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DETERMINING PREGNANCY STATUS IN LARGE AND SMALL HERDS

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Introduction

Pregnancy diagnosis is an important part of reproductive management in productive beef cow-calf operations. Failing to recognize and cull non-pregnant cows or heifers has negative economic implications because these females accrue the same cost of a pregnant cow, but without generating income. Establishing a pregnancy diagnosis program allows producers to make management decisions to increase reproductive efficiency, such as culling of infertile females or potentially resynchronizing non-pregnant females. As the beef cattle industry advances towards more efficient operations and attempts to increase the adoption of reproductive technologies in cattle production, abstaining from pregnancy diagnosis may no longer be economically viable.

In a hypothetical well-managed beef cattle operation with 100 brood cows exposed to a 65-day breeding season, pregnancy rates at the end of the breeding season will often range between 85 and 95%. Considering a cow carrying costs of $800 per cow per year, and final pregnancy rates to be 90%, this operation would spend $8,000 ($800 × 10 open cows = $8,000) per year to maintain cows that failed to become pregnant within the breeding season. Assuming this operation weans 85 calves every year (5% of the pregnant cows fail to wean a calf due to pregnancy loss and calf mortality prior to weaning), if this operation neglects the use of pregnancy diagnosis and fails to recognize the non-pregnant cows after the end of the breeding season, cost of production will increase by $94 per weaned calf ($8,000 divided across 85 calves). Noteworthy, the costs described herein will vary depending on the operations, as cow carrying costs and the amount of time a non-pregnant cow will stay in the herd vary depending on the operation. Yet, failing to recognize non-pregnant cows will most often, if not always, have negative implications to the economic sustainability of cow-calf operations.

Another important consideration is the revenue obtained from marketing non-pregnant cows. These cows normally represent between 15 and 30% of sales revenue in most cow-calf operations. As with many other commodities, cull cow prices undergo seasonal fluctuations. Recognizing non-pregnant cows soon after the end of the breeding season allows producers to develop a market plan for these animals and evaluate the ideal time to sell them in order to optimize profitability. These proceedings provide an overview of the different methods currently available commercially (rectal palpation, transrectal ultrasound, and blood tests) for pregnancy diagnosis. Additionally, it includes a brief overview on recent research developments in pregnancy diagnosis technologies.

Methods Available for Pregnancy Diagnosis

There is no "one size fits all" when it comes to pregnancy diagnosis, and each producer should understand the currently available methods to decide on the most economically viable strategy to diagnose pregnancy in their operations. Another key component of establishing a pregnancy diagnosis and cow-culling strategy is the development of a veterinarian-client-patient-relationship with local veterinarians. This will help producers evaluate the different alternatives available for pregnancy diagnosis and optimize the efficiency and accuracy of pregnancy diagnosis within their herds.

Rectal Palpation

Rectal palpation is the most commonly utilized method of pregnancy diagnosis. This technique relies on the technician identifying physical changes that occur in the uterus during gestation to diagnose pregnancy and estimate gestational age. There are several landmark changes that occur throughout gestation that allow this method to be accurate when employed by a skilled examiner, and these landmarks occur at relatively predictable timepoints during pregnancy. For example, the presence of the embryonic vesicle can be felt around day 32 and 35. On day 45, there is distinct asymmetry between gravid and non-gravid uterine horns, which becomes more pronounced as gestational age increases. Once the pregnancy has surpassed 90 days, placentomes can be palpated as well as different parts of the fetus. Identification of fetal membranes (amnion and chorioallantois), intrauterine fluid, location of the reproductive tract, presence of cervical traction generated by fetal weight, and uterine artery pulsation can also be used during palpation to estimate pregnancy status and age. Overall, rectal palpation is a reliable method for diagnosing pregnancy; however, this technique is dependent on the examiner's experience. Generally, most experienced veterinarians can accurately detect pregnancy as early as 40 days after breeding. Yet, some technicians can accurately distinguish pregnant and non-pregnant cows earlier at approximately 30 to 35 days.

Transrectal ultrasonography

Transrectal ultrasonography is a method widely used by veterinarians and embryologists. It is highly accurate and able to identify pregnant females as early as 26-28 days, depending on the technician's experience and the quality of the machine used. Commercially, transrectal ultrasound is most commonly used between after 30 days of gestation. There are several advantages of transrectal ultrasound when compared to other methods of pregnancy diagnosis. Transrectal ultrasound allows not only to identify the presence of the conceptus, but also to determine its health status and viability. This is accomplished through the evaluation of placental structures (chorioallantois and amnion), chorioallantoic and amniotic fluid echogenicity, placentome morphometries, presence fetal heartbeat, and other factors. Furthermore, ultrasound examination during early gestation (< 100 days) allows for more accurate estimations of conceptus age compared with rectal palpation. During ultrasound examinations, fetal age can be estimated by objectively measuring the embryo/fetus itself (crown to rump length), or fetal structures (crown to nose length, biparietal diameter, eye orbit length, abdominal diameter, and others). More commonly in beef cow-calf herds, gestational age is determined subjectively based on embryo/fetal size and characteristics. The latter approach provides enough accuracy to estimate calving dates and distinguish pregnancies generated via artificial insemination or embryo transfer from pregnancies generate by cleanup sires. **Figure 1** shows representative ultrasound images of bovine fetuses at different ages.

Another advantage of transrectal ultrasound is the ability to determine fetal sex. Fetal sex can be determined between days 60 and 90 of gestation based on the location of the genital tubercle (GT). The GT is the embryonic precursor of the penis (male fetuses) and clitoris (female fetuses). After day 60 of gestation, the position of the genital tubercle (GT) can be easily visualized to distinguish fetal sex. In male fetuses, the GT is located between the umbilical cord and the hind legs. In female fetuses, the GT is located between the hind legs and the tail. Visualization of the fetal scrotum or fetal udder can also be utilized to assist with fetal sex determination. Similar to fetal aging, scheduling fetal sex examinations past the ideal gestational age range (60 to 90 days) is not recommended because the larger fetal size makes sex determination challenging. Moreover, while fetal sexing can be performed up to day 90 of gestation, positioning the transducer to sex fetuses is considerably challenging between days 80 and 90 of gestation. Accuracy of sex determination using ultrasound is greater than 94% (Curran et al., 1989).

The third advantage of ultrasound over manual palpation is the ability to identify uterine and ovarian pathologies that would either be missed by palpation or misdiagnosed. For example, a uterine infection could feel similar to a pregnancy during rectal palpation, but it is easily distinguished from a pregnancy using ultrasound. The ability of veterinarians to identify ovarian structures and pathologies (such as follicular or luteal cysts) can help producers make culling decisions and allocate limited resources more appropriately. Another great advantage of ultrasound is the ability of easily identifying twin pregnancies so that farm personal can be on standby when calving in case assistance is needed.

Blood-based pregnancy diagnosis

In the last few decades, several studies have explored embryo and placental physiology in order to identify biomarkers of pregnancy that can be identified in blood and/or milk. The most successful and currently utilized biomarker for pregnancy diagnosis are pregnancy-associated glycoprotein (PAG; Sasser et al., 1986). These proteins are produced by a population of binucleated trophoblast cells starting on the third week of gestation in cattle (Wallace et al., 2015). Pregnancy-associated glycoproteins make their way into the maternal blood circulation, allowing pregnancy diagnosis to be performed accurately as early as 28-31 days after breeding (Haugejorden et al., 2006). There are currently 3 different companies that provide PAG-based tests for cattle (BioTracking, LLC, Genex Cooperative Inc., and Idexx Laboratories Inc.) and all commercially available blood tests rely on antibodies (ELISA or lateral flow technology) to detect PAG in the sample. Blood tests that are currently available in the market have been shown to result in accurate pregnancy diagnosis for commercial application as long as producers follow the manufacturer recommendations.

Current blood–based pregnancy tests have accuracy rates of 93–96% (reviewed by Wallace et al., 2015). The main factors influencing the accuracy of these tests are human error (e.g., wrong blood tube identification or incorrect sample handling), embryonic mortality, and time interval between previous parturition and blood sampling. Human errors can lead to both false–positive (non-pregnant cow is considered pregnant by the test) and false–negative (considering a pregnant cow not pregnant) results. Cows that experienced embryonic mortality and still have PAG in circulation can generate a false–positive results. Similarly, cows early into the postpartum period have PAG in circulation from their previous pregnancy, leading to a false–positive results. For these reasons, it is recommended that cows are at least 60–90 days postpartum (depending on the blood test) when blood is collected to avoid false–positive results

One of the disadvantages of blood tests compared to rectal palpation or ultrasonography used to be the absence of immediate chute–side results, which represent a logistic challenge for some operations. Recently, chute-side blood testing for pregnancy also became available to cattle producers. Similar to a pharmacy test for human pregnancy, chute-side blood testing with PAG rely on lateral flow technology and can be performed by a non-trained individual. In fact, this is one of the main benefits of blood-based pregnancy tests when compared to ultrasound and rectal palpation. Minimal training is required for producers to learn how to collect blood samples, in contrast to rectal palpation and ultrasonography that requires considerable training for accurate diagnosis.

Strategic Application of Current Pregnancy Diagnosis Technologies

When establishing pregnancy diagnoses and cow culling strategies, it is important for producers to consider what information collected during pregnancy diagnosis will help them make management decisions in their herds.

Determining pregnancy status at the end of the breeding season

For herds that are only determining pregnancy status at the end of the breeding season, there is considerable flexibility with regards to method and timing of pregnancy diagnosis. To determine pregnancy status at the end of the breeding season, blood test or transrectal ultrasonography can be performed starting 28-30 days after bulls were removed from the herd. Rectal palpation can also be utilized starting 40 days after bull removal. Noteworthy, performing pregnancy diagnosis soon after the end of breeding season gives producers more time and flexibility when determining their culling strategy for a given year.

Distinguishing pregnancies generated by artificial insemination or embryo transfer from pregnancies generated by the cleanup bulls

Producers that are taking advantage of artificial insemination or embryo transfer technology are often interested in distinguishing these pregnancies from natural service pregnancies. This can be accomplished when cleanup bulls are turned in with females at least 14 days after artificial insemination or 7 days after embryo transfer. For herds with a breeding season ≤ 70 days, the most strategic way to distinguish these pregnancies is to use ultrasonography between days 90 and 100 of gestation. This allows the veterinarian to differentiate artificial insemination (or embryo transfer) pregnancy from natural service pregnancies based on fetal age. By scheduling the ultrasound examination > 28 days after cleanup bull removal and < 100 days after artificial insemination (93 days after embryo transfer), veterinarians can distinguish pregnancies while also recognizing all females that failed to conceive during the breeding season in a single examination. This is not possible in herds with a breeding season longer than 70 days. In this case, two pregnancy diagnoses would have to be performed to distinguish artificial insemination pregnancies from natural service pregnancies. This further highlights the importance of shortening the breeding season.

Determining fetal sex

Determining fetal sex provides useful information for both marketing and management purposes. Knowing the number of male and female calves gives producers advance notice and time to plan a marketing strategy for the calves before they even hit the ground. This information is particularly valuable for seedstock producers as they are able to make herd management and genetic selection decisions 8 months sooner. The only methods available to determine fetal sex is ultrasonography, which should be performed between 60 and 90 days after breeding.

Determining pregnancy status early to rapidly rebreed non-pregnant females

A potential strategy for these producers is the use of estrus detection and artificially insemination based on estrus expression. In this case, estrus expression is used to recognize non-pregnant females. Estrus detection and artificial insemination are performed between 15 to 25 days after the first fixed-time artificial insemination following the AM-PM rule. When using the AM-PM rule, females detected in estrus in the morning are inseminated in the evening, and females detected in estrus in the evening are inseminated on the following morning (Larson et al., 2009). The utilization of estrus detection aids (tail chalking, Estrotect Breeding Indicators, Kamar Mount Detectors, activity monitors, and others) are recommended to maximize estrus detection rates. Although not required, an intravaginal progesterone device could also be utilized to minimize the number of days spent detecting estrus. In this case, the device is inserted 12 to 14 days after the first fixed-time artificial insemination and removed no later than 19 days after the first insemination. This will result in females exhibiting estrus in a shorter window of time. Overall, this approach is effective to recognize most non-pregnant females and increase the proportion of genetically superior pregnancies. However, it is a labor-intensive strategy and often not feasible for larger herds or herds where technicians are not easily available.

The limitations of estrus detection can be overcome by exposing cows or heifers to a resynchronization protocol. These protocols are similar to conventional estrus synchronization protocols and allow for an additional round of artificial insemination to be performed using a fixed-time approach. In this case, pregnancy diagnosis is performed as early as possible using transrectal ultrasonography 26-28 days after the first fixed-time artificial insemination or a chute-side blood tests performed at least 28 days after insemination. An important consideration when resynchronizing is that females are handled on days that are critical for pregnancy establishment. Therefore, producers should be cautious and focus on minimizing stress during these handling events, as failing to do so could result in pregnancy loss, ultimately defeating the purpose of resynchronization protocols. Another important consideration for resynchronization is the interval between inseminations that might potentially have a negative impact on calving distribution in the subsequent year.

Future Directions on Pregnancy Diagnosis

Diagnosing non-pregnant females earlier than industry-standard methods gives producers the opportunity to utilize resynchronization programs earlier. Continued research in this field will lead to the development of new approaches to resynchronize cattle, allowing producers to increase the number of females pregnant to artificial insemination or embryo transfer earlier in the breeding season.

Color Doppler ultrasonography of the corpus luteum

Color Doppler (CD) ultrasonography is a relatively newer technology in veterinary medicine. This technology allows for the detection of blood perfusion providing clinically relevant information regarding the vascularization of specific organs or structures within these organs. Corpus luteum (CL) is an important ovarian structure that is responsible for progesterone production. Progesterone plays an important role modulating the uterine environment and is required for pregnancy establishment and development. Cows or heifers that fail to become pregnant undergo CL regression starting approximately 16-18 days after breeding. The process of CL regression is characterized by a decreased in luteal blood perfusion, followed by a decrease in luteal size (Pugliesi et al., 2014). Therefore, the use of CD has been proposed as a method to diagnose pregnancy earlier than conventional ultrasound by recognizing females that are undergoing CL regression (Siqueira et al., 2013; Pugliesi et al., 2014; Scully et al., 2015).

Studies using *Bos indicus* beef cattle observed no false-negative results and only 9% false-positive results when CD was utilized 20 days after fixed-time artificial insemination (Pugliesi et al., 2014), indicating that the majority of non-pregnant females can be recognize as early as day 20 of gestation. Yet, several physiological differences exist between *Bos indicus* and *Bos taurus* breeds, including differences in CL morphometry (Sartori et al., 2016) and early embryonic mortality (Reese et al., 2020). Therefore, it was previously unclear if the accuracy of CD examinations in *Bos taurus* and *Bos indicus* beef cattle populations were comparable. More recently, CD studies were replicated in *Bos taurus* beef cows and replacement heifers, resulting in similar accuracy (Holton et al., 2022a,b) and indicating that CD can also accurately recognize most non-pregnant *Bos taurus* females earlier than industry-standard methods (reviewed by Fontes and Oosthuizen et al., 2022). Representative images of cows with high, medium, and low blood perfusion are shown in **Figure 2**. Cows with low blood perfusion on day 20 are non-pregnant, whereas cows with medium and high blood perfusion are considered pregnant (Holton et al., 2022).

Recent developments in CD research have resulted in the development of new resynchronization protocols that reduce the interval between inseminations. In *Bos indicus* cattle, early resynchronization was successfully initiated between 12 to 14 days after fixed-time artificial insemination with pregnancy diagnosis performed via color Doppler on days 20 to 22 of gestation (Silva et al., 2022; Palhão et al., 2020; Pugliesi et al., 2019). In these studies, suitable conception rates were achieved for both the first and second fixed-time artificial insemination, and overall, more females were pregnant to artificial insemination within the breeding season when compared to a single insemination followed by natural service (Palhão et al., 2020). The combination of CD with early resynchronization reduced the time between the first and second fixed-time artificial insemination to 22-25 days compared with 32-35 days when using conventional ultrasound (Epperson et al., 2020). However, these studies were performed in Brazil where the use of estrus synchronization protocols rely on the use of estradiol products. Because estradiol products are not approved for use in estrus synchronization in the U.S., further research is required to establish resynchronization protocols in combination with color Doppler examinations without the use of estradiol products.

Pregnancy associated glycoproteins as an earlier pregnancy diagnosis tool and a predictor of late embryonic mortality

Pregnancy associated glycoproteins significantly increase in the circulation of pregnant females around day 24 of gestation; however, commercially available tests for pregnancy using PAG usually recommend that samples are collected no earlier than 28 days after breeding (reviewed by Wallace et al., 2015). Recent studies have evaluated the use of circulating PAG on days 24 and 25 of gestation in both cows and heifers (Oliveira Filho et al., 2020; Melo et al., 2020; Holton et al., 2022). Although circulating concentrations of PAG are greater in pregnant females compared with non-pregnant, the presence of false-negative results (~7%) limits the use of PAG for diagnosis earlier than current recommendations.

Interestingly, circulating concentrations of PAG around day 30 of gestation in cows that experience pregnancy loss between days 30 and 100 of gestation is decreased compared with cows that successfully maintain their pregnancy (Pohler et al., 2016; Oliveira Filho et al., 2020; Holton et al., 2022). These observations indicate that PAG might serve as a biomarker of placental function and potentially be used to predict pregnancy loss (Pohler et al., 2016). However, while circulating concentrations of PAG during early in gestation differ (day 30) between cows that will later experience pregnancy loss and cows that maintain pregnancy, recent studies have failed to accurately predict pregnancy loss using PAG during early gestation (Holton et al., 2022). Future research and improvements in PAG assays may lead to earlier detection of pregnancy or accurate prediction of pregnancy loss in beef cattle.

MicroRNAs – diagnose pregnancy and/or forecast embryonic mortality

MicroRNAs (miRNA) are small non-coding RNA molecules (18-22 nucleotides) that can regulate gene expression at the post-transcriptional level (Vashisht and Gahlay, 2020). Studies show that these miRNAs have important roles in several physiological and pathological events, such as cell proliferation, apoptosis, stress response, metabolism, and others (Sun et al., 2017). These events might directly or indirectly assist reproductive processes, as miRNAs have been reported to act in embryonic stem cells (Tufekci et al., 2014), participate in estrous cycle events (Almiñana et al., 2018), embryo cleavage and implantation (Fereshteh et al., 2018), follicular growth, and oocyte maturation (Almiñana; Bauersachs, 2020). Due to the fact that miRNAs are associated with several reproductive processes, studies have recently started to evaluate miRNAs as potential candidate biomarkers for pregnancy and embryonic mortality (Pohler et al., 2017; De Bem et al., 2017; Schanzenbach et al., 2017). While current research indicate that miRNA have the potential to serve as relevant biomarkers for pregnancy diagnosis in the future, miRNA extraction techniques are still laborious and logistically challenging for use in the beef industry, having little applicability at the current moment.

Conclusion

In summary, pregnancy diagnosis is one of the most important components of proper reproductive management in beef herds. Failing to identify and cull non-pregnant females have clear negative economic implications. While several pregnancy diagnosis methods are available for producers, understanding the characteristics of each method can help producers identify the most feasible method for their operation. Future research on new methods of pregnancy diagnosis methods, as well as the combination of early diagnosis with novel resynchronzation programs might help producers increase the number of females that become pregnant to assisted reproductive technologies (artificial insemination or embryo transfer) early in the breeding season.

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Figure 1 Ultrasound images of bovine pregnancies at different ages. A: An embryo on day 29 of gestation; B: A fetus on day 49 of gestation; C: A fetus on day 70 of gestation. Images are shown at different scales (Obtained from Fontes et al., 2022).

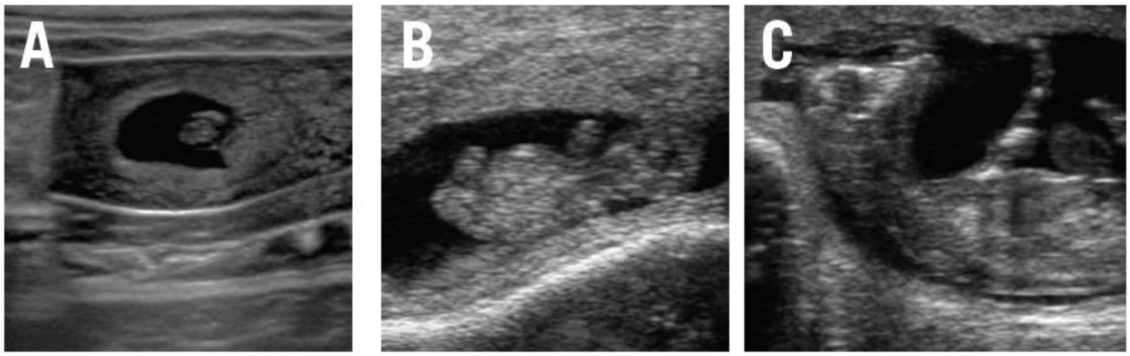

Figure 2. Image collected from a pregnant cow with a highly vascularized CL (**A**) or a non-pregnant cow with a poorly vascularized CL (**B**). Orange and blue images represent sites of CL blood perfusion.