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Technical note

Early pregnancy diagnosis in sheep using near-infrared spectroscopy on blood plasma

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ABSTRACT

The objective of this study was to evaluate the ability of near-infrared reflectance spectroscopy (NIRS) to discriminate between pregnant and nonpregnant ewes in early stages of pregnancy after artificial insemination (AI) from blood plasma. Samples were collected using jugular puncture at 18 and 25 days after AI from 188 Rasa Aragonesa and Ansotana ewes. Plasma samples were analyzed for pregnancy-associated glycoprotein (PAG) and progesterone (P4) using ELISA commercial kits. The spectra of plasma samples were recorded in the visible and near-infrared ranges. The performance of these tests were compared, using as criterion standard the pregnancy status determined using transabdominal ultrasonography at 45 days after AI. Pregnancy rate was 47.9% (90/188). At Day 18, sensitivity was similar in NIRS and P4 tests (98.9% vs. 100%; not significant) and greater than PAG (32.2%; both $P < 0.001$). Specificity was similar in NIRS and PAG tests (both 100%) and greater than that of P4 (84.7%; $P < 0.001$). At Day 25, sensitivity and specificity of NIRS and PAG were both 100%. It can be concluded that NIRS was an accurate method of diagnosis of pregnancy at Days 18 and 25 after AI in ewes.

1. Introduction

An early and practical pregnancy diagnosis test is essential for the efficient reproductive management of an ovine flock. Gestation can be diagnosed using transabdominal ultrasonography from 24 to 25 days of pregnancy (Day 0 = day of breeding), although it is recommended to be performed after Days 40 to 55 because efficiency in counting the number of conceptuses in multiple pregnancies can reach 100% (reviewed by González-Bulnes et al. [1]). An earlier pregnancy diagnosis can be performed using transrectal ultrasonography, but is more time-consuming and requires a more expert operator [1]. The progesterone (P4) assay is accurate as early as Days 17 to 19 [2]. Pregnancy-associated glycoprotein (PAG) determination in plasma using radioimmunoassay with a mixture of ovine and caprine antisera against PAGs allows 95.3% correct pregnancy diagnosis as early as Day 18 [3]. Recently, a new PAG ELISA kit (CER-6900; Marloie) was tested for pregnancy diagnosis in the Rasa Aragonesa breed, showing sensitivity and specificity values of 100% from Day 25 and onward [4].

Near-infrared reflectance spectroscopy (NIRS) technology is currently used in quality assurance analysis for a number of substances, being nondestructive, fast, and suited to online measurements. The objective of this study was to evaluate the ability of NIRS to discriminate between pregnant and nonpregnant ewes at Days 18 and 25 after artificial insemination (AI) from blood plasma.

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2. Materials and methods

2.1. Samples and data collection

This experiment was carried out in the facilities of the Centro de Investigación y Tecnología Agroalimentaria Research Center. A total of 188 adult, multiparous, dry ewes were used. All ewes were identified with ear tags and ruminal boluses for electronic identification. The ewes belonged to two flocks. Flock 1 consisted of 142 Rasa Aragonesa ewes, kept permanently in irrigated pastures of artificial grassland of ryegrass and lucerne, at a stocking rate of 13.4 ewes per hectare. Flock 2 consisted of 46 Ansotana ewes kept in nonirrigated pastures in semiarid areas, grazing permanent grassland of semiarid environment and rainfed lucerne, at a stocking rate of 1.3 ewes per hectare. AI was carried out in March (flock 1) and November (flock 2).

Ewes were treated for 14 days with vaginal sponges containing fluorogestone acetate (Sincropart 30 mg; Ceva Salud Animal S.A., Barcelona, Spain), and 480 IU eCG im (Sincropart PMSG 6000 UI; Ceva Salud Animal S.A.) at sponge withdrawal. Cervical AI was carried out 54.5 $\pm$ 1 hour after sponge withdrawal with semen diluted in skimmed milk and maintained at 15 °C. Each ewe received 400 $\times$ 10^6 total spermatozoa. Fifteen days after AI, five entire adult males were introduced into the flocks for 10 days. Eighteen and 25 days after AI, blood samples were taken from ewes using 5-mL vacuum tubes with heparin. Plasma was collected and frozen after blood centrifugation at 2122 $\times$ *g* for 25 minutes for further PAG, NIRS, and P4 determinations. Analyses of PAG, NIRS, and P4 were performed in all samples taken at Day 18 (N = 188). At Day 25, analyses of PAG and NIRS were performed only in samples from ewes that lambed after AI (N = 90), and from ewes that failed to conceive after AI and natural breeding (N = 29). Samples from ewes that conceived after natural breeding (N = 69) were not analyzed. With the only purpose of knowing whether the spectral differences observed between pregnant and cycling nonpregnant ewes at this day were or not related to the presence of P4, it was analyzed at Day 25 only in ewes that failed to conceive after AI and natural breeding (only in 17 out of the 29 nonpregnant ewes was there was enough plasma left).

After AI, ewes were kept inside and fed ad libitum. Forty-five days after AI, pregnancy diagnosis was performed using transabdominal ultrasonography in standing position, using a real-time B-mode ultrasound scanner (5.0-MHz linear-array transducer; Aloka 500 SSD). At 140 days after AI, pregnant ewes were placed in individual pens and checked daily to allow accurate assessment of the lambing dates. Ewes were determined to have conceived to AI or to natural breeding based on the embryonic vesicle size at ultrasound examination and confirmed according to the lambing dates.

2.2. Criterion standard to determine the pregnancy status at Days 18 and 25 after AI

Because in this study we were focused in pregnancy diagnosis only at Days 18 and 25 of gestation, but not earlier, ewes pregnant at the return estrus were considered as nonpregnant. Ewes diagnosed as pregnant using ultrasound scanning that lambed 149 $\pm$ 7 days after AI were considered pregnant. Ewes diagnosed as pregnant using ultrasonography that lambed later than 156 days after AI were assumed to have conceived after natural breeding and were considered nonpregnant. Finally, ewes diagnosed as pregnant using ultrasonography that did not lamb would be assumed to have suffered fetal death/abortion around/after Day 45 and would have been considered as pregnant at Days 18 and 25. Nevertheless, in the present study all ewes diagnosed as pregnant using ultrasonography lambed.

2.3. Assays of plasma PAG and P4

The PAG assay was performed with a “sandwich” ELISA kit (Ref. Code EG7, CER-6900 Marloie), following the manufacturer’s instructions. The sensitivity of the assay was 0.22 ng/mL. Intra- and interassay coefficients of variation were: for a plasma pool of 2.2 ng/mL, 6.3% and 10.4%, respectively; for a plasma pool of 1.3 ng/mL, 5.3% and 6.7%, respectively. The basal level calculated from 30 samples of 10 nonpregnant ewes was 0.34 ng/mL. The 95% confidence limit (0.8 ng/mL) was considered the threshold for pregnancy diagnosis [4]. Progesterone was analyzed using an ELISA kit designed for ovine plasma (Ridgeway Science, St. Briavels, Gloucestershire, UK), following the manufacturer’s instructions. The sensitivity was 0.27 ng/mL. All samples were analyzed in the same assay. Intra-assay coefficients of variation for sample pools of 0.5, 1, and 2 ng/mL were 8.5%, 9.9%, and 2.3%, respectively. The threshold considered for pregnancy diagnosis was 0.5 ng/mL.

2.4. Reflectance spectrum measurement

Spectra were analyzed using the method previously described [5]. Briefly, after 2 hours at room temperature, 0.5 mL of a plasma sample was placed on a glass microfiber filter (Whatman GF/A, 55 mm, Cat. No. 1820 055; Whatman International, Ltd., Maidstone, UK) and oven-dried at 30 °C for 24 hours. The sample was then placed in a 50-mm diameter ring cup and scanned in reflectance (*R*) mode at 2-nm intervals from 400 to 2498 nm using a Foss NIRSystems 6500 NIR scanning spectrometer (Foss NIRSystems, Silver Spring, MD, USA) equipped with a transport module and controlled via WinISI II version 1.02 software (Infrasoft International, L.L.C., State College, PA, USA). Reflectance was converted into absorbance (*A*) using the formula $A = \log(1/R)$.

2.5. Methods used to discriminate pregnant and nonpregnant ewes

The raw spectra were then transformed applying the standard normal variate and detrending [6] as a scatter correction procedure and also a mathematical first-order derivative treatment. The transformed absorbance spectra of samples for each date representing the two groups (pregnant vs. nonpregnant) underwent partial least squares discriminant analysis (PLS-DA) according to the method described by Dian et al. [7] using the software that piloted

the NIRS instrument (WinISI II version 1.02 software, Infrasoft International, L.L.C.). The PLS-DA model consists of a PLS regression in which the dependent variable is a categorical variable (one for pregnant ewes, two for nonpregnant ewes). Each sample is classified into a group and it is associated to an uncertainty factor which is calculated using a Student *t* test. An uncertainty value of two for this test is recommended by the manufacturer. All samples in this study equaled or exceeded this value. A principal component analysis performed beforehand was used to characterize the factors which better explain the discrimination results. The wavelengths more involved in discriminating between pregnant and nonpregnant ewes were those with greater coefficients in the first two factors. The model was tested via a cross-validation procedure, in which a quarter of randomly chosen samples were temporarily removed from the initial dataset to be used for validation. The PLS-DA model parameters were estimated using the remaining three-quarters (calibration samples), which were used to classify the validation samples. This procedure was repeated four times, i.e., until all dataset samples had been classified using the validation procedure. The cross-validation error of the models was expressed as sensitivity and specificity in relation to the criterion standard for pregnancy status.

2.6. Statistical analysis

Sensitivity (Se) was defined as the ratio between the ewes correctly diagnosed as pregnant and all the pregnant ewes. Specificity (Sp) was the ratio between the ewes correctly diagnosed as nonpregnant and all the nonpregnant ewes. Positive predictive value (PPV) was the ratio between the ewes correctly diagnosed as pregnant and those diagnosed as pregnant. Negative predictive value (NPV) was the ratio of ewes correctly diagnosed as nonpregnant and those diagnosed as nonpregnant. Both PPV and NPV depend on prevalence (Pr) [8,9]. In the present study, Pr is the pregnancy rate after AI. The relationships between PPV and NPV with Se, Sp, and Pr are:

$$\mathrm{PPV} = \mathrm{Pr} \times \mathrm{Se}/[\mathrm{Pr} \times \mathrm{Se} + (1 - \mathrm{Pr}) \times (1 - \mathrm{Sp})];$$
$$\mathrm{NPV} = (1 - \mathrm{Pr}) \times \mathrm{Sp}/[(1 - \mathrm{Pr}) \times \mathrm{Sp} + \mathrm{Pr} \times (1 - \mathrm{Se})]$$

Pairwise differences between groups in the Se, Sp, PPV, and NPV were analyzed using the Freeman-Tukey test and step-down bootstrap adjustment for multiple tests, using the MULTTEST procedure of SAS [10].

Table 1
Sensitivity, specificity, PPV, and NPV of NIRS compared with the plasmatic PAG method (≥0.8 ng/mL) at Days 18 and 25 after AI and P4 method (≥0.5 ng/mL) at Day 18 after AI.

Method	Sensitivity	Specificity	PPV	NPV
Day 18				
NIRS	98.9 (89/90)[a]	100 (98/98)[a]	100 (89/89)[a]	99.0 (98/99)[a]
PAG	32.2 (29/90)[b]	100 (98/98)[a]	100 (29/29)[c]	61.6 (98/159)[b]
P4	100 (90/90)[a]	84.7 (83/98)[b]	85.7 (90/105)[b]	100 (83/83)[a]
Day 25				
NIRS	100 (90/90)[a]	100 (29/29)[a]	100 (90/90)[a]	100 (29/29)[a]
PAG	100 (90/90)[a]	100 (29/29)[a]	100 (90/90)[a]	100 (29/29)[a]

Values within brackets correspond to number of correctly diagnosed ewes/total number of ewes in each category.
Within columns, values with different superscripts differ at: [a,b] $P < 0.001$; [b,c] $P < 0.05$.
Abbreviations: NIRS, near-infrared reflectance spectroscopy; NPV, negative predictive value; P4, progesterone; PAG, pregnancy-associated glycoprotein; PPV, positive predictive value.

3. Results

Results are shown in Table 1. Differences in fertility between flocks 1 and 2 were very low and nonsignificant, so the results were pooled. Ninety ewes out of 188 were pregnant after AI (pregnancy rate: 47.9%). Irrespective of their pregnancy status, all plasma samples showed local peaks at 418, 1502, 1728, 1930, 2056, 2176, 2302, and 2472 nm, and a broad band between 460 nm and 490 nm (Fig. 1). The raw spectra showed decreasing absorbance as wavelength increased from approximately 450 to 1100 nm and continuous increase in absorbance with greater wavelengths. Plasma samples obtained at Day 18 from nonpregnant ewes generally showed slightly higher absorbance than the plasma of pregnant ewes throughout the infrared spectrum, and slightly lower absorbance values throughout the visible part of the spectrum. However, for samples obtained at Day 25, higher absorbances at 418 nm were found in pregnant ewes, whereas similar absorbances between pregnant and nonpregnant ewes were found for the infrared wavelengths. The wavelengths more involved in discriminating between pregnant and nonpregnant ewes were 432, 528, 584, and 1084 (higher coefficients in the first factor) and 448, 464, 1892, and 1964 (higher coefficients in the second factor).

At Day 18, the sensitivity of NIRS was greater than that of PAG (98.9% vs. 32.2%; $P < 0.001$; Table 1), whereas no differences were found in specificity. Sensitivity of NIRS

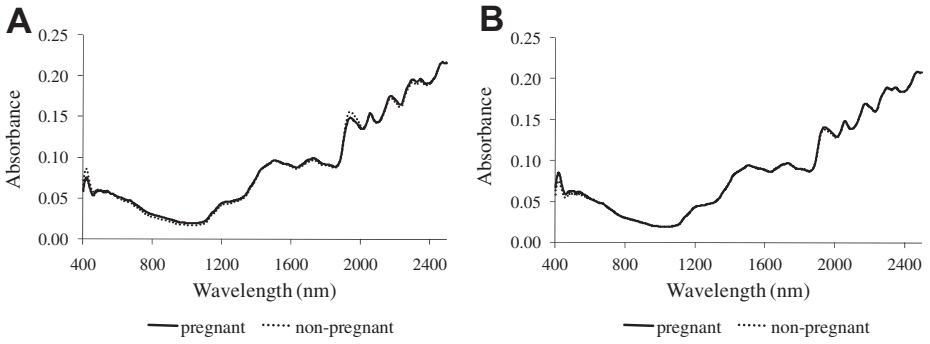

Fig. 1. Plasma raw spectra for pregnant and nonpregnant ewes for samples obtained at 18 days after AI (A) and 25 days after AI (B).

and P4 were similar, whereas specificity was higher in NIRS (100% vs. 84.7%; $P < 0.001$).

At Day 25, no differences were found in any of the studied parameters between PAG and NIRS (100%). Most nonpregnant ewes (15 out of 17) had a high concentration of P4 (≥ 0.5 ng/mL), whereas they were correctly diagnosed as nonpregnant using NIRS and PAG.

4. Discussion

This study describes for the first time the use of NIRS for early pregnancy diagnosis in blood plasma in ewes. The results obtained in our experiment demonstrate the ability of NIRS to detect early pregnancy successfully in two genetically different breeds [11] managed according to different production systems, fed differently, and inseminated in different seasons. As early as 18 days after AI, it allowed the correct classification of 98.9% and 100% of the plasma samples obtained from pregnant and nonpregnant ewes, respectively. Prominent spectral peaks at 1502, 1728, 2056, 2176, 2302, and 2472 nm can be ascribed to the absorption of functional groups related to proteins [12], and absorptions at 1930 nm could arise from functional groups related to carbohydrates. These absorptions agree with previous reports [13,14]. Thus, NIRS could detect in blood plasma the presence of compounds related to the oviductal and uterine fluids to ensure implantation, including micronutrients involved in embryo development and survival [15]. The observed spectral differences do not appear to correspond to P4. Plasma P4 concentration at days 16 to 19 after breeding is low in nonpregnant ewes but high in pregnant ewes because of the persistence of the pregnancy corpus luteum. In our study, 15 nonpregnant ewes, correctly classified as nonpregnant using NIRS, had high P4 concentrations on Day 18. This lower specificity could be due to embryo mortality occurring after the maternal recognition of pregnancy, to either shortened or extended cycles, or to other pathologies such as pyometra, which could affect the P4 level. In addition, the P4 concentration on Day 25 after AI is expected to be high in regularly cycling and pregnant ewes and low in noncycling ewes. In our study, 15 out of 17 nonpregnant ewes had high levels of P4 at Day 25 after AI. Despite their elevated P4 levels, these ewes were correctly diagnosed as nonpregnant using NIRS, reinforcing the absence of any direct relation between P4 and the spectral differences observed between pregnant and nonpregnant ewes.

The performance of P4 and PAG as pregnancy diagnosis tests at Days 18 and 25, respectively, are similar to those previously reported [2]. In the present work, sensitivity and specificity of PAG and NIRS diagnosis at Day 25 was exactly the same (100%). In contrast, at Day 18, the sensitivity of the PAG test was significantly lower, suggesting that the ELISA method used here was not sensitive enough to detect PAG in some pregnant ewes at this age. In fact, sensitivity is greater when measuring PAG with RIA using a mixture of ovine and caprine antisera, allowing for pregnancy diagnosis to be accurately assessed from Day 18 of gestation [3]. In our study, it is not possible to know whether the observed differences between the spectrum of pregnant and nonpregnant ewes corresponds to some of the many existing PAGs in the ruminant placenta [16].

In the present study, ultrasonography at Day 45 was used as the criterion standard to determine the pregnancy status. It must be noted that animals pregnant on Days 18 and 25 can lose the pregnancy before ultrasonography leading to false positive results of pregnancy tests. In the present work, there was no evidence of pregnancy losses, because false positive results were not found in PAG and NIRS tests at Day 25. It must be taken into account that the percentage of ewes losing all their embryos is considered low. In a work carried out in farms, it was found that only 3.8% of ewes lost one or more embryos from Days 25 to 45 and that partial losses were more frequent than complete losses in ewes with a multiple pregnancy [17].

4.1. Conclusions

Near-infrared reflectance spectroscopy is a reliable method that allows an early and efficient pregnancy diagnosis in sheep from 18 days after breeding. Unlike for PAG or P4 determinations, reagents are not required. More research is needed to test the accuracy of NIRS testing earlier than Day 18 after breeding, and to identify the substances responsible for spectral differences between pregnant and nonpregnant ewes.

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