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Revised

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 non-pregnant ewes in early stages of pregnancy after artificial insemination (AI) from blood plasma. Samples were collected by 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 was compared, using as criterion standard the pregnancy status determined by 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%; NS) and higher than PAG (32.2%; both $P < 0.001$). Specificity was similar in NIRS and PAG tests (both 100%) and higher 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 post-AI in ewes.

Keywords: early pregnancy diagnosis, ewe, fertility, NIRS, PAG, progesterone

1. Introduction

An early and practical pregnancy diagnosis test is essential for the efficient reproductive management of an ovine flock. Gestation can be diagnosed by transabdominal ultrasonography from 24 to 25 days of pregnancy, although it is recommended to be performed after days 40 to 55 of gestation as 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 by 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 after breeding [2]. Pregnancy-associated glycoprotein (PAG) determination in plasma by radioimmunoassay using a mixture of ovine and caprine antisera against PAGs allows 95.3% correct pregnancy diagnosis as early as from day 18 of gestation onwards [3]. Recently, a new PAG ELISA kit (CER-6900 Marloie, Belgium) was tested for pregnancy diagnosis in the Rasa Aragonesa breed, showing sensitivity and specificity values of 100% from day 25 onwards [4].

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

2. Materials and methods

2.1. Samples and data collection

This experiment was carried out in the facilities of CITA 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/ha. Flock 2 consisted of 46 Ansotana ewes kept in non-irrigated pastures in semiarid areas, grazing permanent grassland of semiarid environment and rainfed lucerne, at a stocking rate of 1.3 ewes/ha. Artificial insemination (AI) was carried out in March (flock 1) and November (flock 2).

Ewes were treated for 14 days with vaginal sponges containing fluorogestone acetate (FGA; Sincropart 30 mg, CEVA Salud Animal S.A., Barcelona, Spain), and 480 IU eCG im (Sincropart PMSG 6,000 UI, CEVA Salud Animal S.A., Barcelona, Spain) at sponge withdrawal. Cervical AI was carried out 54.5 ± 1 h after sponge withdrawal with semen diluted in skimmed milk and maintained at 15 ºC. Each ewe received $400 \cdot 10^6$ 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 following blood centrifugation at $2{,}122 \times g$ 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 both 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 non-pregnant ewes at this day were or not related to the presence of P4, it was analysed at day 25

only in ewes that failed to conceive after both AI and natural breeding (only in 17 out of the 29 non-pregnant ewes there was enough plasma left).

After AI, ewes were kept inside and fed *ad libitum.* Forty-five days after AI, pregnancy diagnosis was performed by 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 by the lambing dates.

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

As 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 non-pregnant. Ewes diagnosed as pregnant by ultrasound scanning that lambed 149 ± 7 days after AI were considered pregnant. Ewes diagnosed as pregnant by ultrasonography that lambed later than 156 days after AI were assumed to have conceived after natural breeding and were considered non-pregnant. Finally, ewes diagnosed as pregnant by 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 by 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, Belgium), following the manufacturer’s instructions. The sensitivity of the assay was 0.22 ng/mL. Intra and inter-assay 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 non-pregnant 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 by 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 methodology previously described [5]. Briefly, after 2 h 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 h. 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 LLC, 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 non-pregnant ewes.

The raw spectra were then transformed applying the standard normal variate and de-trending [6] as 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.* non-pregnant) underwent partial least squares discriminant analysis (PLS-DA) according to the methodology described by Dian et al. [7] using the software which piloted the NIRS instrument (WinISI II version 1.02 software, Infrasoft International

LLC, State College, PA, USA). The PLS-DA model consists of a PLS regression where the dependent variable is a categorical variable (1 for pregnant ewes, 2 for non-pregnant ewes). Each sample is classified into a group and it is associated to an uncertainty factor which is calculated by a Student's t-test. An uncertainty value of 2 for this test is recommended by the manufacturer. All samples in this study equalled or exceeded this value. A principal component analysis (PCA) performed beforehand was used in order to characterize the factors which better explain the discrimination results. The wavelengths more involved in discriminating between pregnant and non-pregnant 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 through 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 non-pregnant and all the non-pregnant 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 non-pregnant and those diagnosed as non-pregnant. Both PPV and NPV depend on prevalence (P) [8,9]. In the present study, prevalence is the pregnancy rate after AI. The relationships between PPV and NPV with Se, Sp and P are:

$$PPV = P \cdot Se/[P \cdot Se+(1-P) \cdot (1-Sp)]; \quad NPV=(1-P) \cdot Sp/[(1-P) \cdot Sp+P \cdot (1-Se)]$$

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

3. Results

Results are shown in Table 1. Differences in fertility between flocks 1 and 2 were very low and non-significant, 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 (Figure 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 non-pregnant 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 non-pregnant ewes were found for the infrared wavelengths. The wavelengths more involved in discriminating between pregnant and non-pregnant 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).

(Approximate location of Figure 1)

At day 18, the sensitivity of NIRS was higher than that of PAG (98.9 *vs.* 32.2%; $P < 0.001$; Table 1), whereas no differences were found in specificity. Sensitivity of NIRS 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 non-pregnant ewes (15 out of 17) had a high concentration of P4 (≥ 0.5 ng/mL), whereas they were correctly diagnosed as non-pregnant by NIRS and PAG.

(Approximate location of Table 1)

4. Discussion

This study describes for 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 NIR spectroscopy 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 non-pregnant 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 non-pregnant ewes but high in pregnant ewes due to the persistence of pregnancy corpus luteum. In our study, 15 non-pregnant ewes, correctly classified as non-pregnant by 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 post-AI is expected to be high in both regularly cycling and pregnant

ewes and low in non-cycling ewes. In our study, 15 out of 17 non-pregnant ewes had high levels of P4 at day 25 post-AI. In spite of their elevated P4 levels, these ewes were correctly diagnosed as non-pregnant by NIRS, reinforcing the absence of any direct relation between P4 and the spectral differences observed between pregnant and non-pregnant 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 both PAG and NIRS diagnosis at day 25 was exactly the same (100%). By 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 higher when measuring PAG by 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 is not possible to know whether the observed differences between the spectrum of pregnant and non-pregnant 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 females pregnant on days 18 and 25 can loose the pregnancy before ultrasonography leading to false positive results of pregnancy tests. In the present work there was no evidence of pregnancy losses, as 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 1 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].

In conclusion, NIRS 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 test earlier than day 18 after breeding, and to identify the substances responsible for spectral differences between pregnant and non-pregnant ewes.

5. Acknowledgments

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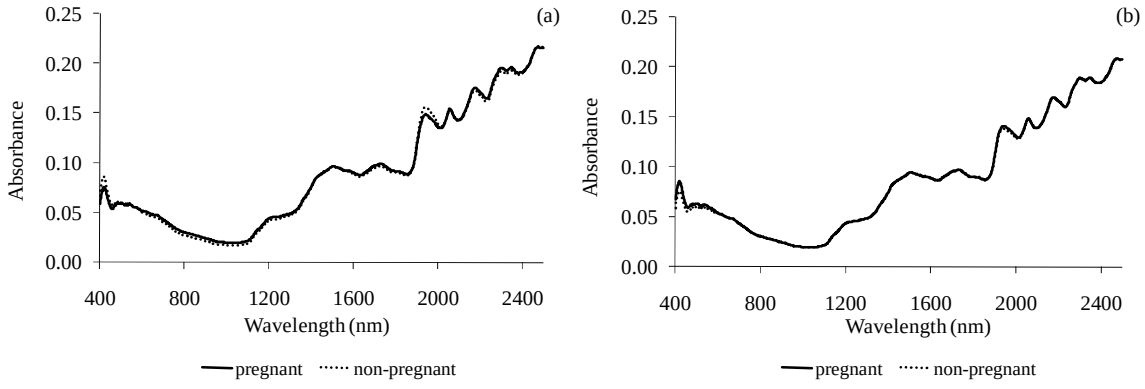
269

270 **Table 1.** Sensitivity, specificity, positive predictive value (PPV) and negative predictive value
271 (NPV) of near-infrared reflectance spectroscopy (NIRS) compared with the plasmatic
272 pregnancy-associated glycoprotein (PAG) method (≥0.8 ng/mL) at days 18 and 25 after AI
273 and progesterone (P4) method (≥0.5 ng/mL) at day 18 after AI. Values within brackets
274 correspond to number of successes/total number of ewes in each category.

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]

275 Within columns: **[a,b]**: $P < 0.001$; **[b,c]**: $P < 0.05$.

276



277

278 Figure 1. Plasma raw spectra for pregnant and non-pregnant ewes for samples obtained at 18
279 days after AI (a) and 25 days after AI (b).