Review of pregnancy diagnosis techniques in cattle and small ruminants
Brian K. Whitlock, Elizabeth A. Coffman
Department of Large Animal Clinical Sciences, College of Veterinary Medicine, The University of Tennessee, Knoxville, TN

Abstract
Pregnancy diagnosis is a common management practice for ruminants and there are a number of methods available, with each having advantage(s) and/or disadvantage(s). A brief review of pregnancy diagnosis techniques in cattle, goats, and sheep is provided.

Keywords: Cattle, goat, sheep, pregnancy, diagnosis

Introduction
Pregnancy diagnosis is a common management practice for the purpose of identifying pregnant and non-pregnant ruminants. Early and accurate pregnancy diagnosis is of considerable value in improving efficiency of production. By discriminating between pregnant and non-pregnant animals, better management control is made possible. There are a number of methods available for diagnosis of pregnancy in ruminants and the method(s) of choice will depend on equipment availability, number of days post-breeding the animals are examined, and the desired accuracy. This manuscript describes different methods of pregnancy diagnosis in cattle and small ruminants and assesses and compares their usefulness.

Observation
Traditional methods of pregnancy diagnosis are presumptive based on observation of the animals and include non-return to estrus, abdominal enlargement, and onset of lactation. These methods are non-invasive and require only basic knowledge and training.

Non-return to estrus
In non-return to estrus, it is assumed that animals that are not observed to return to estrus within the normal interval (21 days average in cows and does, 17 days average in ewes) are pregnant. This approach is advantageous over the other two observational methods in that it occurs earlier in gestation and thereby limits the time before re-breeding, treatment, or culling of open animals. Provided that adequate time, records, and knowledge are applied, non-return to estrus yields the potential to be early and accurate. In the bovine, standing to be mounted is the most reliable indicator of heat and is referred to as ‘standing heat’. Secondary signs include vulvar discharge, mounting of other cows, vocalization, restlessness, swelling or reddening of the vulva, and lip curling. In does, increased vocalization, tail-flagging, frequent urination, decreased appetite, decreased milk production, seeking of interaction with bucks, and standing to be mounted are all indicators of estrus. In ewes, a marking harness or teaser ram is most commonly used to aid in estrus detection.

Several techniques have been developed to aid in detection of heat to alleviate this problem. In a review of electronic devices used in cattle to identify estrus animals, Rorie, et al. reported that mount detectors, pedometry, and electrical resistance of reproductive tract fluids increase efficiency of heat detection compared to visual identification. Estrus detection animals may serve as additional aids in estrus detection. While androgen treatment of steers, freemartins, and cows could be employed, its application is illegal in the United States. Instead, surgically-altered bulls are frequently used. Epididectomy, penile or preputial deviation, and penile or preputial fixation are the most common modifications in the preparation of teaser bulls. Tailhead markers also serve to indicate animals in standing heat. In goats, testosterone-treatment of wethers or does, epididectomy and vasectomy of bucks, and intersex animals as well as marking harnesses and ‘buck rags’ act to aid in heat detection. Similarly, vasectomized rams with marking harnesses are frequently used in sheep. In one study of 177 ewes, a harnessed vasectomized ram raddled only 1 of 118 pregnant ewes (99% accuracy); however, only 31 of 59 (53%) non-pregnant animals were marked, demonstrating an the limited sensitivity of this test in detecting non-pregnant animals.

Efficiency of heat detection (sensitivity) and accuracy (positive predictive value) serve as limitations in diagnosis pregnancy in this manner. In a study of estrus detection in dairy operations, the efficiency of detection is usually less than 50%; in beef cattle, detection rates are often even lower due to less frequent observation and less intensive management. Similarly mediocrite efficiency is also likely in sheep and goats. The length of estrus in cows is reported to range from 0.5-24 hours or 33 minutes to 35.8 hours, and 24% of dairy cows have estrus periods characterized as low intensity and short duration (<1.5 mounts per hour and <7 hours duration). Duration in does
is 12-48 hours. Based on this information, it is possible to understand how animals in estrus could be overlooked. Other factors affect display of estrous behavior in cows, including type of housing, footing, lameness issues, number and reproductive status of herdmates, time of day, environmental temperature, nutrition, and level of milk production. Indeed, malnutrition may be a common cause of failure to cycle, particularly in beef breeds at the end of the cold season. Similar limitations exist in small ruminants. Additionally, some does will not stand for particular bucks (especially if they are young or de-scented), even if the does are in heat. As all these factors contribute to the degree of estrous behavior displayed, they have the potential to limit efficiency of heat detection.

Accuracy may also be limited by confounding factors that prevent return to estrus. Cystic ovarian disease is the second most common reason (besides pregnancy) for failure to return to estrus in the bovine. Any other form of ovarian disease that alters cyclical activity may similarly result in lack of behavioral signs despite absence of pregnancy. Other reproductive abnormalities that disrupt the estrous cycle may also complicate diagnosis. Hydrometra, which has a reported incidence of 3.0 to 20.8% in dairy does, is one example of a condition that could lead to a false positive. Additionally, sheep and goats show seasonal anestrous, and breeding prior to the transitional period or outside of the normal breeding season via a synchronization and induced-ovulation protocol limit the potential application of non-return to estrus in determination of reproductive status.

It should also be noted that false negatives may occur in using this criterion for determination of pregnancy status. Some females continue to exhibit signs of behavioral estrus despite being pregnant. This can be problematic as administration of prostaglandins or repeat insemination usually results in abortion. Additionally, teaser animals with very high libido may mount non-receptive animals; this is particularly significant when relying on marking to indicate estrus rather than observation of standing heat.

Weight gain

Weight gain, particularly abdominal weight in the third trimester, and abdominal contour may be used as indicators of pregnancy. In ewes at four months of gestation, a body weight increase of 6-12% was documented in animals carrying singletons and 13-16% in animals with twins. While waiting for observation of this change is very non-invasive, serious limitations are evident. First of all, application of this approach implies a noteworthy delay in arriving at a presumptive diagnosis, and the cost of retaining and feeding an open animal while waiting for weight gain to manifest can be significant. Also, alternative causes of distended abdomen such as hydrometra or stretched abdominal muscles may account for apparent weight gain and lead to false positives. False negatives may also occur, particularly in primiparous females carrying a single fetus. In a review of 24 diagnostic methods used in sheep, increase in body weight was considered unreliable or unsatisfactory in its ability to identify pregnant animals.

Onset of lactation

Onset of lactation or udder development may be used as suggestive signs of pregnancy. As with weight gain, a major drawback of reliance on this physiological change is that it does not occur until late gestation and therefore could result in significant economic losses through lost time and retention of open animals. It does exhibit some reliability. In a comparison of several methods of pregnancy diagnosis, at 130 days of gestation, 84% of ewes showed enlarged, firm udders and 14% demonstrated slight to moderate swelling. Elimination of the dry period and continuous lactation may be applied in dairy cows or goats, as it does not appear to adversely affect milk yield in does and decreases metabolic imbalances despite a slight decrease in production in cows. In these systems, onset of lactation or udder development is eliminated as a marker of pregnancy status. False positives may occur in the application of this approach. Inappropriate lactation syndrome has been described in small ruminants, with numerous anecdotal reports in addition to those in the literature.

Despite their relative ease of application, observational forms of pregnancy detection lack the sensitivity and specificity required for diagnostic precision. In fact, the errors and limitations probably make these tests more expensive in the long-term, through retention of open animals, as compared to other tests that cost more initially. Due to great advances over the last few decades, these approaches are being applied with decreasing frequency as techniques with lower error rates are adopted.

Palpation

Abdominal palpation/ballottement

Abdominal palpation for pregnancy diagnosis in ewes and does is a traditional management method used by producers throughout the world. Tentative diagnosis is done by balloting at the base of the udder. The gravid uterus or fetus can sometimes be palpated through the abdominal wall by placing a hand on either side of the
abdomen and lifting upwards. If a fetus is felt to drop on the palpating hand the animal is pregnant. This technique becomes easier and more reliable as pregnancy advances and is easier in thinner ewes and does. Pregnancy was diagnosed in does with a 76% accuracy after 80 days of gestation by the abdominal palpation method. At 61 to 70 days of gestation, the accuracy of abdominal palpation method has been reported to be 70%. Accuracies of 80 to 95% were reported in ewes at 90 to 130 days of pregnancy and it was estimated that an experienced operator could handle sheep at a rate of 200 per hour provided adequate assistance. A fetus can sometimes be balloted low in the right flank during the last month of gestation. The examination can be made easier by withholding feed and water for at least 12 h before examining the animals. Since this method is simple and does not involve any equipment it can be used by sheep and goat owners to screen their flock. Although frequently used by sheep and goat owners this technique is not an acceptable method of pregnancy determination because of less than ideal accuracy and the inability to determine pregnancy status early.

Rectal-abdominal palpation
Rectal-abdominal palpation technique for diagnosing pregnancy has been used in the ewe and doe. It is recommended that ewes and does be held off feed overnight prior to the examination to improve speed and accuracy of this technique. Animals are placed in dorsal recumbency (e.g., a laparotomy cradle) for examination. A soap enema is injected into the rectum and a lubricated rod with a rounded tip is inserted into the rectum to a depth of 30 to 35 cm. The free hand is placed on the caudal abdomen while the rod is manipulated with the other hand. The rod is moved ventral and dorsal and from medial to lateral until an obstruction is encountered and palpated against the abdominal wall or a decision is reached that the ewe or doe is not pregnant. The method is approximately 97% accurate at 60 to 64 days post-mating and requires less than 30 seconds per ewe allowing potentially 200 ewes per hour to be examined using this technique. This technique is 94 to 97% accurate in diagnosing pregnancy in does 55 days after breeding. However, this procedure is not without risk. Rectal trauma, abortion, and even death have been reported following rectal-abdominal palpation. It takes significantly more time to make a diagnosis in ewes which are not pregnant compared to those which are pregnant and the longer time taken for the non-pregnant animals may explain the higher incidence of perforation amongst them.

Palpation of the uterus via laparotomy
The uterus can be palpated directly through a small laparotomy incision made cranial to the mammary gland and approximately 1.5 cm lateral to the midline. The incision should be sufficient to allow the introduction of two fingers to palpate the uterus. An enlarged thin-walled uterus containing fluid is taken as positive evidence of pregnancy. Direct palpation of the uterus gave more than 92% accuracy in diagnosing pregnancy in ewes four to five weeks pregnant. An experienced operator may detect cotyledons at 42 days after service. Direct palpation of the uterus approaches 100% accuracy after 42 days of gestation. Although this method is very accurate, it is not applicable under field conditions due to surgical risks which reduce the subsequent fertility of animals being tested. Its use is limited to experimental purposes or during abdominal exploratory for other purposes.

Palpation of the cervix
Palpation of the cervix involves digital palpation of the external cervical os per vagina at 50 days or more post-breeding. A very soft, blunted cervix or inability to reach the cervix is suggestive of pregnancy while a firm conical-shaped cervix projecting into the vagina is suggestive of non-pregnancy. In addition to palpation of the cervix, cervical mucus examination has been described with limited success. The Estroscope was used to determine changes in the color and flow elasticity of cervical mucus. Changes in color of cervical mucus from clear and colorless to opaque and pale yellow along with increased elasticity were obvious by the fourth week of pregnancy. Unfortunately, for both techniques similar observations would be made in pseudopregnant animals, and animals would be erroneously categorized as pregnant.

Transrectal palpation
Transrectal palpation of the uterine contents and the ovaries is the most common method used by veterinarians for diagnosis of pregnancy and ovarian structures in cattle. Transrectal palpation had been the standard method of detecting pregnancy in cattle until ultrasonography of the reproductive tract became more widespread. However, transrectal palpation is still a very important diagnostic tool.

Palpation of the ovaries per rectum. Detection of a corpus luteum (CL) is important for making decisions regarding treatment of cattle. The CL persists as a result of the maternal recognition of pregnancy. The presence of a full-size (20 to 25 mm diameter), mature CL 20 to 22 days after a cow has been inseminated is suggestive of
pregnancy. Follow-up examinations conducted at a later date show this method to be 85 to 90% accurate as a means of predicting the outcome of pregnancy.\(^1\) The limitations of this technique for the detection of pregnancy are the same as those which apply to the use of plasma or milk progesterone concentrations. Unfortunately the technique of transrectal palpation to detect a functional CL is inaccurate.\(^28\) Previous studies have determined that the sensitivity of transrectal palpation for detection of CL’s ranges from 33 to 96%, and specificity ranges from 22 to 100%.\(^28,29\) Detection of ovarian structures by transrectal palpation requires skill and in general more experienced clinicians are more accurate than less experienced ones.\(^30\) Lastly, there may be some risk of enucleating CL’s during transrectal palpation of the ovaries thus causing the termination of a diagnosed pregnancy. Due to the inaccuracy of this technique and the risk of enucleating a corpus luteum of pregnancy, the authors do not recommend transrectal palpation for persistence of a CL to diagnose pregnancy in cattle.

### Palpation of the uterus per rectum.

The traditional method for diagnosis of pregnancy in cattle for the past 150 years has been examination of the uterus by transrectal palpation.\(^31\) Descriptions of the technique have been published previously.\(^1,32\) Palpation of the bovine uterus per rectum is one of the most frequent procedures performed and is the most frequent method used for pregnancy diagnosis and a skilled practitioner is able to detect pregnancy in cattle as early as day 35.\(^33,34\) The importance of a systematic and non-traumatic technique of palpation per rectum cannot be overemphasized. Embryonic and fetal deaths can be induced accidentally by this procedure.\(^35-38\) However, there is contradictory information regarding the potential deleterious effects of palpation per rectum for early pregnancy diagnosis on embryo/fetal viability with some studies suggesting a possible adverse\(^39-43\) effect of early palpation per rectum and other studies suggesting that the time at which the first palpation per rectum was performed after insemination had little effect on the calving rate.\(^44,45\) A positive diagnosis of pregnancy can be made by palpation and identification of: the amniotic vesicle (AV); the fetal membrane slip (chorioallantoic membrane); placentomes; and the fetus (Table 1).\(^1\) The latter two positive signs are detected at an advanced stage when it is of less economic importance.

### Palpation of the amniotic vesicle.

The AV can be detected by transrectal palpation of the bovine uterus as early as 30 days of gestation when the AV is about 10 mm in diameter; by 35 days, it is about 17 mm in diameter. The uterine horn is palpated along its length from base to tip; the AV can be identified as a distinct spherical, turgid object floating in the allantoic fluid. From 30 to 60 days of gestation the AV can be detected by transrectal palpation of the bovine uterus and the diameter of the vesicle is useful in determining approximate stage of gestation (Table 1). Palpation of the AV may not be without risk as some studies have implicated it as a cause of atresia coli and jejuni in calves.\(^46,47\)

### Palpation of the chorioallantois.

From 35 to 40 days of gestation it is possible to palpate the chorioallantois (fetal membrane slip) in the horn ipsilateral to the AV and embryo of cattle. The uterine horn is grasped through the rectal wall between the thumb and a finger, and squeezed gently, allowing the grasped structures to fall away. The chorioallantoic membrane is the first to ‘slip away’, followed by the thicker uterine and rectal walls. The fetal membrane slip can be detected until about day 90 of gestation in the cow (Table 1). Unlike palpation of the AV, palpation per rectum of the chorioallantois for early pregnancy diagnosis is not associated with birth defects of calves.\(^48\) Also, palpation per rectum for early pregnancy diagnosis in dairy cattle using fetal membrane slip does not affect embryo/fetal viability.\(^49\)

### Palpation of the fetus.

From about 60 or 65 days of gestation, the AV loses its turgidity. It is therefore possible to palpate the fetus directly and estimate the stage of gestation from the size of the fetus (Table 1). However, the fetus is not always within the reach of the examiner. Depending on the size of the dam and the examiner, the fetus may be out of reach (descended into the abdomen) between 120 and 210 days of gestation. While the fetus is not accessible, other positive/cardinal signs of pregnancy must be sought to make a definitive diagnosis.

### Palpation of the placentomes.

Placentomes (caruncles and cotyledons) can first be palpated from about ten to 11 weeks of gestation as irregularities on the surface of the uterine wall, particularly along the greater curvature of the gravid horn. From three months onward, the placentomes can be identified as discrete structures, notably in the uterine body at the base of the horns just over the pelvic brim. Placentomes become progressively larger as the pregnancy advances until approximately day 150 (Table 1).
Indications/suggestions of pregnancy

From about 30 to 35 days of gestation, the uterine horn ipsilateral to the ovary with the CL increases in size relative to the non-gravid horn. At the same time, the uterine wall is thinner because of the accumulation of fluid within the uterus. Unfortunately other factors (e.g. post-partum uterus before complete involution, mucometra, hydrometra, and pyometra) can be responsible for a disparity in the size of the uterine horns.

The middle uterine artery provides the main blood supply to the gravid uterus. To satisfy the demands for increased blood during pregnancy, the middle uterine artery enlarges. As the artery becomes larger, the turbulence in the blood flow results in a change in the pulse character so that the artery feels as if it is ‘buzzing’ or vibrating; this is called fremitus. The uterine arteries are in the broad ligaments and can be located by palpating along the shaft of the ilium. Unilateral fremitus develops on the gravid side from about three to four months of gestation, and is likely to be bilateral from about six to seven months onward in a singleton pregnancy. There is variation in the time of onset and, in some animals, where the blood supply to the uterus is largely from one side, it may only be unilateral. Since fremitus does not develop until the third or fourth month of gestation (delays pregnancy determination) and other factors may be responsible for fremitus developing, fremitus alone should not be used to determine pregnancy status.

Imaging

The application of various imaging modalities in pregnancy diagnosis and monitoring is a mainstay in human reproductive medicine and has been increasingly adapted to animal application. The benefits are numerous and include direct visualization of the fetus and heart rate; monitoring of viability; determination of fetal number, gender, and gestational age; and identification of pathological processes and fetal death. Great advances have been made—especially regarding ultrasonography—in the preceding decades, leading to an increase in its utilization in veterinary medicine.

Radiography

Radiography can be applied for suggestive diagnosis based on the presence of a fluid-filled uterus and as a definitive diagnosis after ossification of fetal bones occurs which allows direct visualization of the fetus. Its use is limited to impossible in the bovine due to the sheer size of the abdomen and necessity of expensive equipment and near-dangerous levels of x-rays required to achieve an image; however, it can be used in small ruminants to diagnose pregnancy and determine fetal numbers. The work of Benzie achieved 96% accuracy from day 43 of gestation in black face sheep.50 A study in Clun Forest ewes suggests accuracy may be lower—closer to 79%—in larger ewes; however, greater than 90% accuracy was obtained in determining number of lambs after day 90 of gestation.51 In Saanen and Toggenburg goats, ossification and fetal skeleton may be observed as early as day 58 and are more reliably evident by day 65; it is suggested that radiographs after day 70 can lead to accuracy in diagnosis of pregnancy and fetal numbers approaching 100%.52 Alternately, it has been recommended to wait until day 90 in the doe to decrease false negatives; hydrometra may also be diagnosed at this point if the uterus is large and fluid-filled without detection of fetal skeletons.53

Radiography is highly accurate in pregnancy diagnosis. The ability to determine fetal number should not be underestimated in its importance, especially in small ruminants, as it allows identification of ewes and does carrying multiples and subsequent implementation of nutritional management to minimize risk of metabolic disorders (pregnancy toxemia). Disadvantages include expense of equipment and procedure, radiation exposure, limited applicability in larger ewes and does, and necessity of waiting until late in gestation (following fetal ossification).

Ultrasonography

Great strides have been made in the application of ultrasonography to pregnancy diagnosis in ruminants over the last several decades. Decreasing cost and increasing availability of equipment; potential for earlier diagnosis of pregnancy; immediate diagnosis with performance of the procedure; and ability to determine fetal gender, viability, approximate fetal age, and existence of some maternal and fetal abnormalities have led to commonplace use of ultrasound in reproductive applications. In addition, transrectal ultrasound is very safe.54,55 An experienced operator can work very efficiently, with reports that up to 300 animals per hour may be examined (60 to 120 if looking for number of fetuses) with transabdominal scanning; transrectal viewing requires more time.56 While cost may still limit its use in production animal medicine, decreasing cost of technology coupled with increasing applicability is likely to ensure growing application in the future.
**A-mode.** A-scan ultrasonography relies on the differential reflection of sound waves, particularly fluid versus tissue, and it has been applied in the past to diagnose pregnancy. A characteristic light, sound, or oscilloscopic blip pattern is produced by the unit with the detection of a fluid-filled structure; when encountered in the caudal abdomen, a diagnosis of pregnancy is made. It displays some reliability in sheep and goats. Additional advantages include use as early as 40 to 50 days of gestation and accuracy approaching 95% at 60 to 80 days of gestation. The shortcomings of this method are evident in a potentially high rate of false positives, such as those that may be misdiagnosed due to a full urinary bladder, pyometra, or hydrometra.

**Doppler.** Detection of pregnancy via the application of the Doppler principle to detect fetal pulse has been applied historically. This technique relies on changes in sound waves produced when they are reflected off of moving objects (blood, heart wall, etc.). Detection of the middle uterine artery, fetal heartbeat, umbilical cord, and fetal movement may be indicators of pregnancy with this procedure. It is safe and reliable and has been applied successfully in cows, ewes, and does. In comparison to A-mode ultrasonography, it is more accurate and allows earlier pregnancy diagnosis in ewes and it also allows detection of multiple fetuses, albeit with low accuracy. Transrectal application in goats achieved 94 to 100% correct diagnoses. However, it has fallen out of favor with increasing utilization of B-mode ultrasound due to the many advantages it presents.

**B-mode ultrasound.** The observation of real-time images based upon the differential reflection of sound waves to an ultrasound probe that serves to both emit and receive signals is the most clinically applicable (and currently the most regularly-utilized) form of ultrasonography applied in reproductive management. Most frequently, a 5.0 to 8.0 MHz linear array or curvilinear probe is employed; 3.5 MHz probes are most useful for transabdominal use, particularly in the bovine, due to their greater tissue penetration. Both transrectal and transabdominal (in the right flank) approaches are described. The transrectal approach has been employed in all ruminant species. In the bovine, the probe can be carried in manually after removing fecal material while the transducer is usually fixed to a rigid extension rod for use in small ruminants. Because the uterus remains in the pelvic cavity during early gestation, this technique is advantageous in that it allows earlier detection of pregnancy. The transabdominal approach is performed high in the right flank. Some operators restrain animals in dorsal or lateral recumbency for this procedure, but it can be performed standing, which is more clinically useful.

In the bovine, with a 5.0 MHz transducer applied transrectally, an AV can be detected at day 13 to 14 and an embryo by day 26 to 29. Accuracy may approach 100% by day 22 in ideal circumstances, and heifers may be diagnosed up to three days earlier than cows. It is suggested that a 5.0 MHz transrectal probe be used after day 24 in the cow, such that a heartbeat may be detected. More recently, monitoring for CL regression by day 20 has been investigated as a potential indicator of pregnancy; greater than 25% regression from day 14 to day 20 may be considered as diagnostic of not pregnant while less than 10% regression denotes pregnancy. This approach has only recently gained attention and is not yet suitable for routine application; however, it is noteworthy in that it allows diagnosis prior to return to estrus and may allow earlier re-breeding. Transcutaneous ultrasound results in very low sensitivity in early pregnancy diagnosis compared to transrectal ultrasound, but it may be used as a non-invasive technique in mid- to late-gestation. In addition to identifying pregnant animals quickly and non-invasively, this approach allows assessment of uterine fluids, placenta, vasculature, and the fetus which may identify potential pathologies.

Generally, in small ruminants, fluid pockets in the uterus representing the embryonic vesicle are evident by transrectal ultrasonography at day 20 of pregnancy, the beginning of formation of placentomes at day 21, and the embryo proper at day 25 to 30. However, visualization of the fluid in membranes has been reported as early as day 15 and observation of heartbeat at days 18 to 19; with a 7.5 MHz transrectal probe, all sheep embryos were counted accurately at day 25. In goats, transrectal ultrasound can reach 98.7% sensitivity and 100% specificity at day 26. Similarly, transrectal scanning of sheep results in high accuracy from days 24 to 34, with specificity of 98% for days 32 to 34. Another report indicated accuracy may reach 99% in the ewe at least 45 days post-breeding. Buckrell suggested that day 45 to 50 may be the ideal time to diagnose pregnancy in sheep and goats. Some studies suggest that after day 25, the transabdominal method is more accurate than transrectal in determining reproductive status. Because transabdominal ultrasound may be done even as the uterus falls over the pelvic brim into the abdomen, it is suggested days 40 to 75 in goats and days 45 to 90 in sheep are acceptable periods to obtain a high degree of accuracy. When transrectal ultrasound is used, fasting the animals for 12 hours and lifting the abdomen may significantly improve diagnostic accuracy in the very early (day 18 to 24) and later (day 41 to 50) periods.

Identification of fetal number is important, especially as it aids in nutritional management to reduce impact of metabolic disorders associated with multiple fetuses. It is possible to recognize twins (and sometimes triplets or quadruplets) via ultrasound in all ruminants as the presence of multiple embryonic vesicles or fetuses. Fowler and
Wilkins determined multiple versus single pregnancies with 97% accuracy in sheep; however, recognition of twins versus triplets was less accurate than determination of singles versus multiples, and ewes were restrained in dorsal recumbency and clipped for their analysis. In small ruminants, it is recommended that days 45 to 100 are optimal for estimation of fetal number.

Fetal gender determination, or fetal sexing, is useful in that it allows planning for future offspring and marketing of animals that are still in utero. In cows, Muller and Wittkowski described fetal gender determination from 73 to 120 days of gestation with 94% accuracy based on observation of the mammary teats or scrotum. Curran, et al. reported earlier diagnosis, at day 55 to 60, based on location of the genital tubercle. The genital tubercle can be recognized as a hyperechoic, bi-lobed structure that migrates to a position between the hindlimbs beneath the tail in the female and just caudal to the umbilicus in the male. In small ruminants, the two lobes may not be discernable, and the genital tubercle may appear as a single echogenic spot on ultrasound; nonetheless, it migrates similarly. Most application has focused on this structure for fetal gender determination. All found gender determination possible in all cases in 15 multiparous Friesian cows from days 56 to 98 of gestation, with an overall accuracy of 97.3%. Accuracy of fetal gender determination in goats is highest at 40 to 60 days gestation, with 100% and 85.7% accuracy in singletons and multiple pregnancies, respectively. However, genital tubercle migration may show breed and individual variation, so it is usually suggested that diagnosis be sought between 55 and 70 days of gestation. Also, genital tubercle migration can occur significantly later in animals produced by embryo transfer as compared to those resulting from natural mating. Sex determination can also be carried out by the transabdominal approach, but with both methods, position of fetus can hinder diagnosis due to difficulty visualizing the necessary structures.

Estimation of gestational stage and development is also possible and provides a potential benefit of ultrasound. Crown-rump length, bi-parietal diameter, eye-socket diameter, trunk diameter, head length, nose diameter, abdominal diameter, umbilical diameter, and meta-carpal length have been applied with some success in estimating fetal age. Simultaneous measurement and application of two different measurements of bones with different growth allometry can increase estimate reliability. Transcutaneous ultrasound may be sufficient to identify structures to aid in estimation late in gestation. Crown-rump length and bi-parietal diameter as well as thoracic, skull, and placentome measurements are correlated with developmental stage in the goat. The relationship of placentome area to gestational age is stronger in does as compared to ewes. Full discussion of findings and regression equations used in stage determination is beyond the scope of this article, but it merits mention that the ability to estimate provides an advantage of pregnancy diagnosis via ultrasonography in cases where exact breeding date is unknown. Furthermore, tracking of fetal development and comparison to established normal values can be particularly useful in the area of advanced reproductive technologies. For further information on the topic, the reader is referred elsewhere.

Observation of the fetus and evaluation for viability and potential abnormalities is a particularly useful aspect of ultrasound in comparison to the other modalities. Irregular contours of the developing embryo or fetus, debris on surfaces or in fluid, breaks in the amniotic membrane, and reduction in fluid volume are a few abnormalities associated with death of the developing conceptus. Cessation of embryonic heartbeat and subsequent loss has been documented in studies of early diagnosis. Abnormalities associated with late gestation and associated ultrasound findings have been reviewed. Hydrallantois, enlarged placentomes, hyperechoic debris, fetal inactivity, and large offspring syndrome are abnormalities that may be recognized on ultrasound. For example, a study involving serial scans allowed identification of hydrops allantois in a ewe at 110 days gestation. This is especially important in its application to advanced reproductive technologies (cloning, in-vitro fertilization, etc.), which are associated with increased incidence of some of the aforementioned issues. It also merits mention that ability to view the uterus and ovaries may allow identification of pathological processes in the non-pregnant animal, many of which may have direct bearing on fertility.

A final note is made that the ability to use color flow Doppler is drawing attention as it may apply to pregnancy diagnosis. Significant changes in blood flow are evident in the pregnant versus the non-pregnant uterus at day 18 of gestation and attempts have been to correlate CL blood flow to pregnancy status in embryo recipients (with low sensitivity and specificity thus far). Currently, these approaches are interesting from a research standpoint but they do not yet have a clinical application. Nonetheless, it will be interesting to observe their development and information they may yield in the future.

There are multiple advantages of using ultrasound to determine pregnancy status, and very accurate results may be obtained by the experienced practitioner. The ability to directly view the embryo or fetus imparts important information regarding its stage, viability, and gender as well as potential recognition of the presence of multiples.
**Chemical assays**

**Estrone sulfate**

Estrone sulfate (ES) is the major estrogen produced by the fetoplacental unit during pregnancy and is conjugated in the fetal liver. Measurement of ES has been used to diagnose pregnancy in cattle, goats, and sheep. Estrone sulfate levels in maternal plasma of cattle are undetectable until approximately day 72 of gestation. Because of the late stage at which it is reliable, it is of little value in the early identification of non-pregnant animals. Estrone sulfate can be detected in sheep plasma from 70 days after conception, whereas in does it can be detected 40 to 50 days post-breeding. Estrone sulfate concentrations measured in the milk of does using an enzyme-linked immunosorbent assay (ELISA) test were able to diagnose pregnancy with an accuracy of 82% and non-pregnancy with an accuracy of 83%. Unfortunately, a positive ES test may or may not indicate the presence of a viable fetus.

**Progesterone assay**

Measurement of concentrations of progesterone in blood and milk is one method for diagnosing pregnancy. The CL is the principal source of progesterone in the cyclic ruminants and is required to maintain early pregnancy. Concentrations of progesterone are also elevated during the luteal phase of non-fertile estrous cycles and thus are not specific indicators of pregnancy. However, determining progesterone concentrations in milk or blood after breeding has been somewhat successful. If ruminants are pregnant, the CL persists and progesterone concentrations remain high while in non-pregnant animals, luteolysis occurs at the end of a non-fertile cycle and progesterone concentrations are low.

Concentrations of plasma progesterone are determined 18 days post-breeding in ewes and 21 to 24 days post-breeding in does and cattle. Concentrations of plasma progesterone measured in ewes on day 18 post-breeding showed that all females diagnosed non-pregnant did not lamb, while 84% of those diagnosed pregnant did lamb and is similar in does as accuracy of diagnosing pregnancy and non-pregnancy in does by determining serum progesterone concentrations was 86% and 100%, respectively.

Concentrations of plasma progesterone in milk generally reflect plasma concentrations; however, concentrations of progesterone in milk are much higher. Milk progesterone concentrations are above 10 ng per ml from 22 and 26 days after breeding in does classified as positive for pregnancy. Like plasma progesterone concentrations, an accuracy of 86% for detecting pregnancy and 100% for detecting non-pregnancy was reported in does. Unfortunately milk progesterone concentration varies from day to day and also with the type of milk sample obtained. Analysis of milk progesterone has been used as a method of pregnancy diagnosis in cattle at 20 to 23 days with accuracy similar to rectal palpation. Measurement of concentrations of progesterone in milk is reasonably accurate for identification of non-pregnant cows (94%) but is not sufficiently accurate (77%) for recognition of pregnant cows. Consequently, serum and plasma concentrations of progesterone tend to be more reliable and accurate predictors of pregnancy than concentrations determined from milk.

Overall progesterone testing accurately predicts non-pregnancy but is only a fair test for diagnosing pregnancy. Increased progesterone concentrations only indicate the presence of a functional CL and there are several conditions that may extend the luteal life and result in false positive results. A benefit of determining circulating or milk progesterone concentrations as an indicator of extended CL life—and possibly pregnancy—is that false negatives for pregnancy almost never occur. However, false positives for pregnancy diagnosis are common and measurement of progesterone concentrations in blood and milk requires expensive technology.

**Early pregnancy factor/early conception factor**

Early pregnancy factor is a glycoprotein that is produced as a result of fertilization and can be detected in serum or milk of pregnant cattle as early as one to two days after insemination. Early pregnancy factor is present in plasma within hours of conception and concentrations fall below detectable levels soon after death or removal of the embryo. Initially the assay for detecting early pregnancy factor was sensitive but time-consuming and not suitable for routine use. More recently, the early conception factor test was marketed as a “cow side” immunoassay capable of diagnosing the non-pregnant cow within 12 to 48 h after ovulation. Unfortunately, this test has been reported as inaccurate in cattle and horses.

**Pregnancy associated glycoprotein**

The pregnancy-associated glycoproteins (PAGs) are abundantly expressed in the outer cell layer of the placenta of ruminants from the time the placenta attaches until parturition. Pregnancy-associated glycoproteins...
are separated into two groups (PAG-1 and PAG-2) and at least 21 bovine PAGs have been identified.\textsuperscript{124,125} In cattle, some PAGs from the PAG-1 subgroup become detectable in the maternal circulation beginning at approximately the time of implantation (day 25). Concentrations of these glycoproteins steadily increased throughout pregnancy, peaking just before parturition.

The most widely recognized work with PAGs has been the development and commercial application of pregnancy-specific protein B (PSP-B) as a biochemical marker of pregnancy; BioPryn\textsuperscript{a} (BioTracking, LLC, Moscow, ID) is a commercially available ELISA designed to detect PSP-B.\textsuperscript{126-129} Immunooassays for PAGs other than PSP-B that are likely based on similar antigens have been developed.\textsuperscript{130-135} Mean PAG concentrations in cattle begin to increase from days 15 to 35, but variation in serum PAG concentrations among cows precludes their use as a reliable indicator of pregnancy until days 26 to 30.\textsuperscript{136,137} The first report using specific antisera for detecting pregnancy-associated glycoproteins secreted by the binucleate cells of the trophectoderm into the maternal plasma, allowed for the accurate discrimination between pregnant and non-pregnant goats from 21 days after breeding.\textsuperscript{138} Subsequent study indicated PAG determination was highly accurate (≥ 99%) on days 24 and 26 after mating in goats.\textsuperscript{65} Detection of PAG’s has also been used in sheep to accurately detect pregnancy. Pregnancy diagnosis by assay of PSP-B in does and ewes is also available commercially (BioPRYN\textsuperscript{a}) and samples can be shipped to several different affiliated laboratories. According to the manufacturer’s recommendation serum samples should be taken from does and ewes 30 and 22 days or more after breeding, respectively.\textsuperscript{126}

Variations in inter-assay agreement of PAG occur and may be due to variations in the antiserum utilized. In two studies, one designed to determine factors affecting plasma PAG concentrations in pregnant high-producing dairy cows and the other to assess the predictive importance of maternal PAG concentrations in recipients carrying somatic clones to pregnancy outcome, PAG concentrations varied with the assay and more specifically the primary antibody being used.\textsuperscript{130,140} It is likely that the different polyclonal antisera used in radioimmunoassay (RIA) systems to measure PAG recognize other PAG molecules, and because more than 100 genes encode various PAG molecules in ruminant placenta, varying affinities would explain the inconsistent or varying inter-assay results.\textsuperscript{141} Assays utilizing heterologous PAG RIA systems may be more sensitive and demonstrate higher PAG concentrations, improving ability to detect early pregnancy.\textsuperscript{132} Sensitivity and specificity were improved when a monoclonal antibody was used to detect PAG.\textsuperscript{131} The monoclonal antibody detected only a few PAG-1 family members belonging to the binucleate trophoblasts cell-specific group; use of the monoclonal antibody permitted pregnancy to be detected in all animals by 28 days after artificial insemination.

Persistence of PAG concentrations in the postpartum period or after pregnancy loss is one shortcoming of pregnancy determination based on PAG. Managers and practitioners must be aware of this limitation of current testing technology. The proteins reach a maximum concentration in plasma at approximately the time of parturition; with their long half-life, they remain in circulation for two to three months after calving.\textsuperscript{133,137,143,144} When using plasma PAG for pregnancy diagnosis, the concentrations expected at various times following conception and previous parturition must be taken into account.\textsuperscript{137,143} The current manufacturer’s recommendation is that serum samples be taken from cattle 90 days or more after parturition and 30 days post-breeding.\textsuperscript{126} Cows less than two months postpartum may have PAG concentrations above the pregnancy/open cut-off value, even if they had not been inseminated. An important consideration is that PAG detected by monoclonal antibodies have a relatively shorter half-life, averaging only 4.3 days during the post-calving period, and PAG detected with monoclonal antibodies were below pregnancy threshold concentrations by week eight postpartum and 2.7-7.0 days after induction of embryonic mortality.\textsuperscript{131,146,147}

More recently a serum PAG-based, rapid ELISA pregnancy test for cattle has been described.\textsuperscript{148} The advantages of the rapid ELISA PAG test are: 1) that the test can be performed on the farm or at a nearby facility in approximately 90 min and requires only a simple thermostatically controlled water bath, 2) the diagnosis of pregnant or non-pregnant can be made by a subjective visual assessment of blue color with a high degree of accuracy (sensitivity and negative predictive value approached 100% and the specificity and positive predictive value were greater than 92%), 3) because of the relatively short half-life of the PAG detected by the assay, pregnancy detection with the rapid ELISA test could be performed as early as 50 days postpartum, and 4) the rapid ELISA test was highly accurate (95%) when used as early as 25 to 29 days after insemination.\textsuperscript{148} Lastly, an ELISA for measuring PAG in milk has also been tested.\textsuperscript{149} Pregnancy could reliably be diagnosed from day 28 onwards by testing serum and from day 150 onwards by testing milk.\textsuperscript{149} Thus, PAG determination in milk is possible, but, due to the low concentrations, a more sensitive assay is needed before this test has practical relevance.

Interferon-stimulated gene 15

Trophoblast cells of bovine, caprine, and ovine embryo secrete interferon tau (IFN-τ). This substance is responsible for the maternal recognition of pregnancy, and for preventing the release of prostaglandin F₂α,
regression of the CL, and the subsequent return to estrus. In the absence of a viable embryo, there will be no or insufficient INF-τ secretion, resulting in normal luteolysis and a return to estrus. Interferon tau also induces synthesis and secretion of interferon-stimulated gene 15 (ISG15) in the uterus of cattle. Subsequently ISG15 is released by the endometrium when IFN-τ is released from the conceptus and ISG15 expression in peripheral white blood cells was greater in pregnant cows than in non-pregnant cows 18 days after insemination. Identification of non-pregnant cows on days 18 to 20 after first insemination would facilitate a second insemination of non-pregnant cows approximately 10 days earlier than waiting to use current imaging and/or chemical detection techniques. Prediction of non-pregnant cows based on low ISG15 was 100% accurate when examining serial collections (days 15 to 32 after insemination). However, low ISG15 mRNA was 89% accurate in correctly predicting non-pregnant cows when examined on a single day, day 18, following insemination. In the same study, progesterone on a single day, day 18, had a negative predictive value of 100%. This is an important practical issue, since tests with a high negative predictive value will avoid inadvertent treatment of pregnant females with luteolytic agents and reduce the chances of iatrogenic abortions.

Table 1. Guide to transrectal palpation of cattle for pregnancy diagnosis (modified and used here with permission courtesy of R.L. Carson).

<table>
<thead>
<tr>
<th>Days*</th>
<th>Amniotic Vesicle Size†</th>
<th>Fetal Size†</th>
<th>Fetal Membrane Slip§</th>
<th>Placentomes†</th>
<th>Conceptus Location</th>
<th>Fremitus**</th>
</tr>
</thead>
<tbody>
<tr>
<td>35</td>
<td>Pea</td>
<td>½</td>
<td>Relative</td>
<td>Pelvis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>42</td>
<td>Grape</td>
<td>1</td>
<td>Relative</td>
<td>Pelvis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>48</td>
<td>Plum</td>
<td>2</td>
<td>Bilateral</td>
<td>Pelvis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>53</td>
<td>Bantam Egg</td>
<td>3</td>
<td>Bilateral</td>
<td>Pelvis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>58</td>
<td>Grade A Egg</td>
<td>4</td>
<td>Bilateral</td>
<td>Pelvis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>Duck Egg</td>
<td>Palm</td>
<td>Bilateral</td>
<td>Pelvis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>65</td>
<td>Hand</td>
<td>Mouse</td>
<td>1 Bilateral</td>
<td>Pelvis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>75</td>
<td>Rat</td>
<td>2</td>
<td>Bilateral</td>
<td>Pea</td>
<td></td>
<td></td>
</tr>
<tr>
<td>90</td>
<td>Rat</td>
<td>3</td>
<td>Bilateral</td>
<td>Dime</td>
<td></td>
<td></td>
</tr>
<tr>
<td>105</td>
<td>Cat</td>
<td>4</td>
<td>Nickel</td>
<td>Abdomen</td>
<td></td>
<td></td>
</tr>
<tr>
<td>120</td>
<td>Cat</td>
<td>Hand</td>
<td>Quarter</td>
<td>Abdomen</td>
<td>Ipsilateral</td>
<td></td>
</tr>
<tr>
<td>130</td>
<td>Beagle</td>
<td>Fifty</td>
<td>Fifty</td>
<td>Abdomen</td>
<td>Ipsilateral</td>
<td></td>
</tr>
<tr>
<td>180</td>
<td>Brittany</td>
<td>Fifty</td>
<td>Fifty</td>
<td>Abdomen</td>
<td>Ipsilateral</td>
<td></td>
</tr>
<tr>
<td>210</td>
<td>Pointer</td>
<td>Fifty</td>
<td>Fifty</td>
<td>Pelvis</td>
<td>Bilateral</td>
<td></td>
</tr>
<tr>
<td>240</td>
<td>Doberman</td>
<td>Fifty</td>
<td>Fifty</td>
<td>Pelvis</td>
<td>Bilateral</td>
<td></td>
</tr>
<tr>
<td>270</td>
<td>German Shepherd</td>
<td>Fifty</td>
<td>Fifty</td>
<td>Pelvis</td>
<td>Bilateral</td>
<td></td>
</tr>
</tbody>
</table>

*Day of gestation. †Positive/Cardinal signs of pregnancy. ‡Finger approximately 1.5 to 2.0 cm wide. §Finger and hand widths. 
Palpation of the chorioallantoic membrane (fetal membrane slip) will initially be detected in the gravid horn but later in gestation is detected in the non-gravid horn as well. **Depending on the stage of pregnancy there will be fremitus in the middle uterine artery ipsilateral to the gravid horn or bilateral.

References


