Video Article

Transabdominal Ultrasound for Pregnancy Diagnosis in Reeves' Muntjac Deer

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Abstract

Reeves' muntjac deer (*Muntiacus reevesi*) are a small cervid species native to southeast Asia, and are currently being investigated as a potential model of prion disease transmission and pathogenesis. Vertical transmission is an area of interest among researchers studying infectious diseases, including prion disease, and these investigations require efficient methods for evaluating the effects of maternal infection on reproductive performance. Ultrasonographic examination is a well-established tool for diagnosing pregnancy and assessing fetal health in many animal species[1-7], including several species of farmed cervids[8-19], however this technique has not been described in Reeves' muntjac deer. Here we describe the application of transabdominal ultrasound to detect pregnancy in muntjac does and to evaluate fetal growth and development throughout the gestational period. Using this procedure, pregnant animals were identified as early as 35 days following doe-buck pairing and this was an effective means to safely monitor the pregnancy at regular intervals. Future goals of this work will include establishing normal fetal measurement references for estimation of gestational age, determining sensitivity and specificity of the technique for diagnosing pregnancy at various stages of gestation, and identifying variations in fetal growth and development under different experimental conditions.

Video Link

The video component of this article can be found at http://www.jove.com/video/50855/

Introduction

Ultrasonographic examination is a well-described method of pregnancy diagnosis in cattle[1], sheep[2,3], goats[4], horses[5], companion animals[6,7], and other domestic animal species. This imaging modality has also been identified as a valuable tool for pregnancy detection and fetal age estimation in several species of captive cervids, including fallow deer[8-11], red deer[12-16], Hokkaido sika deer[17], and reindeer[18,19]. In many cases, ultrasonography has produced higher accuracy rates than other mechanisms of pregnancy detection, such as serum progesterone or pregnancy-associated glycoprotein analysis[9,18]. Furthermore, ultrasonographic visualization of the reproductive tract can provide additional information regarding fetal viability, gestational age, and identification of developmental abnormalities that would otherwise be difficult to assess. The utility of ultrasonography for evaluating reproductive status suggests that this methodology should be adapted to other species in which active breeding programs are used.

Reeves' muntjac deer (*Muntiacus reevesi*) are a small cervid species native to southeast Asia[20]. While the reproductive cycle of these animals is not well-characterized in their native territories, studies of feral populations in parts of southern England have improved understanding of their breeding patterns[20]. Unlike most cervids, Reeves' muntjac appear to breed year-round, with no apparent seasonal fawning peaks[20-22]. Female muntjac typically give birth to a single fawn following a 210-day gestation period, and reliably enter a postpartum estrus within 24 hr[20-22]. Does that do not conceive during this postpartum period will return to estrus approximately 24-25 days later[20]. In captive situations, reproduction is typically achieved by housing does with one or more intact male. This system produces satisfactory conception rates and requires little technical skill for personnel, however exact breeding dates are difficult, if not impossible, to determine.

Muntjac are commonly used in cytogenetic studies due to the wide range of diploid chromosome numbers and high rate of karyotypic diversification among the different species[23]. These animals are also currently being investigated as a model of prion disease pathogenesis and transmission, with particular interest placed on the study of vertical transmission of chronic wasting disease (CWD) in cervids. The importance of the muntjac in a number of different research applications suggests that improved reproductive technologies should be established to complement current investigative procedures. Specifically, noninvasive mechanisms to assess the gestational period could potentially elucidate critical variations in reproductive performance and fetal growth and development under different experimental conditions. Furthermore, the development of real-time fetal measurement references would be potentially valuable in estimating gestational age in situations where breeding dates cannot be determined. Previous studies have described mechanisms for using fetal measurements, such as crown-rump length (CRL), chest depth (CD), and head length (HL) to estimate the approximate stage of gestation in cervid species[11-14, 16,17]. Additionally, some of these studies have also established guidelines describing when specific developmental milestones, such as skeletal mineralization or fetal heartbeat, can be identified[8-19]. While these data may be useful to help guide reproductive ultrasound analysis in the muntjac, most of the other cervid species studied have much larger body size and longer gestational periods, reducing the translational value of these studies for muntjac applications.

The protocol outlined here describes transabdominal ultrasonography of the female muntjac for the purposes of diagnosing and monitoring pregnancy. Successful execution of this protocol can facilitate early pregnancy detection and evaluation of fetal growth and development. This technique has valuable applications in the conduct of studies investigating reproduction, *in utero* development, or vertical transmission of infectious diseases in a small cervid model and may also be useful for clinical purposes in captive breeding operations.

Protocol

1. Procedural Preparation

All animal procedures described have been reviewed and approved by the Institutional Animal Care and Use Committee at Colorado State University, an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC), Intl. accredited institution.

1. Prior to examination, immobilize animals using appropriate sedation or anesthesia protocols to provide relaxation and allow for appropriate positioning. One handler restrains the deer while a second person administers the sedation to the animal.

   Note: The authors have found the following combination to provide effective sedation in this species: butorphanol (0.45 mg/kg), azaperone (0.035 mg/kg), and medetomidine (0.04 mg/kg), administered intramuscularly in the gluteal muscles.
2. Once the doe has reached a plane of sedation that allows for compliant handling, position her in either dorsal or lateral recumbency on an examination table or within a V-trough. Elevate the head, neck, and thorax slightly (approximately 20-30°) to reduce the risk of regurgitation and aspiration of rumenal contents and to facilitate proper ventilation during the procedure.

   Note: In most cervids or other ruminant species, abdominal ultrasound is performed in standing animals; however, the small size and behavioral considerations of this species requires heavy sedation for examination and sternal positioning is therefore more difficult and may preclude effective imaging technique. Positioning the animal midway between sternal and lateral recumbency may be ideal for reducing the risk of regurgitation and hypoventilation and should be considered if possible. In any recumbent position, it is critical to carefully monitor the animals' respirations and immediately stop the examination and place the animal in sternal recumbency if regurgitation or hypoventilation is noted.
3. If necessary, clip hair from the area of the abdomen to be examined in order to achieve adequate contact between the footprint of the transducer and the skin.

   Note: In early gestation, evidence of pregnancy can be detected in the caudal abdomen near the mammary glands. This region of the muntjac is typically hairless, and therefore shaving may not be indicated at this time. As the fetus grows and the uterus distends to occupy a more cranial position in the abdomen, clipping of the hair will be necessary prior to imaging. Care should be taken to avoid causing any mechanical trauma to the skin, including lacerations from razor blades or thermal injury from electrical clippers.
4. Position the ultrasound unit in a location that allows the technician to easily visualize the monitor while conducting the examination with his or her dominant hand. Input animal identification information (name, identification number, *etc.*) into the system to automatically label saved images and video loops.

2. Transabdominal Reproductive Ultrasound Examination

1. Apply a generous amount of acoustic coupling gel to the caudal abdomen.
2. Place a 5-10 MHz curved linear transducer on the skin of the caudal abdomen, immediately cranial to the pubis, and move it slowly in a cranial direction to evaluate the structures within the pelvic cavity. Scan the left and right quadrants of the caudal abdomen to assess both horns of the muntjac's bicornuate uterus.

   Note: Comprehensive abdominal ultrasound involves assessment of all abdominal viscera, including the liver, kidneys, spleen, gastrointestinal tract, urinary bladder, and reproductive tract. For the purposes of this protocol, only examination of pregnancy will be discussed in detail; however, the normal appearance of other caudal abdominal organs should be recognized as these structures are likely to be encountered during the course of the study. Organs most commonly seen in this location include segments of the gastrointestinal tract, the urinary bladder, and the reproductive tract. The large and small intestines are identified by the characteristically layered appearance of the intestinal wall. The lumen may or may not be pronounced depending on the type and amount of contents, which may include fluid, gas, mucous, or food particles. The urinary bladder may be difficult to identify unless distended with urine, and the layers of the bladder wall are significantly less distinct than those of the intestines. Apart from the uterus, the ovaries may also be found on ultrasound of the reproductive tract of the female muntjac. The ovaries will appear as ovoid structures near the caudal pole of the kidneys, and their appearance may change over the course of the estrous cycle. The presence of multiple mature, fluid-filled follicles within the cortex is suggestive of estrus.

   Note: By rotating and/or "fanning" the scanhead of the transducer, structures can be evaluated in transverse or sagittal planes.
   1. If needed to differentiate a gravid uterus from the urinary bladder, empty the bladder manually by applying gentle pressure to the caudal abdomen or by placing a urinary catheter and repeating the scan.

      Note: Manual expression of the bladder should only be attempted when the animal is in a sternal or upright position, which facilitates a more natural orientation of the abdominal viscera and reduces the risk of trauma to other structures. The uterus may also be differentiated from the urinary bladder by assessing the thickness of the organ's wall. The typical thickness of a moderately distended bladder is approximately 1.4 mm[24], while the authors have found that the more muscular wall of the gravid uterus in the muntjac generally measures between 7-8 mm.

Note: Early pregnancy is difficult to diagnose definitively due to a lack of identifiable fetal structures, and in fact the first indication of pregnancy may simply be a small fluid pocket in the caudal abdomen. If seen at this time, the developing embryo will appear as a hyperechoic, ovoid to amorphous structure within the uterus (**Figure 1**).

3. Make adjustments to the depth, frequency, and gain as necessary to improve contrast and image detail.

   Note: higher frequencies are associated with shorter wavelengths, which are necessary for improved image resolution. Most transducers are capable of operating within a range of frequencies (for example, 6-10 MHz)[24]. Frequency can be adjusted using the scanner controls on the ultrasound unit, and increasing frequency will result in a clearer image. Increasing frequency concurrently decreases the depth of sound wave penetration; therefore, reducing the frequency may be necessary to visualize deeper structures, but resolution may become compromised[24]. For this reason it is important to select the highest frequency possible to reach the desired tissue depth. Adjustments to the gain controls will alter the amplification of sound waves returning to the transducer from the tissues and affect the degree of contrast in the image[24]. The gain should be set to a point where optimal contrast between hyperechoic and hypoechoic tissues is achieved, and these settings may need to be adjusted depending on the structures that are being imaged.

3. Obtaining Fetal Measurements

Note: Gestational age in many domestic animal species can be estimated using established reference ranges for fetal measurements obtained from ultrasound examination. The most commonly used measurements include straight crown-rump length (SCRL), chest depth (CD), and head length (HL)[17]. While sufficient data has not yet been collected to develop reliable gestational age guidelines in Reeves' muntjac deer, measurements may be collected in order to begin establishing these references. Fetal heart rate (FHR) has been shown in other species of cervid and ruminant to increase linearly as gestation progresses to a certain point (typically around day 60 of gestation), at which time the heart rate begins to decrease[17]. FHR may be used to estimate gestational age in conjunction with other ultrasound observations, however it is more commonly used as a measure of fetal viability[17,24]. FHR has been calculated in Reeves' muntjac as early as 35 days post-observed mating.

1. To measure SCRL, locate the uterus on the monitor and make fine adjustments to the position of the transducer until a sagittal view of the entire fetus is obtained (**Figures 2** and **3**). Freeze the image and measure the distance between the most dorsal aspect of the skull and the base of the sacrum (**Figures 2** and **3**).
2. To measure CD, obtain a sagittal view of the fetus (the same image may be used to measure SCRL) and measure the distance between the dorsal and ventral aspects of the thorax (**Figures 2** and **3**).
3. To measure HL, using the same sagittal view of the fetus as described for the SCRL and CD measurements, measure the distance from the occipital crest to the base of nose (**Figures 2** and **3**).
4. To calculate fetal heart rate, identify the heart within the thoracic cavity of the fetus by visualizing its rhythmical contractions. Calculate the heart rate by counting the number of contractions (heart beats) that occur within a 1 min period of time.

   Note: If the ultrasound unit being used has Doppler capabilities, this feature may be employed to further assist in distinguishing the heart from other nonspecific fetal movements.
5. Verify that all saved images and video loops include all necessary identifying information, including animal name or number, date, measurements, and appropriate structure labels.

4. Procedural Conclusions and Recovery

1. Remove all residual ultrasound contact gel from the animal's abdomen using a soft cloth or paper towels. Clean the gel from the transducer prior to storage.
2. If using the sedation protocol described in step 1.1, administer atipamezole subcutaneously at a dose of 0.25 mg/kg to reverse the effects of medetomidine. If using a different sedation protocol in which an antagonist is available, administer this agent to the animal according to published dosages at the conclusion of the procedure to speed recovery.
3. Place the muntjac on a soft bedded surface in sternal recumbency, with the head slightly elevated to reduce the risk of regurgitation and aspiration of rumenal contents. Monitor the animal closely until fully recovered and able to ambulate around the enclosure without assistance.

Representative Results

Using the protocol described here, pregnancy can be diagnosed and monitored in Reeve's muntjac deer with minimally invasive techniques. The earliest time point that pregnancy could be detected by the authors was approximately 35 days following observed mating behavior.

In the early stages of gestation, pregnancy was diagnosed by visualizing a fluid-filled uterus identified as an anechoic space in the caudal abdomen. Within the uterus, the developing embryo appeared as a hyperechoic, ovoid structure with few identifiable features (**Figure 1**). As pregnancy progressed, fetal structures became apparent and a conclusive diagnosis could be made following the observation of skeletal mineralization and fetal heartbeat (**Figures 2** and **3**).

Fetal measurements can be obtained and used to establish reference guidelines for estimating gestational age in cases where an exact breeding date is not known, although we have not yet fully developed this system in the muntjac. Straight crown-rump length (SCRL), HL, and CD are standard fetal measurements that are commonly used to estimate fetal age in many species[17], and these measurements were obtained from ultrasound images of muntjac pregnancies using the protocol described here. All three of these measurements can be obtained from the same properly positioned sagittal image (**Figures 2** and **3**), but separate pictures can be used if the entire fetus is not able to be captured in a single sagittal view. In the future, these techniques can also be used to identify variations in growth and development observed under different experimental conditions and to determine fetal viability.

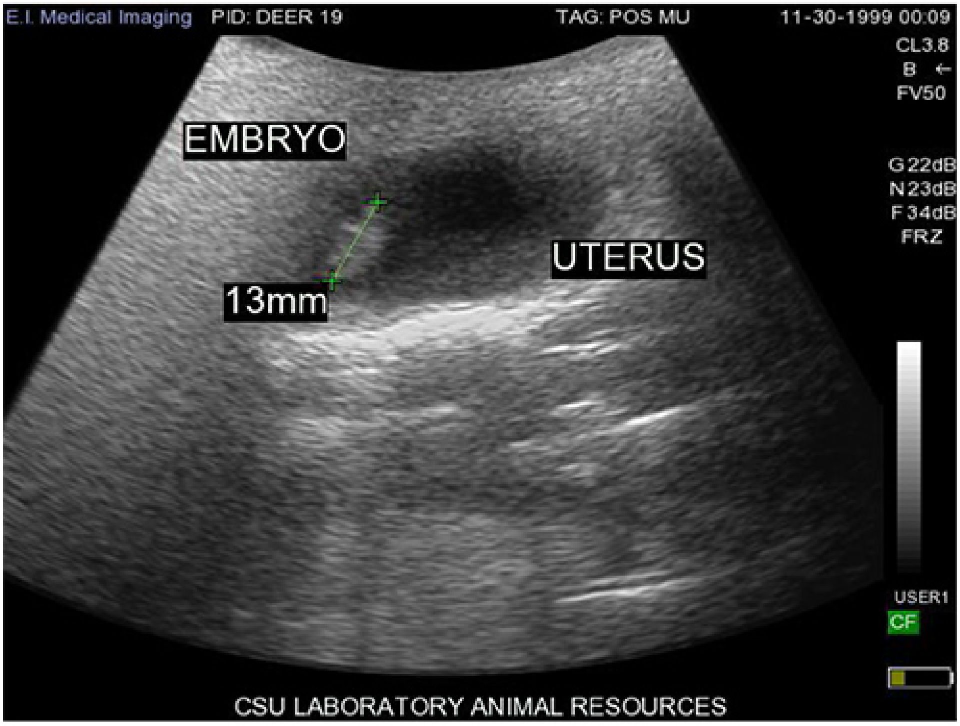

Figure 1. Ultrasound image demonstrating early pregnancy in a Reeves' muntjac deer. The doe in this study had been housed with an intact male muntjac for 46 days, however an exact breeding date is not known. Fetal features are not distinguishable at this stage of gestation.

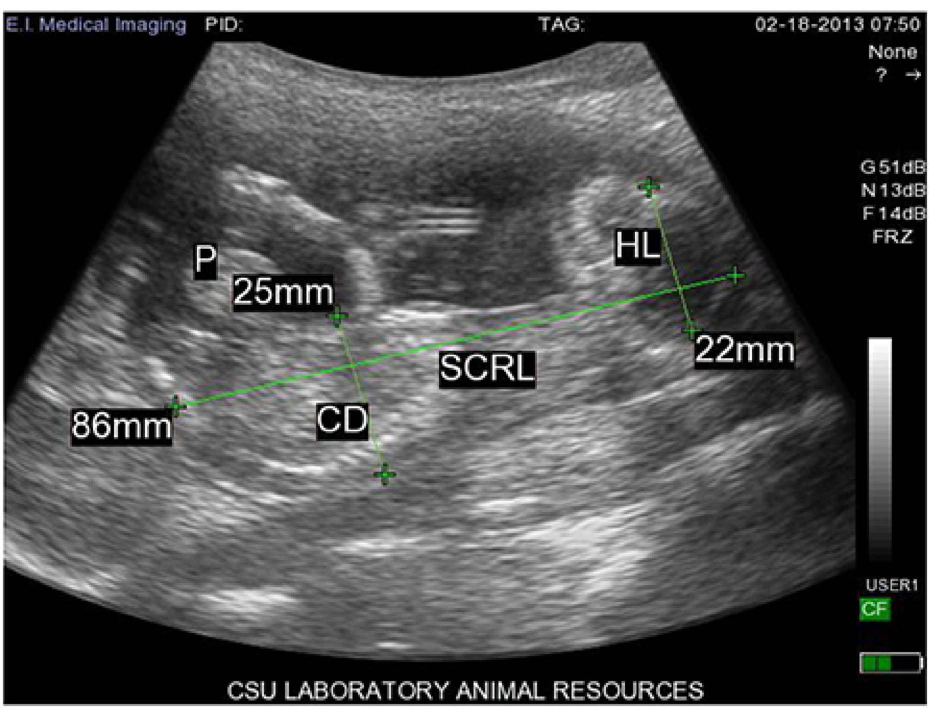

Figure 2. Ultrasound image of the same pregnancy depicted in Figure 1, 91 days following introduction of the doe to the buck. (CD - chest depth, HL - head length, P - placentome, SCRL - straight crown-rump length).

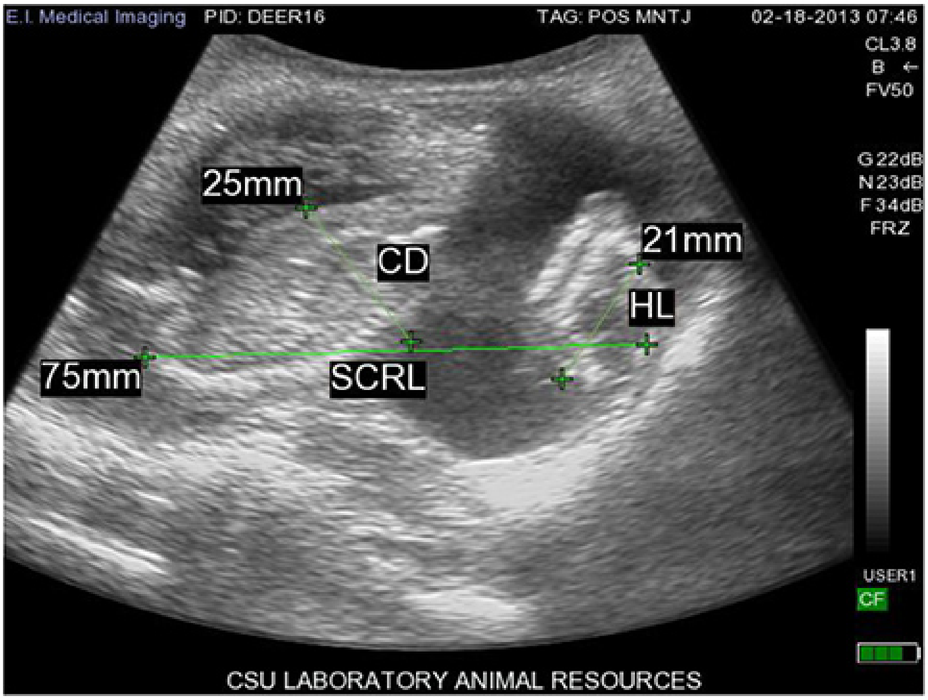

Figure 3. Ultrasound image of a second pregnancy demonstrating appropriate positioning for obtaining fetal measurements. Image taken 91 days following introduction of the doe to the buck. (CD - chest depth, HL - head length, P - placentome, SCRL - straight crown-rump length).

Discussion

This protocol describes the application of transabdominal ultrasonography for pregnancy diagnosis in Reeves' muntjac deer. Consistent with other ultrasound studies, appropriate equipment and technical proficiency are imperative to a successful reproductive examination of the muntjac doe. Careful review of basic mammalian anatomy, the physical principles of diagnostic ultrasound, and the typical ultrasonographic appearance of the abdominal viscera will aid in the reduction of mistaken diagnoses. Furthermore, selection of appropriate transducers and familiarity with the instrumentation and scanner controls is critical for obtaining high-quality images and accurate interpretation.

For many inexperienced ultrasonographers, imaging artifacts are a common cause of poor image resolution and inaccurate assessment of structures. Reverberation at the skin-transducer interface is one of the most frequently encountered artifacts in veterinary abdominal ultrasound, and can be significantly reduced by eliminating the presence of gas between the two surfaces. Adequate hair removal and the generous application of acoustic gel to the skin should be sufficient to minimize the impact of this artifact. Discussions of other imaging artifacts can be found in various texts of human and veterinary ultrasonography, and will therefore not be discussed in further detail here; however, a general knowledge of these phenomena will allow the ultrasonographer to control them more effectively[24].

If conducting a reproductive examination, it should be understood that the uterus may be difficult to visualize in a normal, nonpregnant animal. In early pregnancy, the lumen of the uterus may become identifiable as a hypoechoic to anechoic space as the gestational sac forms. At this stage in the muntjac, the uterus can typically be found by scanning at the level of, or slightly caudal to, the mammary glands. Prior to the skeletal mineralization or the appearance of a fetal heartbeat, pregnancy may be misdiagnosed in the presence of other reproductive conditions causing fluid to accumulate in the uterus, which may include pathology such as pyometra, hydrometra, or mucometra. Ultrasound diagnoses should be made in conjunction with a complete clinical assessment of the animal prior to sedation for the procedure. In early gestation, the conceptus will appear as a spherical to ovoid, hyperechoic structure within the amniotic sac. Specific developmental time points are not well-characterized in this species; however skeletal mineralization and fetal cardiac motion appear to be consistently detectable at approximately 8 weeks post-mating.

Under ideal conditions, transabdominal ultrasound can be an incredibly useful tool for evaluation of reproductive structures in many species, including Reeves' muntjac. The value of this technique, however, is highly dependent upon the specific capabilities of the animals' housing facilities. Unlike more passive mechanisms of pregnancy detection, such as visual observation of mating behavior and subsequent abdominal distention of the doe, ultrasound requires physical and/or chemical restraint of the animal for a period of time lasting 5-10 min or longer. In many facilities, this is not possible or practical, and the necessity of the examination should be considered based on the specific objectives of the study. In addition, certain physiological or pathological states may suggest that ultrasonography is contraindicated in certain animals due to the potentially detrimental effects of distress related to capture and sedation. It is important to assess the suitability of each animal for ultrasound examination prior to initiating the study, and a veterinarian should be consulted in the planning stages of these projects.

Current practices for pregnancy detection in Reeves' muntjac and other wildlife species are typically passive in nature (observation of mating behavior and abdominal distention in does). Serum progesterone or pregnancy-associated glycoprotein levels can be measured to confirm the diagnosis[18,25], however the results of these assays do not provide any information regarding stage of gestation or fetal viability. Ultrasound offers the benefit of permitting real-time assessment of reproductive status with accuracy similar to or greater than that of serum hormone assays[18], while maintaining minimal invasiveness and distress to the animals. In operations where the breeding date is difficult or impossible to determine, ultrasound may provide a more accurate mechanism by which to estimate stage of gestation than hormonal assays, even in the absence of published gestational tables. Furthermore, physical and chemical restraint for ultrasound examination appears to cause minimal distress to healthy muntjac and provides an ideal opportunity to perform a thorough physical examination and collect additional samples (blood, urine, *etc.*) for general health assessments.

Gestational age estimation through ultrasonographic measurements has been established in many species, including cattle[1], sheep[2,3], goats[4], and horses[5], and has been described in a limited number of cervid species as well[8-19]. In the absence of exact breeding dates or established gestation tables, fetal age of Reeves' muntjac can only be roughly estimated based on fetal development parameters outlined for other species. A specific goal of the work described here is to establish more formal guidelines for fetal aging in this species. With sufficient normative data in place, future applications of ultrasonography in this colony will include identifying abnormalities in fetal development in the presence of different experimental variables and assessing fetal viability. In addition, sensitivity and specificity of transabdominal ultrasound can be determined and compared to other commonly employed mechanisms for detecting pregnancy in cervid species.

Disclosures

The authors have nothing to disclose.

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