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Technical note

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

Donato Andueza [a,b,*], José L. Alabart [c], Belén Lahoz [c], Fernando Muñoz [c], José Folch [c]

[a] INRA, UMR1213 Herbivores, Saint-Genès-Champanelle, France
[b] Clermont Université, VetAgro Sup, UMR Herbivores, Clermont-Ferrand, France
[c] Centro de Investigación y Tecnología Agroalimentaria (CITA) de Aragón, Zaragoza, Spain

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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.

* Corresponding author. Tel.: +33 4 73 62 40 71; fax: +33 4 73 62 41 18.
E-mail address: donato.andueza@clermont.inra.fr (D. Andueza).

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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 ± 1 hour after sponge withdrawal with semen diluted in skimmed milk and maintained at 15 °C. Each ewe received 400×10^6 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 × *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 ± 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