A comparison of pregnancy diagnosis methods in commercial sheep using lambing as a gold standard

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Abstract

Several methods are used for pregnancy diagnosis in sheep, including ultrasonography and detection of pregnancy associated glycoproteins (PAG) in blood, using either an IDEXX Rapid Visual Pregnancy Test (RVPT) kit or IDEXX Bovine Pregnancy Test (BPT). The objective was to compare transabdominal ultrasonography, RVPT and BPT for pregnancy diagnosis in sheep, with lambing results as a gold standard. Crossbred ewes (n = 196) from 3 commercial flocks were exposed to a ram for a 60-day breeding period. Transabdominal ultrasonography was performed 30 days after ram removal and a blood sample was concurrently collected. Results of transabdominal ultrasonography, RVPT, and BPT were compared to lambing results. For ultrasonography, RVPT and BPT, sensitivities were 98.6, 97.2, and 95.1%, respectively (95% CI were 95 - 99.8, 93 - 99.2 and 90.1 - 98,) and specificities were 100, 94.3 and 98.1% (95% CI were 93.2 - 100, 84.3 - 98.8 and 89.9 - 99.9). Seven pregnant ewes were misclassified as not pregnant on the BPT, whereas 4 of these ewes were also misclassified as not pregnant on the RVPT. We concluded that RVPT, BPT, and transabdominal ultrasonography had similar sensitivity and specificity when compared to lambing results.

Keywords: Pregnancy-associated glycoproteins, sheep, pregnancy diagnosis, transabdominal ultrasonography

Introduction

Global sheep production continues to rise to meet consumer demand for sheep meat.[1] Therefore, it is imperative to improve reproductive efficiency in sheep production. Early and accurate diagnosis of pregnancy enables producers to implement management interventions to attain pregnancy in non-pregnant females and aid culling decisions. Common methods of ovine pregnancy diagnosis include transrectal or transabdominal ultrasonography and assays for pregnancy associated glycoproteins (PAG).

Ultrasonography is a standard method for pregnancy diagnosis in sheep. One study indicated accuracy of 100% for transrectal ultrasonographic pregnancy diagnosis performed at least 20 days after mating when compared to a second ultrasound examination.[2] In another study, specificity, and accuracy of transabdominal ultrasonography in sheep was 100% at 39 days compared to necropsy examinations or lambing.[3] Despite the high diagnostic accuracy of ultrasonography for pregnancy diagnosis in ewes, limitations include handling facilities, ewe positioning for examination, equipment availability, portability and cost, as well as transducer type and frequency.

Pregnancy-associated glycoproteins are inactive aspartic proteinases produced by mono- and bi-nucleate giant cells in the trophoblast layer of the ruminant placenta.[4,5] These proteins cross into maternal circulation via passive diffusion and have been used to detect pregnancy in ruminants, including sheep.[6-8] Pregnancy-associated glycoproteins is detected in pregnant ewes as early as 20 days after insemination.[6]

Although commercially available ELISAs use bovine anti-PAG antibodies for detection of maternal PAG, structural similarities between bovine and ovine PAG enable detection of ovine PAG using bovine assays.[9,10]

The IDEXX Rapid Visual Pregnancy Test (RVPT) and IDEXX Bovine Pregnancy Test (BPT) (IDEXX Laboratories, Inc., Westbrook, ME) detect PAG in blood of pregnant ruminants. Sensitivity and specificity of BPT for pregnancy diagnosis in sheep was 93.5 and 98.9%, respectively, when compared to ultrasonographic pregnancy diagnosis.[9] Similarly, RVPT had 97.56% sensitivity and 93.75% specificity for pregnancy diagnosis in sheep when using transabdominal ultrasonography as the gold standard at known pregnancy timepoints.[11]

Veterinarians advising clients on methods for pregnancy diagnosis for their flock may consider factors such as flock size, available facilities or equipment, cost and timeliness of results. Whereas results of ultrasonography are immediately available, the BPT requires submission to a laboratory for analysis and a subsequent delay in obtaining results. The RVPT is a sandwich ELISA that detects PAG in maternal samples of whole blood or serum, providing more flexibility in sample collection and processing. The RVPT relies on visual readout of results, can be performed in veterinary clinics by trained personnel, without expensive laboratory equipment, with results available in < 1 hour. The ease of performing the test in the veterinary clinic is appealing for veterinarians in rural areas working with small-holder livestock farms and aiming to provide value-added services.

Blood PAG concentrations vary throughout pregnancy, with unique profiles expressed by various assays.[12] One study indicated decreased sensitivity of RVPT (compared to transabdominal ultrasonography) at the end of the first trimester of pregnancy in ewes.[13] Many factors affect PAG concentrations in maternal circulation, including maternal nutrition and placental mass, breed, sex of the fetus, stage of pregnancy and fetal number.[12, 14-16] Due to wide variations in flock management, breeds and stages of pregnancy in reproductive management of sheep, evaluation of available methods of pregnancy diagnosis compared to lambing results is warranted.

The objective was to evaluate diagnostic sensitivity and specificity of transabdominal ultrasonography, RVPT, and BPT in commercial sheep at varying stages of pregnancy, based on a single ultrasonographic examination and concurrently collected blood sample, using lambing as the gold standard. To the authors' knowledge, this was the first study to evaluate all 3 methods of pregnancy diagnosis under conditions encountered by veterinarians in the field where gestational days were variable but estimated, using lambing results at the gold standard.

Materials and Methods

All animals used in this study, conducted from November 2016 to July 2017, were breeding age ewes (1 - 7 years) from two privately owned flocks and Michigan State University Teaching and Research Center, with Dorper, Shropshire, Hampshire, and crossbred (Dorset X Polypay) sheep breeds. All ewes were managed on grazing pastures with supplemental feeds. All procedures performed during this study were approved by the Institutional Animal Care and Use Committee at Michigan State University (AUF #07/16-107-00).

Transabdominal ultrasonography using an Ovi-Scan scanner with variable 3.5 - 5.0 MHz, 170° axial scan transducer (BCF Technology, Rochester, MN) was performed on all ewes at least 30 days after ram exposure. Gestational age was estimated based on fetal measurements and fetal count was obtained during examination.[17] Concurrently, blood samples were collected by jugular venipunture into an 8.5 ml serum separator tube (BD Diagnostics, Franklin Lakes, NJ). Samples were transported on ice to the

laboratory and centrifuged at 400 x g for 5 minutes. Serum was pipetted into 1.5 ml polypropylene microcentrifuge tubes and stored for 2 weeks at -20°C until analysis.

Serum samples were analyzed using the IDEXX Rapid Visual Pregnancy Test (IDEXX Laboratories, Inc., Westbrook, ME) according to the manufacturer's instructions. Briefly, 100 µl of sheep serum samples and assay controls were pipetted into anti-PAG antibody coated wells with detector solution and incubated for 7 minutes at room temperature (18 - 26°C). Following incubation, wells were washed 3 times with distilled water. After washing, conjugate solution was added to each well and incubated for 7 minutes at room temperature. Wells were washed 3 times with distilled water, followed by addition of substrate solution. After 7 minutes of incubation at room temperature, stop solution was added to each well and color in each well was compared to the positive and negative controls. Color development deemed greater than the negative control was considered a positive result, whereas color development equal to or less than the negative control was considered a negative result. Only a dichotomous result was reported for RVPT (none of the samples were classified inconclusive). The reader of the RVPT results was blinded to the results of the ultrasound and BPT.

The IDEXX Bovine Pregnancy Test was performed as described,[12] using a commercial antigen capture enzyme-linked immunosorbent assay (IDEXX Laboratories, Inc., Westbrook, ME).[1] Briefly, 100 µl of ovine serum and assay controls were pipetted into anti-PAG antibody coated plates with 25 µl sample diluent. Plates were sealed and incubated at 37°C for 60 minutes, then washed four times using 350 µl wash solution. Detector solution (100 µl) was added to each well, covered and incubated at room temperature (18 - 26°C) for 30 minutes, followed by four washes. Then, 100 µl of conjugate solution was added, the plate covered and incubated at room temperature for 30 minutes, followed by four washes. The ELISA was developed by adding 100 µl of substrate and incubating covered for 15 minutes at room temperature. Development was stopped with addition of 100 µl stop solution and absorbance recorded at 450 nm using a microtiter plate spectrophotometer. Sample OD (optical density) values were reported as absorbance of serum samples, minus absorbance of negative controls. Samples with OD $\geq$ 0.3 were considered pregnant, whereas values $\leq$ 0.3 were reported as not pregnant. Intra-plate and inter-plate coefficients of variation (CV) for positive controls were 2.7 and 6.3%, respectively.

Lambing records were maintained for all ewes by producers or flock managers. Ewes were included in the final analysis if all data for ultrasonography, RVPT result, BPT result, and lambing date or confirmation of no lambs delivered were recorded. Sensitivity, specificity, positive and negative predictive values, and accuracy of each method of pregnancy diagnosis were calculated using lambing as the gold standard. Accuracy (Acc) was calculated using the formula Acc = TP+TN/(TP+FP+FN+TN) where TP = true positive, FP = false positive, TN = true negative, and FN = false negative. Kappa analyses were conducted to determine agreement between lambing and each method of pregnancy diagnosis. A McNemar's test was computed to test the hypothesis of no significant difference between results of each method of pregnancy diagnosis and lambing. A simple regression analysis was performed for comparison of BPT ELISA OD values and estimated gestational days.

Results

All ewes (n = 196) examined were either classified as pregnant (n = 141) or not pregnant (n = 55) following transabdominal ultrasound examination. Pregnant females ranged from 35 to 92 days estimated gestational age, based on fetal measurements obtained via ultrasonography.

For ultrasonography, RVPT and BPT, sensitivity was 98.6, 97.2, and 95.1%, respectively, whereas specificity was 100, 98.11, and 94.3%, when compared to lambing results (Table 1). For all 3

methods, Kappa analyses indicated significant agreements with lambing (Table 2). For McNemar's analysis, there were no significant differences between either ultrasonography or RVPT and lambing results, but there was a difference ($p = 0.0339$) between results of BPT and lambing results. The ELISA OD values from the BPT are shown (Figure). Seven pregnant ewes were misclassified as not pregnant on the BPT, whereas 4 of these ewes were also misclassified as not pregnant on the RVPT.

Discussion

Based on results, we concluded that a single blood sample obtained from ewes at least 30 days after breeding and analyzed using the RVPT can provide timely and valuable information for management of reproduction in sheep flocks. The most useful aspect of this test may be the ability to easily diagnose pregnancy in sheep raised on small-holder livestock farms or in developing countries where access to veterinary care and ultrasonography equipment is limited, but reliable information on pregnancy status of production animals can impact the producer's livelihood. In addition to ultrasonography or laboratory ELISA, veterinarians have the option to expand their billable services by providing pregnancy diagnosis in sheep via in-house PAG testing using the RVPT.

In the current study, sensitivity of transabdominal ultrasonography was 98.6%, whereas sensitivity and specificity of transabdominal ultrasonography to detect pregnancy in sheep 30 days after insemination was 83 and 100%, respectively, with sensitivity reaching 100% at 39 days after insemination.[3] Sensitivity of the RVPT and BPT were 97.2 and 95.1%, respectively, comparable to ultrasonography. Perhaps some ewes were too early in pregnancy for transabdominal ultrasonography, since the ram was removed only 30 days prior to the examination. However, ultrasonography can provide valuable information about fetal number and viability. Furthermore, PAG can persist in maternal circulation after pregnancy loss in ruminants and for up to 10 weeks postpartum in sheep, which may result in false positives if the test is performed too soon after pregnancy loss or lambing.[12,13,18]

False positives obtained during interpretation of the RVPT were likely due to the visual readout and interpretation error of negative wells located adjacent to strongly positive wells or a slight color change in the well due to residual PAG following pregnancy loss. In a previous study, specificity of the RVPT increased from 85 to 93% when read using a microtiter plate reader rather than solely relying on visual interpretation.[19] The cost of a microtiter plate reader ranges from $4,000 to $5,000 and may be too costly of an investment for a private practice if visual readout results are acceptable. Although a few ewes may be misclassified using only visual readout, in the present study, RVPT test closely agreed with lambing results and has potential to provide valuable management information to sheep producers and veterinarians.

Seven ewes in this study were diagnosed pregnant based on ultrasonography and subsequently lambed but were classified not pregnant with OD values ≤ 0.3 on the BPT (Figure 1). Of these 7 ewes, 4 were also classified as not pregnant using the RVPT and were between 35 and 55 days, based on ultrasonographic fetal measurements. In cows, the profile of PAG secretion in early pregnancy declined before increasing again and continuing to rise until calving.[20] Perhaps PAG secretion in sheep has a similar phenomenon that may account for the misclassifications of the 7 ewes in early pregnancy. In this regard, decreased sensitivity of the RVPT at 49 days in ewes was reported.[13] Future studies are needed to fully characterize the pattern of secretion of PAG throughout pregnancy in sheep and evaluate effects on test performance when diagnosing pregnancy in sheep using an assay targeted at bovine PAG.

There was strong agreement with lambing for 3 common methods of pregnancy diagnosis in sheep. This information provides veterinary practitioners with the evidence needed to counsel clients who are trying to select the optimal method of pregnancy diagnosis for reproductive management of their

flock, based on available resources and the timeframe for desired results. It is important for veterinarians to recognize the limitations of reading results of the RVPT and potential for misclassification of open or pregnant animals when using PAG assays such as the BPT due to changes in PAG secretion throughout pregnancy or persistence of PAG in maternal circulation after pregnancy loss. Misclassifications during pregnancy diagnosis may impact the economic value of the test due to housing a non-pregnant female until the next breeding season; however, implementation of pregnancy diagnosis via any available method is a critical component of sound reproductive management of the flock. While the chosen method of pregnancy diagnosis will depend on many factors including cost, accessibility of services, technical expertise, and flock management, veterinarians can be confident that, based on this study, transabdominal ultrasonography, RVPT, or BPT at varying stages of pregnancy yield results in close agreement with pregnancy outcomes.

Conflict of Interest

None.

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Table 1. Sensitivity (Se), specificity (Sp), positive predictive value (PPV), negative predictive value (NPV), and accuracy with 95% confidence interval (CI) of the IDEXX Rapid Visual Pregnancy Test (RVPT), IDEXX Bovine Pregnancy Test (BPT) and transabdominal ultrasonography (US) compared to lambing in commercial ewes (n = 196).

	US	**RVPT**	**BPT**
Se (%)	98.6	97.20	95.1
95% CI	95-99.8	93-99.2	90.1-98
Sp (%)	100	94.34	98.11
95% CI	93.2-100	84.3-98.8	89.9-99.9
PPV (%)	100	97.89	99.27
95% CI	-	93.9-99.2	95.1-99.8
NPV (%)	96.36	92.59	88.14
95% CI	87-99	82.6-97	78.2-93.8
Accuracy (%)	98.98	96.43	95.92
95% CI	96.3 – 99.8	92.7-98.5	92.1-98.2

Table 2. Kappa analysis of transabdominal ultrasonography (US), IDEXX Rapid Visual Pregnancy Test (RVPT) and IDEXX Bovine Pregnancy Test (BPT) versus lambing for detection of pregnancy in 196 commercial ewes.

	US	RVPT	BPT
Degree of Agreement	0.974	0.900	0.910
95% CI	0.939 - 1	0.832-0.967	0.844-0.975
p-value	< 0.0001	< 0.0001	< 0.0001

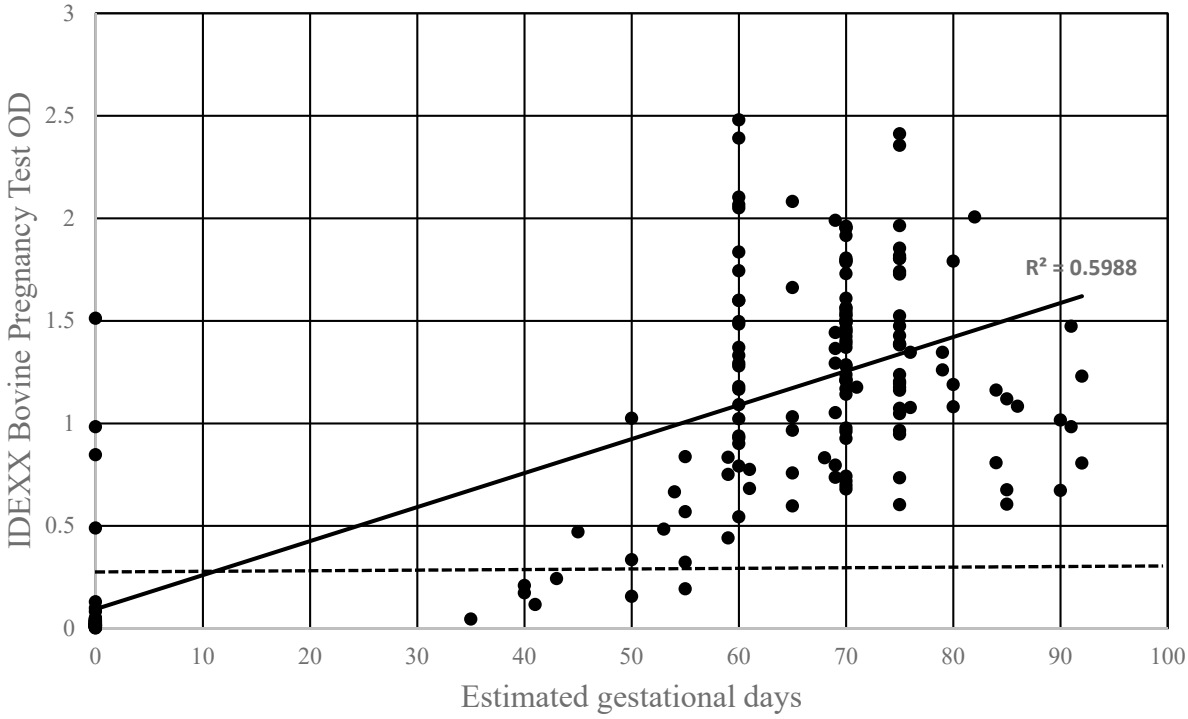

Figure. ELISA optical density (OD) values for samples tested using IDEXX Bovine Pregnancy Test (BPT) (n = 196) by estimated gestational days. Horizontal dashed line indicates the OD cut off value of 0.3 for classification of pregnant or not pregnant on test interpretation. Seven ewes diagnosed pregnant by ultrasonography were reported as not pregnant, whereas 4 of these ewes were diagnosed as not pregnant on the RVPT.