gestation to avoid false positive diagnosis.

Curran et al. (1986) characterized growth of the embryo from day 20 through 60 of gestation and determined when characteristics, such as the heartbeat (day 22), spinal cord (day 28), placentomes (day 35), split hooves (day 44) and ribs (day 58) first become detectable by US and can be used to estimate age of the fetus. Death of the embryo can be clearly diagnosed by no detectable heartbeat, membrane detachment/disorganization, reduced amounts of intrauterine fluid or hyperechoic floating structures, including remnants of the conceptus (Lopez-Gatius F.; Garcia-Ispierto, 2010). This method also requires a trained technician and the major disadvantage of US is that accuracy is limited before 28-30 days of gestation and pregnancy status is only guaranteed at the time of diagnosis.

Doppler ultrasonography/3D

The establishment and initial 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 (Mann, 1999; Lucy, 2001). Thus, measuring blood flow to the CL may be useful for early diagnosis of pregnancy in cattle particularly if performed at 19 to 21 days of gestation (Matsui and Miyamoto, 2009; Quintela et al., 2012). The incorporation of new ultrasound technologies, such as Doppler and 3D ultrasound, enable a more detailed assessment of the uterus, ovarian follicles, and corpus luteum. Doppler ultrasound utilizes color-flow mode (CFM) which allows for 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).

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 timed AI and 64.8% in predicting pregnant ones. The angle at which the probe is held in relation to the tissue, artery or vein will affect the resulting calculations of blood flow velocity (Herzog et al., 2010). Thus, for Doppler ultrasound to be truly effective, the operator's skill to consistently achieve the same scan and the definition of clear criteria for positive and negative diagnoses are critical for successful outcomes. Improving the software to generate better imaging and tools that use pixel density to categorize ovaries may help overcome this limitations and improve diagnostic capabilities.

Three-dimensional (3D)/four-dimensional (4D) imaging is the most recent advancement in ultrasound technology. It has been widely used in human medicine and some small animal clinics providing new information about pregnancy status, birth prediction and improving diagnostic confidence. Currently, its use is limited in large animals, mainly due to the high cost of instruments and system, operation difficulties due to animal movement and lack of knowledge for capturing high-quality volume images. The first studies characterizing development of ovarian structures in beef heifers (dominant follicle and corpus luteum) using a 3D Doppler ultrasonography showed similar findings compared to 2D images. However, the extra time required and limited additional information obtained may restrict its use beyond a research purpose (Scully et al., 2014).

Research advances using this new imaging technology may help determine physiological states of both follicles and corpus luteum; such as distinguishing between a newly formed and established CLs, or whether a large follicle on the ovary is in the growth or regression stage of development, through analysis of blood flow and internal structures. Additionally, the ability to improve measurements through embryo development can increase the accuracy of early pregnancy diagnosis and help elucidate the mechanisms of pregnancy loss.

Chemical blood tests

Chemical based tests are indirect pregnancy detection methods that have been developed to improve early diagnosis using either milk or blood samples. The adoption of these tests in beef farms is slow compared to dairy farms. However, the ease and increasing efficiency of testing has increased adoption by producers. There are two categories of biomarkers detected for cattle pregnancy tests: non pregnancy specific (progesterone, interferon stimulated genes, early pregnancy factor) and pregnancy specific (pregnancy associated glycoproteins). The effectiveness of the test varies greatly based on the biomarker used for diagnosis.

Non Pregnant Specific Markers

Early pregnancy factor (EPF) was initially a popular candidate for pregnancy diagnostics due to its target sampling time of 2-7 days post conception. In cattle, EPF is thought to suppress the maternal immune system to prepare for uterine attachment 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 their use in pregnancy detection. Combined with the high incidence of embryonic loss that occurs after day 2 of gestation, EPF is generally not regarded as an option for pregnancy diagnosis in cattle.

Progesterone is a steroid hormone produced by the CL during the luteal phase of the estrous cycle and by the placenta during pregnancy. The differences in progesterone levels between a pregnant and non-pregnant cow are used as a biomarker for early pregnancy detection. In the 1980s, rapid on-farm qualitative tests for assessing progesterone concentration were commercialized for pregnancy diagnosis in dairy cows. Cows with low progesterone 18 to 24 days after insemination would be classified as non-pregnant and cows with high progesterone would be classified as pregnant. Although this method is fairly accurate in diagnosing non pregnant cows (81-100%), the efficacy of positive diagnosis (60-100%) is questionable (Nebel et al., 1987; Sasser and Ruder, 1987; Nebel, 1988). The non-specificity and resultant high prevalence of false positives has limited its use as pregnancy marker in research and commercial farms.

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. It has been hypothesized that they are involved with processing of growth factors found at the placental-uterine interface or assisting with adhesion of 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 and encompasses more than 20 individual proteins and two dozen genes (Wallace et al., 2015). 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; 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 98 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.

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), a recent study in dairy heifers undergoing embryo transfer have 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.

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.), 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. In our most recent study, we compared the small-RNA profiles of purified extracellular vesicles from serum of pregnant, embryonic-mortality, and control cows. From 214 miRNA identified, 32 had differentially abundant loci, representing 27 differentially abundant mature miRNA. The differential abundance of 3 of 4 mature miRNAs (miR-16a/b, -25, and -3596; not -100) at day 17 of gestation was confirmed using reverse-transcription quantitative PCR, with all 3 present at lower levels in the pregnant cows versus those with embryonic mortality (Pohler et al., 2017). These recent findings support the idea that miRNAs are a good biomarker candidate for early pregnancy detection. But, additional research is needed to simplify methods of detection and identify specific miRNA most effective for pregnancy diagnosis.

Table 1. Comparison of three methods of pregnancy test in cattle

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	21 minutes

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 embryonic 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 embryonic mortality decreases. In a more recent study, late embryonic 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 embryonic viability. Cows that experience embryonic 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 specific miRNA most applicable for embryo viability analysis.

Conclusion

In summary, many improvements have been made toward developing an ideal pregnancy diagnosis method where viable pregnancies can be detected as early as possible and also provide additional information such as future embryonic loss. As technology develops, we expect blood based pregnancy testing to increase in popularity due to low cost and ease of application. However, as has been noted, the practice of pregnancy diagnosis in beef farms is low and increasing producer awareness about the economic and production advantages of knowing pregnancy status of the herd is essential to increase reproductive efficiency.

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